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RESEARCH ARTICLE

Variation in Mycosis of Entomopathogenic Fungi on Mealybug, *Paracoccus marginatus* (Homoptera: Pseudococcidae)

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Abstract External symptoms of mycosis, chronological developments and duration of mycosis by entomopathogenic fungi, *Beauveria bassiana, Verticillium lecanii* and *Metarhizium anisopliae* on mealybug, *Paracoccus marginatus* was studied at in vitro condition. Variations were observed in timing and duration of the different phases of mycosis. The mycosis cycle of entomopathogenic fungi was faster in *M. anisopliae* and *B. bassiana* treated *P. marginatus* but it was slower in *V. lecanii*. The *M. anisopliae* killed the insect faster than *B. bassiana* and *V. lecanii*. There were no variations observed in conidiogenesis phase in all the three entomopathogenic fungi. Disintegrated cuticle structure was observed at the end of the mycosis cycle.

Keywords Entomopathogenic fungi · *P. marginatus* · External mycosis

Introduction

In India, cotton cultivation is faced with twin problems of inflated pesticide bill and development of pest resistance. Widespread incidence of mealybug in almost all cotton growing regions in India was reported [1]. About 5000 species of mealybugs have been recorded and 56 species have been reported to feed on 15 genera of family, including cotton and many other plants of economic importance [2]. The mealybug, *Paracoccus marginatus* (Williams and Granara de Willink) (Homoptera: Pseudococcidae) is a soft bodied sap sucking insect pest. It recently attained the status of a serious pest in the cotton growing areas in India. Outbreak of mealy bugs on agricultural and horticultural crops caused severe damage and huge loss to economically important crops [3, 4].

Detrimental consequences of insecticides for the pest management are numerous and include concerns over workers, food safety, environmental contamination and declining biodiversity in agro-ecosystem, development of insecticide resistance, resurgence of sucking pests, deposition of residues in/on food and environment and ecological hazards. Hence, there is an urgent need to reduce pesticide usage while meeting human needs for food and good quality fiber. It paves way for the development of cost effective alternatives to conventional chemical pesticides [5]. Moreover, mealybugs are difficult to control with insecticides due to their cryptic nature, waxy-coat around the body and life-style of forming dense colonies of multiple and overlapping generations [6]. Microbial insecticides such as entomopathogenic fungi can provide the best alternative because it is more environment friendly option to control this insect pest. More than 750 species of fungi are pathogenic to insects and many of them offer great potential for the management of sucking pests [7].

In microbial pest management studies the duration of the different phases of mycosis development on insects are relatively scarce. It is particularly important because it provides the basic information about entomopathogenic fungal mycosis. Hence, the present study was carried out with the objective to study the external mycosis of entomopathogenic fungi, *Beauveria bassiana*, (Bals.) *Verticillium lecanii* (Zimmerman) and *Metarhizium anisopliae* (Metsch.) Sorokin. on *P. marginatus*. In addition to this

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duration of the different phases of mycosis of entomopathogenic fungi on mealy bug was also studied.

The present study describes the infection process of the entomopathogenic fungi, B. bassiana, V. lecanii and M. anisopliae on mealybug, P. marginatus. These studies were used to describe the chronological events leading to complete host invasion by the entomopathogenic fungi. These events include (i) adherence of conidia to the host cuticle (ii) germination of the conidia (iii) production of either penetrant hyphae or germ tubes that colonize the surface of the cuticle (iv) disappearance of waxy mealy coating on the integument and penetration of germ tubes due to action of cuticle degrading enzymes (v) extensive lateral colonisation of hyphae on the insect procuticle and (vi) re-emergence or production of conidiophores through cadavers integument.

Material and Methods

Source of Fungus

The present study was conducted at the ICAR-Central Institute for Cotton Research (CICR), Regional station, Coimbatore, Tamil Nadu, India. Homogeneous culture of P. marginatus was maintained in the glass house used for this experiment. The entomopathogenic fungi isolates viz., B. bassiana, V. lecanii and M. anisopliae were obtained from ICAR-National Bureau of Agricultural Important Insects (NBAIR), Bengaluru, India. The fungal isolates were maintained on Saboured Dextrose Agar Yeast (SDAY) medium for 10 days at 25 °C before inoculation to P. marginatus.

