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Histopathological Protocol To Find Out The Mode Of Action Of Fungus *Beauveria bassiana* (Balsamo) Vuillemin Preparation On Cuticle Of Termite Worker Of *Odontotermes Obesus* (R.)

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ABSTRACT

Odontotermes obesus (Rambur) (Isoptera: Termitidae) is a common termite pest found in almost all crop plants including cash crops such as maize, wheat, groundnut, rice etc. in South East part of Rajasthan (India). Presently, the control methods mostly swing towards chemical that has led to high cost, persistence and adverse effects to the environment. Biological control with pathogenic fungi is a promising alternative to chemical control against the subterranean termite. *Beauveria bassiana* (Balsamo) is one of the several natural agents for controlling the subterranean termite by direct penetration of the insect cuticle. Workers of the termite *Odontotermes* spp. (Isoptera: Termitidae) were inoculated with the entomopathogenic fungus, *Beauveria bassiana* by exposing the termite to viable conidia in a petri dish. The most effective dose 4.5×10^8 conidia/ml against worker was used to see its effect on cuticle. It was done by using standard histopathological protocol. Termite workers were sacrificed after 48 hrs. treating with highest dose of 4.5×10^8 conidia/ml. Transverse sections of body revealed marked changes in various insect tissues including cuticle. The target of fungus was of course the cuticular part, which was well evident by damaged exocuticle, endocuticle and deposition of conidia in large amount on the external surfaces. The analysis of the colonized termites provided an overview of the infection, showing that the hydrophobic conidia were able to adhere all over the external surface of body wall with a preference for surfaces containing spines.

KEY WORDS: Termite, *Beauveria bassiana*, worker, histopathology, cuticle

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INTRODUCTION

Termites are ancient insect, having evolved approximately 200 million years ago¹. The evolution and existence of termites were associated with their nasty problems. Consequently the control of the termites was inevitable and necessary. Termites are social insects found mainly in the tropics between 45° North and 45° South latitudes. These distribution areas cover over two-thirds of the landmass, involving some 100 countries².

Termites have been reported worldwide as one of the most important group of insects that cause significant and serious damages to crops, structures and buildings^{3,4,5}. The spread of termites is enhanced by human activities such as movement of wood products from termite-infested to termite-free locations. Around US\$ 22 billion are spent annually for termite control and repairing the damages⁶.

Insecticide resistance and the demand for reduced chemical inputs in agriculture have provided an impetus to the development of alternative forms of pest control. Biological control offers an attractive alternate or supplement to the use of chemical pesticides. Microbial biological control agents are naturally occurring organisms and perceived as being less damaging to the environment. Furthermore, their generally complex mode of action makes it unlikely that resistance could be developed to a bio-pesticide. Biological pest control agents include viruses, bacteria, fungi, and nematodes. The use of microorganisms as selective pesticides has notable successes⁷.

The organisms used in microbial insecticides are essentially nontoxic and non pathogenic to wildlife, humans, and other organisms not closely related to the target pest. The safety offered by microbial insecticides is their greatest strength. The toxic action of microbial insecticides is often specific to a single group or species of insects and this specificity means that most microbial insecticides do not directly affect the beneficial insects (including predators or parasites of pests) in treated areas.

Entomopathogenic fungi can be used as a short-term control measure to eliminate active termites (such as fungal dust or liquid applications directly to galleries and workings of active infestations in the structures to be protected) or as a longer-term control measure where the fungi are used to suppress or eliminate the active colonies (such as direct dosing of termite nests, or used in baiting programmes) and also as a protective treatment to prevent termite infestations (such as under slab or soil perimeter applications of a repellent formulation) .

Beauveria bassiana (Balsamo) is a fungus which causes a disease known as the white muscardine disease in insects. When spores of this fungus come in contact with the cuticle (skin) of susceptible insects, they germinate and grow directly through the cuticle to the inner body of their

host. Here the fungus proliferates throughout the insect's body, producing toxins and draining the insect's nutrients, eventually killing it. Therefore, unlike bacterial and viral pathogens of insects, *Beauveria* and other fungal pathogens infect the insect with contact and do not need to be consumed by their host to cause infection⁸. Once the fungus has killed its host, it grows back out through the softer portions of the cuticle, covering the insect with a layer of white mold (hence the name white muscardine disease). This downy mold produces millions of new infective spores that are released to the environment. *Beauveria* is also a naturally occurring fungus in soils throughout the world, and has been researched for control of insects.