Preparation of Conidial Suspensions

Fungal inoculum was prepared from the conidia harvested from the three week old cultures by scraping the surface of the culture plate that was flooded with distilled water containing 0.02 % Tween 80® as surfactant. A Neubauer haemocytometer was used to estimate the conidial concentration and the resulting suspension was standardised to 1×10^8 conidia ml^{-1}. A standard dose of 1×10^8 conidia ml^{-1} of M. anisopliae, B. bassiana and V. lecanii in 0.02 % Tween 80® was prepared.

Inoculation of P. marginatus with Entomopathogenic Fungi

A sample of thirty P. marginatus adults were surface sterilized with 0.1 % sodium hypochlorite solution and inoculated by immersing them in 10 ml of conidial suspension of entomopathogenic fungi for 10 s. For untreated

check, insects were immersed in the 0.02 % Tween 80® [8]. The treated insects were carefully transferred to petridishes with cotton leaves moistened with filter paper to maintain the turgidity. The petridishes were incubated for 24 h in a moist chamber and were monitored for hyphal emergence; mycelia from six randomly selected cadavers were sampled and cultured on SDAY plates for confirming the identity. External symptoms of mycosis were observed at 24, 48, 72, 96, 120, 144 and 168 h after inoculation. Pathological changes in each sample were observed under a microscope and photographs were documented. Variations in duration of different phases of mycosis were compared.

Results and Discussion

Mycosis of B. bassiana on P. marginatus

The result from the Fig. 1 revealed that, conidial adhesion and initiation of germination of spores were observed at 24 h of inoculation (Fig. 1A). Penetration was observed at 48–96 h. At this stage, the germ tube formed the hole at the point of penetration on the integument of P. marginatus and also disappearance of waxy mealy coating of cuticle was recorded (Fig. 1B, C). This might be due to enzymatic action of entomopathogens as reported [9–17].

Colonization was noticed at 72–120 h after inoculation. During the colonization, hyphae penetrated and invaded the cuticle both by mechanical and enzymatic action. At 96–144 h after inoculation insect mortality, oozing out of fluids from cadavers and mummification were observed with the haemocoel completely filled with fungal growth (Fig. 1D, E, F). Conidiogenesis and hyphal re-emergence were recorded at 120–168 h after inoculation. At this stage of mycosis white cottony mealy mould layer 'white bloom' was observed on integument of P. marginatus (Fig. 1G, H).

Successful infection and germination of conidia depend primarily on the virulence of fungi to adhere and penetrate the host integument [9, 10, 17–20]. EI-Sinary [21] and Quesada-Moraga [22] explained that the effectiveness of the entomopathogenic fungi began clearly after 48 h after inoculation and the hyphae penetrated the integument inside the trachea and the epithelial and epidermal cells. After 72 h the fat tissues were damaged and lethality was reached to 100 % after 96 h. These findings are in conformity with Ramlee et al. [23] who stated that, after inoculation with B. bassiana, the hyphae penetrated the integument inside the whole body cavity to reach the fat, neural and muscle tissues and damaged them. Also it reached to malpighian tubule, epithelial cells and finally colonized the gut lumen. By the time the infected insects were already dead.