Taking these facts into considerations, current research programme has been undertaken to evaluate the impact of one important fungal entomopathogen *Beauveria bassiana* (Balsamo) Vuillemin against termite *Odontotermes obesus* (Rambur) which is one of important pest species of termite in Udaipur district of South Rajasthan infesting many crops and commodities.

MATERIALS AND METHODS

Termite collection

Underground nests of *Odontotermes obesus* (R.) located at crop fields were opened up, termites consisting of workers, soldiers and nymphs together with nest material were collected in a tray and brought to the laboratory. The termite workers along with the nesting mound soil were put in plastic containers and were observed in the laboratory for 1-2 months. The rearing container was covered with lids. The size of the containers ranged from 0.03 - 0.07 m². Water was added to the containers every 3-7 days to maintain moisture. These termites were kept in petri dishes containing wet filter paper for 24 h before inoculation.

Culture of Beauveria bassiana (balsamo) vuillemin

Beauveria bassiana (Balsamo) was collected from Maharana Pratap University of Agriculture and Technology, Udaipur Rajasthan and subcultured on Sabouraud Dextrose Agar (SDA) media, in the laboratory.

Spore concentration of Beauveria bassiana (Balsamo)

A spore suspension was prepared by harvesting conidia from the SDA plates just before application. Conidial count was done using Neubauer Improved Haemocytometer under a microscope for conidia/ml of dilution and recorded separately.

Inoculation

Thirty workers were placed on different petri plates that contained 1 ml conidial suspension of *B. bassiana* at different concentration of 4.5×10^8 , 4.5×10^7 , 4.5×10^6 and 4.5×10^5 conidia/ml on Whatman's filter paper disc (9 cm X 1mm) or 1 ml of control suspension solution which contained sterile water. The termites were exposed to conidial suspension for 24 hours. The treated termites caste were then transferred in sterile glass Petri dishes (10 cm in diameter), lined with sterile moistened Whatman No. 1 filter paper. A wood piece was placed in the petri plate for food. Both experimental and control petri dishes were kept at $28^\circ\text{C} \pm 2^\circ\text{C}$ and relative humidity 75 ± 5 percent in BOD in the dark.

Histology

After 48 hours of treatment, 10 workers were removed from the petri dish and fixed in an alcoholic Bouin's fluid. The chitinized cuticle of the insect body is nearly impermeable to fixing fluids, so that for fixing of the insect whole body, the insect drop into boiling fixative, alcoholic Bouin's; leave them in fixative for an one or two minutes, then transferred to normal fixative for 24 hours at 4°C . The treated and control termites workers were dehydrated in a graded series of ethyl alcohol, cleared in xylene, embedded in paraffin. Serial sections of 6-7 μ thick were cut and stained in Hematoxyline and Eosin stain. These treated sections were compared with controlled untreated sections to observe the effect of the fungus on cuticle and body wall. Images were captured using a digital camera (Sony -Cybershot) mounted on the trinocular microscope.

RESULT

Structure of normal body wall (Fig: 1)

The body wall is reinforced by a cuticle covering its outer surface. Light microscopy investigation of sections from control termite clearly identified the three layers of the integument: the cuticle, the epidermal cells and the basal membrane. The cuticle has a stratified appearance in sections. The cuticle is divided into three layers: (inside to outside) i.e. Endocuticle, Exocuticle and Epicuticle.