Fig. 1 Symptoms of external mycosis of *B. bassiana* on *P. marginatus*. **A** Adhesion and germination of conidia on the integument of *P. marginatus* at 12–24 h after inoculation. **B** Penetration of conidia at 48–72 h after inoculation. The germ tube creates a hole at the point of penetration. **C** Disappearance of mealy coating of cuticle due to enzymatic action at 48–72 h. **D**, **E**, **F** At 96–120 h after inoculation, death, oozing out of fluids from cadavers and mummification. **G**, **H** Hyphal re-emergence and conidiogenesis at 120 h and it extended up to 168 h after inoculation—cadaver is covered by a white cottony mealy mold layer
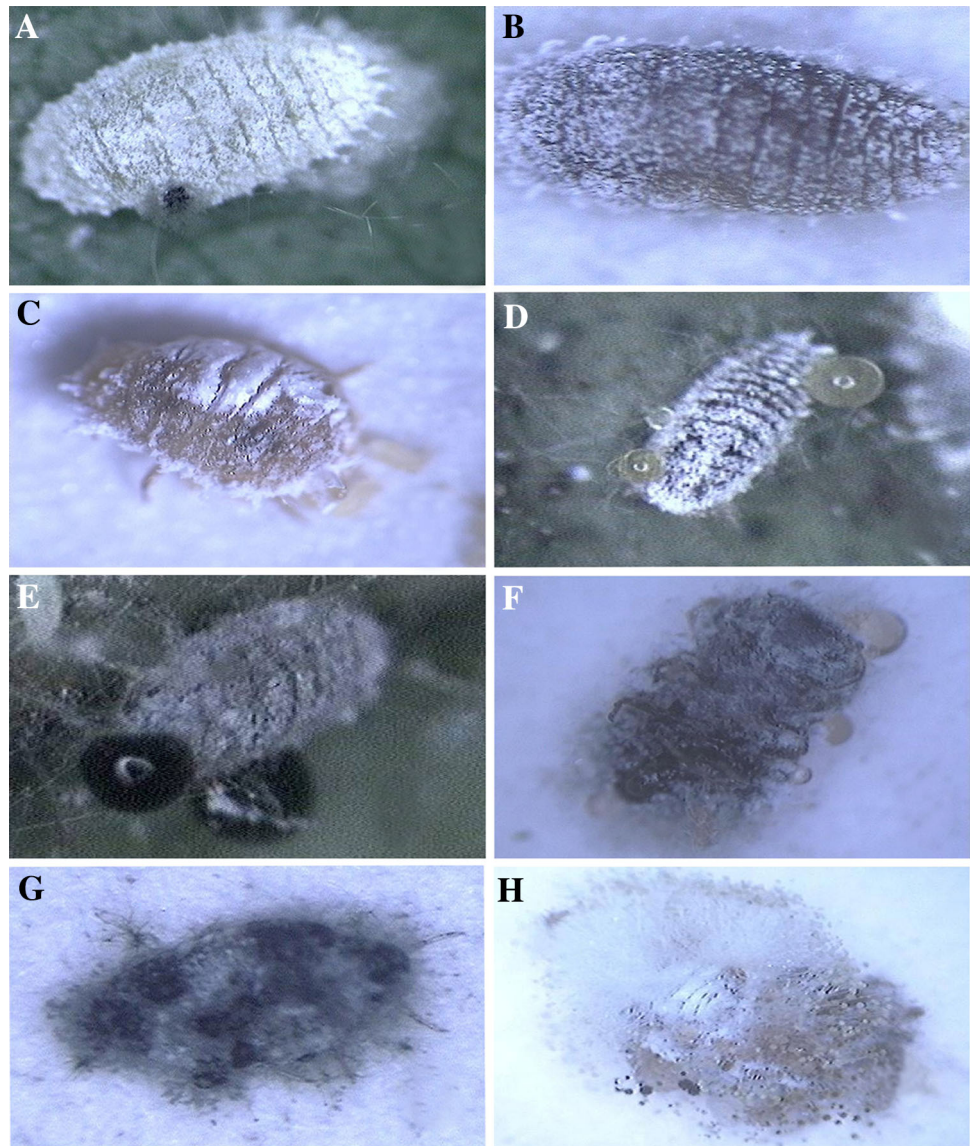
The penetration of entomopathogenic fungi through the cuticle is some times preceded by the formation of an appressorium, providing the fulcrum for the mechanical and enzymatic processes that mediate penetration [24]. Appressoria characterized by a thickening of the extremity of the germ-tubes, probably due to the translocation of the conidial cytoplasmatic contents facilitates the enzymatic synthesis necessary for the penetration phase [25].

Mycosis of *V. lecanii* on *P. marginatus*

The mycosis on *P. marginatus* by *V. lecanii* followed a sequence of events ranging from the exposure of the host to conidia to the release of conidiophores from cadavers is studied. The adhesion and germination of conidia on the integument was prolonged up to 72 h (Fig. 2A). Penetration of germ tube was started at 48 h and prolonged up to 120 h

after inoculation. At this stage, disappearance of waxy mealy coating was observed (Fig. 2B, C). Only in *V. lecanii* the colonization was prolonged up to 144 h. This indicated the slow rate of colonization. During colonization oozing of fluids from cadavers was noticed (Fig. 2D, E). Insect mortality was recorded at 96 h and it was prolonged up to 168 h. Conidiogenesis was recorded at 120–168 h (Fig. 2F, G). Hyphae emergence showed white to yellowish cottony appearance on the cadavers at 144–168 h (Fig. 2H). Fungi are constrained by the complex structure of the insect cuticle and typically, a variety of extracellular enzymes are involved in the degradation of proteins, chitin and lipids [10, 15, 26]. Furthermore, once the host hemocoel has been invaded, a number of other determinants such as the fungal capacity to fend off the host defence reactions and feed on the host tissues could also affect the efficacy of the pathogen [27]. In agreement with earlier observations by Schreiter et al. [28],

but atypical of other entomophagous hyphomycete fungi, *V. lecanii* hyphae extensively colonized the insect cuticle prior and concomitant with host penetration and infection. These findings are in agreement with the present study.

Mycosis of *M. anisopliae* on *P. marginatus*

The results of external mycosis of *M. anisopliae* revealed that the conidial adhesion and germination was noticed at 24 h (Fig. 3A) and due to enzymatic action mealy coating got disappeared (Fig. 3B). Penetration of germ tube started simultaneously during adhesion and germination process. It was recorded up to 96 h after inoculation (Fig. 3C). The penetration site was random, indicating that *M. anisopliae* does not require specific orientation. The phase of host colonization occurred between 72 and 120 h, and most of the insects died between 72 and 144 h after inoculation. Oozing out of the fluid from cadavers and mummification was observed (Fig. 3D).

Interestingly the *M. anisopliae* killed the insect faster than the *B. bassiana* and *V. lecanii*. It might be due to faster rate of penetration and colonization. Conidiogenesis was observed between 120 and 168 h after inoculation. Initial points of re-emergence were noticed through the membranous intersegmental regions (Fig. 3E, F). Sporulation started soon thereafter. Subsequently, cadavers were completely covered by greenish conidia (Fig. 3G, H). Observation of the development of *M. anisopliae* on termite, *Heterotermes tenuis* revealed many similarities with the events reported in the present study [29].

Fig. 2 Symptoms of external mycosis of *V. lecanii* on *P. marginatus*. **A** Adhesion and germination of conidia on the integument at 24 h after inoculation. **B**, **C** Disappearance of mealy coating due to enzymatic action at 48–72 h. **D**, **E** Oozing of fluids from cadavers at 72–96 h after inoculation. **F**, **G** Conidiogenesis at 120–168 h after inoculation. **H** Hyphae re-emergence as white to yellowish cottony appearance at 148–168 h
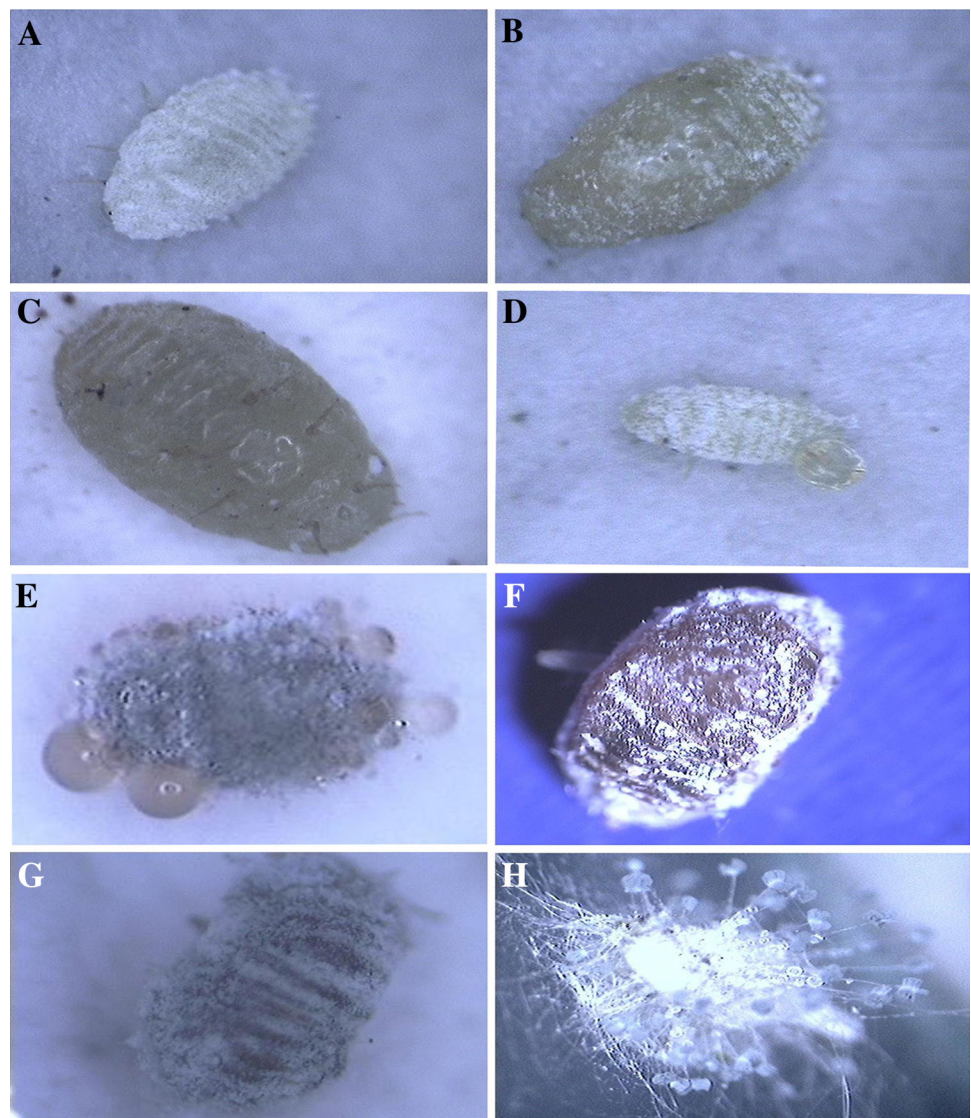
Fig. 3 Symptoms of external mycosis of *M. anisopliae* on *P. marginatus*. **A** Adhesion and germination of conidia on the integument of *P. marginatus* at 24 h after inoculation. **B** Mealy coating was disappeared on integument due to enzymatic action. **C** Penetration by the germ tubes at 24–72 h after inoculation on different regions of cuticle. **D** Oozing out of fluids from cadavers and mummification. **E**, **F** Re-emergence of hyphae through the membranous intersegmental region. **G**, **H** The hyphae emerged from the cuticle of the dead adult and grew all over the body forming greenish mycelial mat and conidia
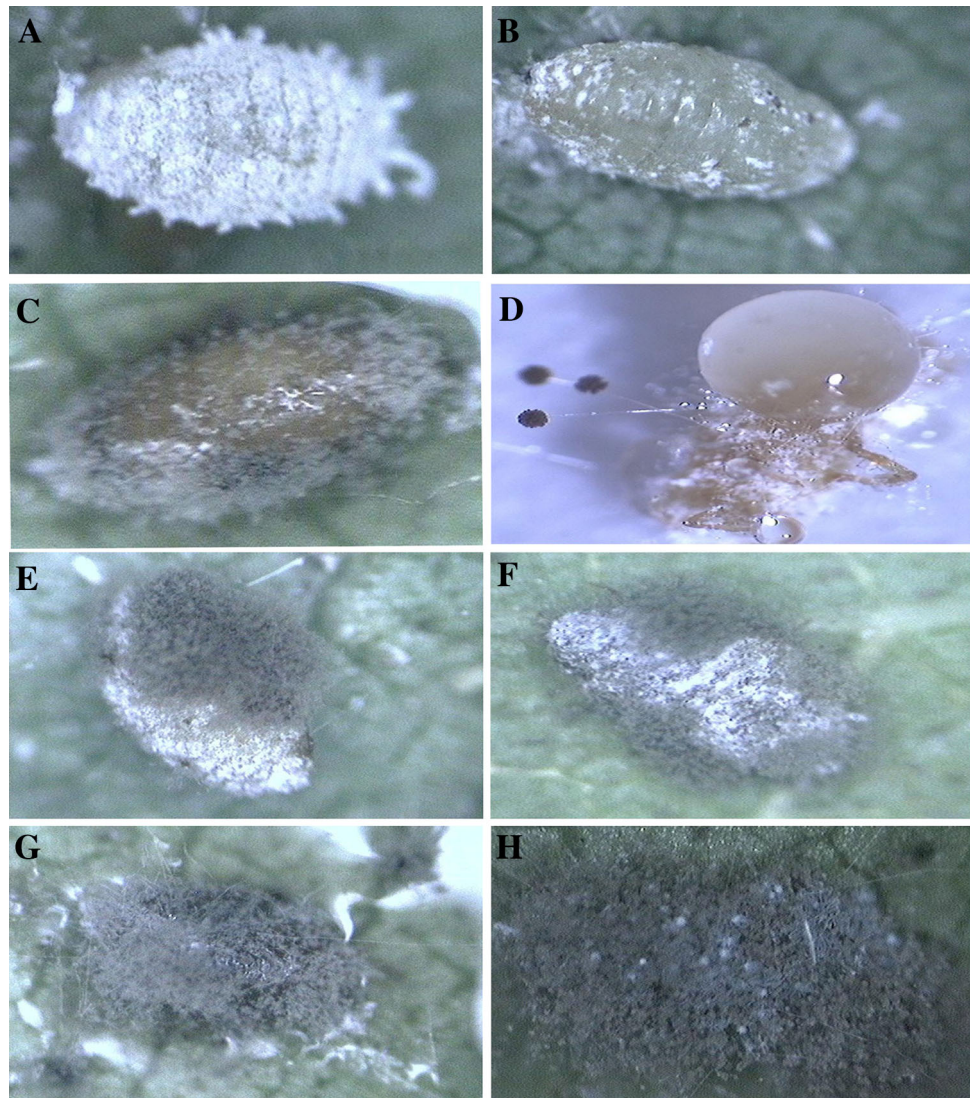
Comparison of Variations in Mycosis of Entomopathogenic Fungi on *P. marginatus*