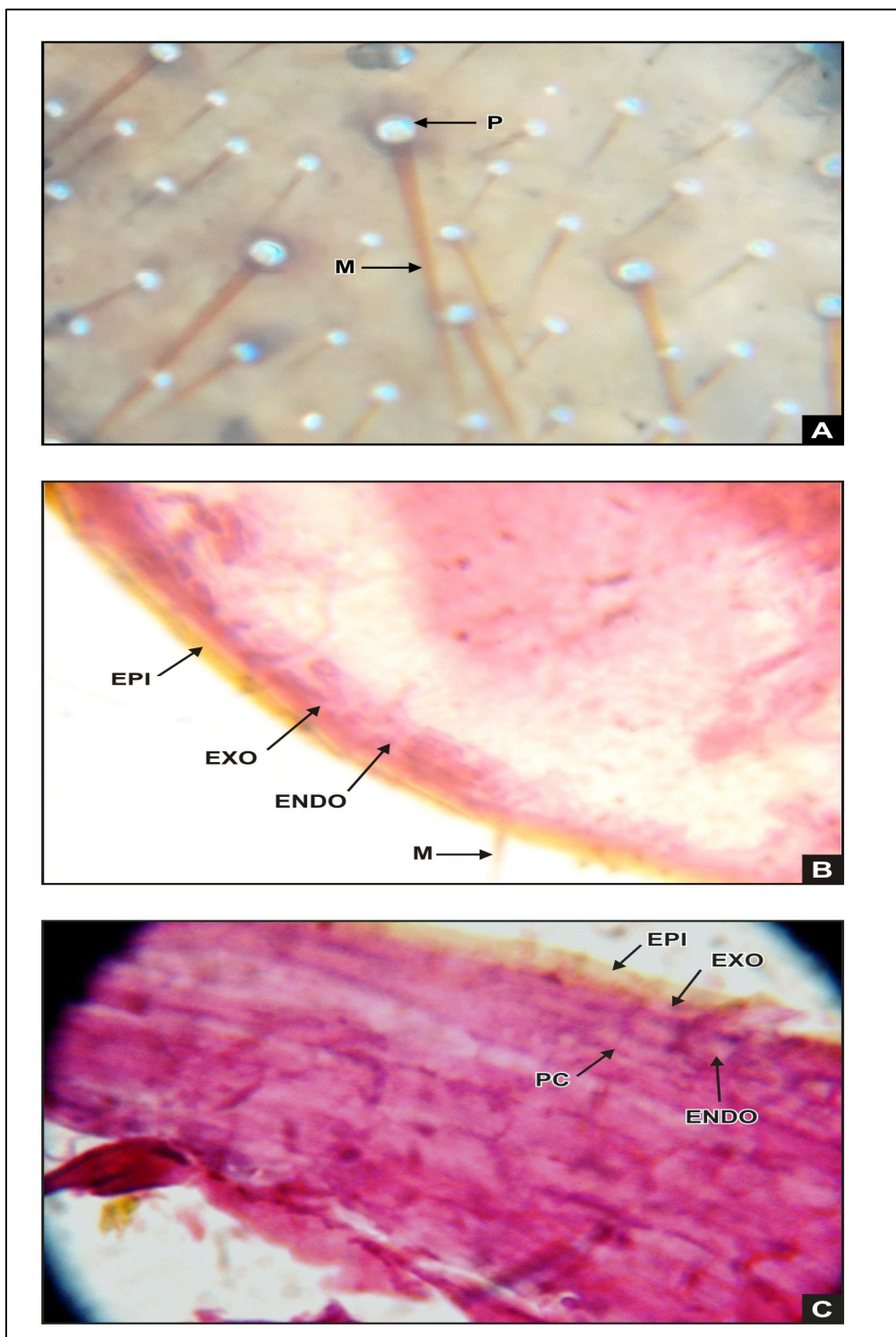


Fig: 1 Histoarchitecture of worker of *Odontotermes obesus* (R.) showing cuticular layers and their arrangements:

- (A) Magnified view of body wall showing microtrichia (M), Pit (P)
- (B) T.S. of cuticle showing different layers Epicuticle (EPI), Exocuticle (EXO), Endocuticle (ENDO), Epidermis (EPD) and Microtrichia (M)
- (C) Further magnification of a portion of cuticle showing different layers in detail. Epicuticle (EPI), Exocuticle (EXO), Endocuticle (ENDO), Epidermis (EPD), and Pore Canals (PC)

The characteristic constituent of the cuticle is chitin, but the exocuticle distinguish from the endocuticle by dark pigmentation and denser structure. The epicuticle is a thin nonchitinous layer covering the other two, which together form the procuticle. Endocuticle has a horizontal lamellate structure, in which fine vertical striations were observed.