Variations were observed in timing and duration of the different phases of infection cycle of entomopathogenic fungi (Fig. 4). In case of *B. bassiana* and *M. anisopliea*, conidial adhesion on the integument was observed immediately after inoculation and conidial germination was observed at 24 h after inoculation whereas, in *V. lecanii* conidial germination was prolonged up to 72 h due to slow rate of germination.

The penetration of hyphae of *B. bassiana* on the host integument was noticed during 48–96 h, whereas in *M. anisopliae* the penetration started from 24 h onwards. In *V. lecanii* penetration of hyphae on integument of *P. marginatus* was at 48 h and was extended up to 120 h after inoculation. *V. lecanii* differed in the colonization process also. The colonization started at 72 h and prolonged up to
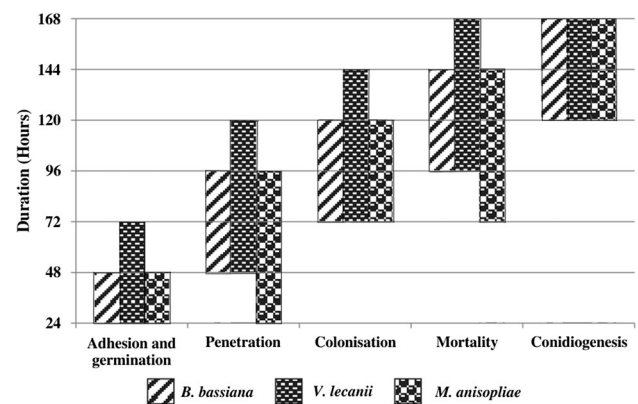

Fig. 4 Phases of mycosis by entomopathogenic fungi on *P. marginatus*

144 h. This indicates the slow rate of colonization, whereas, in *B. bassiana* and *M. anisopliae*, colonization occurred during 72–120 h. The mortality of *P. marginatus*

due to *M. anisopliae* infection, observed at 72 h and it was extended up to 144 h. The insect mortality due to *V. lecanii* infection was recorded after 96 h and it were prolonged up to 168 h. In *B. bassiana* it ranged from 96 to 144 h (Fig. 4).

Conidiogenesis was recorded between 120 and 168 h after inoculation in all the three entomopathogenic fungi. The mycelial extrusion was more intense for *M. anisopliae* than for *B. bassiana*. Faster rate of insect mortality was observed in *M. anisopliae* than *B. bassiana* and *V. lecanii*, it might be due to more mycelial extrusion, faster penetration and colonization. Towards the end of pathogenesis, no evidence of cuticular structure could be seen because of disintegration of cuticular membrane and the muscular tissues surrounding the hyphae were under lysis due to enzymatic action.

Future Perspectives

Major gaps exist in the knowledge on the mechanisms underlying parasitic fungal host specificity and molecular and biochemical determinants related to fungal host specificity. Efforts should also be directed towards better understanding of the role, physical properties, and biochemical composition of the mucilaginous matrix at the interface between entomopathogens and the host. An important component of future studies should also be devoted to the role of phylogenetic and ecological constraints in the expression of host range by parasitic fungi.

Conclusion

The possible mechanism of pathogenicity of entomopathogenic fungi on *P. marginatus* could involve invasion of penetrating hyphae through cuticle and reach haemocoelic tissues and/or organs leading to physiological malfunctioning of vital organs and result in fatality by the entomopathogenic fungi during mycosis. Variations in infection cycle of entomopathogenic fungi on *P. marginatus* with respect to timing and duration of different phases were also recorded. The characteristics of mycelial extrusion, faster penetration and colonization of entomopathogens decide the efficacy of the pathogen. The present study demonstrated the importance of understanding of these phases in selecting suitable isolates for biological control of insects.

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