Several epidermal cells, arranged in a single layer, are visualized beneath the cuticle. All the epidermal cells are glandular and secrete cuticle and the enzymes involved in production and digestion of old cuticle during moulting.

The thin basement membrane that forms the inner lining of the body wall is closely adherent to the epidermis. It is the basal parts of the body wall formed from degenerated epidermal cells and appear as non-living amorphous (shapeless) granular layer of integument. The basement membrane forms a continuous sheet beneath the epidermis, where muscles are attached and become continuous with sarcolemma of the muscles.

Conidial adhesion penetration and germination through the insect cuticle (Fig: 2)

The analysis of the colonized termites provided an overview of the infection, showing that the hydrophobic conidia were able to adhere all over the external surface of body wall with a preference for surfaces containing spines. There were dense depositions of large number of conidia all over the external surface of the body. Fungal conidia were aggregated at the basal regions of microtrichia. The microtrichia are structural barriers on the insect body generally secreted by underlying epidermal cells. The attack on microtrichia in our studies showed that a mechano receptor property of the skin was hampered that might lead to poor articulate. The conidia of *Beauveria bassiana* germinated on the cuticle and the germ-tube penetrated into the cuticle of the termite. The appressorium was formed by elongated germ tube at the point of fungal entry in the cuticle. This appressorium truly attached the fungus firmly to the cuticle while the germ tube penetrated into the integument. The strong cuticle lost its mechanical strength and all three cuticular layers i.e. epicuticle, exocuticle and endocuticle lost their identities and became fragile. The epidermis and cuticle of infected termite worker was separated. Hyphae cutting the cuticle and growing laterally within the cuticle were also observed. The hyphae continue their development actively invading the successive procuticle layers, the hemocoel and adjacent tissues. Numerous hyphae can be seen in the adjacent tissues of the host cuticle. After the fungus had penetrated the integument, the hyphae began to ramify inside the termite. A prominent layer of closely packed conidiophores with their intertwining branches was formed around the termite.

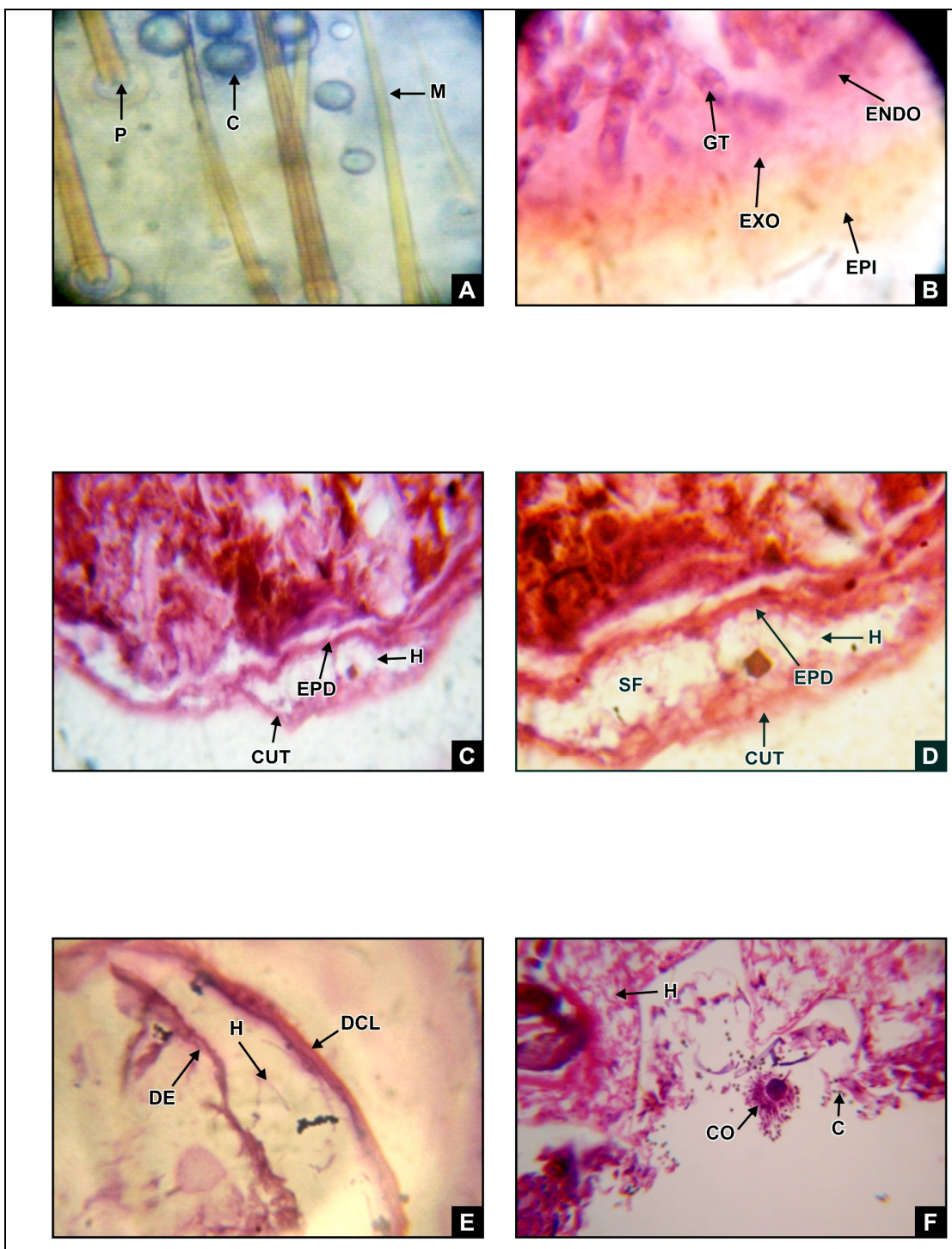


Fig 2: Histoarchitecture of worker of *Odontotermes obesus* (R.) treated with *Beauveria bassiana* (Balsamo) Vuillemin showing changes in the cuticular layer

- (A) Conidia (C) of fungus adhere to the body wall, Pit (P), Microtrichia (M) and Conidia (C)
- (B) T.S. of cuticle showing establishment of mycelia of fungus in between Exocuticle (EXO) and Endocuticle (ENDO), Fungal invasion and Germ tube (GT) penetrate and grown in cuticular layer
- (C) T.S. of cuticle showing Hyphae (H) penetrating between the Cuticle (CUT) and Epidermis (EPD) growing laterally within the cuticle

(D) Magnified view of T.S. of treated worker cuticle showing Cuticle (CUT) lose its entire architecture with Space formation (SF), growth of Hyphae(H) between Cuticle (CUT) and Epidermis (EPD) separating the layer.

(E) T.S. of body wall of treated worker showing degenerate Cuticular layers (DCL), Hyphae (H) and Degenerated epidermis (DE)

(F) Conidiophore (CO), Conidia (C) formation on the degenerate cuticle of cadaver and Hyphae (H) are apparent.

DISCUSSION

Chemical pesticides have been the practical method used by growers for the control of economically important pests for many decades, but their effects on non-target organisms, groundwater contamination, residues on food crops and the development of insect resistance to chemicals have forced the industry and scientists to focus on the development of alternative control measures. Alternative methods including biological control with reference to entomopathogenic fungi in the genera of *Metarhizium*, have been sought. Control of insect pests, particularly by their natural enemies comprising parasitoids, predators and pathogens in agro-ecosystems, is a continuous process. In the search for new avenues in biological control, the importance of entomopathogens has been highlighted as an environmentally friendly pest control method.

The insect's outer integument is composed of a layer of epidermal cells overlies with the procuticle. This consists of protein and chitin⁹ which is a polymer of N-acetyl glucosamine. Over this lies the epicuticle, which amongst other materials typically contains wax.

It is difficult to introduce conidia into the body and gut without contaminating the cuticle. The only real proof that conidia attacks from outside is actually to observe penetration by entomopathogenic fungus in cuticle by histological study.

The invasion of fungal conidia by adhering on the cuticular surface, entering inside and germinating into, the epidermal layer thereby destroying whole body tissues and again profusely germinating on the insect's cuticle i.e. re-infection and it is the specific characteristic feature of microbial pathogens.

In the cuticular section we observed the exact mode of action of *Beauveria bassiana* (Balsamo) Vuill. against the worker of *Odontotermes obesus* (R.) treated topically with 4.5×10^8 conidia /ml concentration. When the cuticle was studied under low (x10) and high (x40 and x 100) magnifications, the cuticular layers depicted severe damage.

The mechanical strength of cuticular layers became reduced due to its weak and fragile nature. The epicuticle became thin, exocuticle and endocuticle detached from epidermis having space in between. This space formation further made cuticle soft and fragile^{10,11}.

We also observed that conidia were aggregated at the basal regions of microtrichia. The microtrichia are structural barriers on the insects' body generally secreted by underlying epidermal cells. The attack on microtrichia in our studies showed that mechano-receptor properties of the skin were hampered that might lead to poor articulate. The hydrophobic conidia of fungal species were attached themselves to all body regions, with a preference for the surfaces containing hairs, as was also reported by ¹². *Beauveria bassiana* (Balsamo) Vuill. conidia were found especially to be trapped by and tightly bound to microtrachia ¹³.

The virulence of fungal entomopathogens involves four steps: adhesion, germination, differentiation and penetration. Each step is influenced by a range of integrated intrinsic and external factors, which ultimately determine the pathogenicity. A successful infection is achieved by the attachment or adhesion of spores to the host. Adhesion is necessary and normally achieved through the secretion of mucilage ¹⁴. A wide range of factors such as water, ions, fatty acids and nutrients on the cuticle surface and the physiological state of the host influence spore germination and behavior ¹⁵. Successful germination requires the assimilation of utilizable nutrients and a tolerance to any toxic compound present on the surface ¹⁶.

After germination, appresoria appeared at the end of short germ tubes, subterminally or on the side branches. Asexually produced fungal spores or conidia are generally responsible for infection and are dispersed throughout the environment in which the insect hosts are present.

The conidia started germinating, germ tubes were apparent evolving through body wall. The body wall and muscular tissues inside epidermis, all were meshed up with no identities. Germ tubes were clearly seen entering and establishing hyphae were prominent inside the body cavity.

When conidia attach on the cuticle of a host, it germinate, initiating cascades of recognition and enzyme activation reactions, by both the host and the fungal parasite. Invasion of the insects body and circulatory system (hemolymph) occurs once the fungus has passed through the cuticle of the external insect skeleton ¹⁷. Structures and processes for the invasion of insect tissues are including the formation of germ tubes, appresoria and penetration pegs ¹⁸.

Colonization of the dead insect concluded in the formation of a compact, hard, mass of mycelia within the integument. After 1 to 4 days of the insect's death hyphae emerged through weaker parts of the integuments such as articulating membranes, intersegmented space.

Entomopathogenic fungus are exceptional in their pathogenesis, where the infection of the insect commences by the attachment of the conidia on the cuticle and subsequent development of the infectious structures called appresoria, which releases an array of cuticle solubilizing enzymes ¹⁹.

Chouvenc and Nan-Yao²⁰ observed that when termites were left for decomposition several days after death caused by an external infection of *M. anisopliae*, the hyphal growth was generalized in the body cavity of the cadaver and observed thousands of conidia in the termite's gut.

Shortly after host death, the fungal hyphae penetrate the cuticle from within and terminate in the formation of sporophores (usually conidiophores) that yield asexual spores (conidia) which function as dispersive and infective units²¹.

In many species of fungi, the production of conidia is highly dependent on moisture²². Conidia are the infective propagates of secondary infection, determining disease development and spreading within a season. Proteins are major components of insect cuticle and a recyclable resource for the insect. Therefore, both insects and entomopathogenic fungi produce a variety of cuticle-degrading proteases²³. A number of cuticle-degrading enzymes are produced during penetration of the host, including proteases, lipases and chitinases²⁴. Proteases have been shown to play a key role in the penetration process and a wide range has been identified, including trypsin, chymotrypsin, elastases, collagenase and chymoelastase^{25,26}.

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