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Research Article

Pathogenesis of entomopathogenic fungus, *Metarhizium anisopliae* (Metsch.) Sorokin., on mealybug, *Paracoccus marginatus* (Williams and Granara de Willink) (Homoptera: Pseudococcidae)

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ABSTRACT: A study was conducted to investigate the basis and mode of infection of the entomopathogenic green muscardine fungus, *Metarhizium anisopliae* on the mealybug, *Paracoccus marginatus.* The pathogenesis of *M. anisopliae* on *P. marginatus* was studied at 24, 48, 72, 96, 120, 144 and 168 hours after inoculation. The conidial adhesion and germination process of *M. anisopliae* occurred within 24 hours after inoculation. The hyphae penetrated the epicuticle and reached the endocuticle within 48 to 72 hours after inoculation. Lysis of the endocuticle occurred while the penetrant hyphae invaded into the epidermis. Invasion and colonization of hyphal bodies into the haemocoel of *P. marginatus* was observed at 72 to 120 hours after inoculation. By 120 to 144 hours after inoculation, there was considerable abundance of hyphae that extensively colonized on the host and complete invasion occurred at 168 hours after inoculation. At this stage, the larvae became moribund and died. Hyphae re-emerged out of the cuticle after 168 hours after inoculation, and grew all over the surface forming a green mycelial mat. The developmental cycle of *M. anisopliae* on *P. marginatus* took 172 to 196 hours to disintegrate and kill the insect from the day of inoculation.

KEY WORDS: Histopathology, *Metarhizium anisopliae*, mealybug, *Paracoccus marginatus*, pathogenicity

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INTRODUCTION

The mealybug, *Paracoccus marginatus* (Williams and Granara de Willink) (Homoptera: Pseudococcidae) is a soft bodied sap sucking and a polyphagous pest attacking many field and horticultural crops. Persistent increase in the population of mealybugs, has threatened the cultivation of many crops including cotton (Arif *et al*., 2009; Dhawan *et al*., 2009). Both the nymphs and adults of *P. marginatus* suck sap from leaves, stems, bolls and twigs. Severe infestation leads to stunted growth and drying. Mealybugs are difficult to control with insecticides due to their cryptic nature, waxy-coat and life-style of forming dense colonies of multiple and overlapping generations (Blumberg and Van Driesche, 2001). Moreover, indiscriminate use of insecticide leads to development of insecticide resistance, resurgence of sucking pests, deposition of residues in/on food and environment and cause ecological hazards.

Considering the adverse effect of insecticides, the use of entomopathogenic fungi as biological control agents, is a viable alternative to conventional insecticides. More than 750 species of fungi are pathogenic to insects and many of them offer great potential for the management of sucking pests (Rabindra and Ramanujam, 2007). The entomopathogenic green muscardine fungus, *Metarhizium anisopliae* is a well known, broad-range entomopathogen, which has been evaluated for the biological control of several insect pests (Toledo *et al*., 2008).

In the present study, adults of *P. marginatus* were infected with *M. anisopliae* and studied under microscope to determine the histopathology and development of *M. anisopliae*, before and after death of the host. Ultra-structural observations were used to describe the chronological events leading to complete invasion by *M. anisopliae* on mealybug. This work provides the basis for further understanding on the mode of action of *M. anisopliae.*

MATERIALS AND METHODS

Source of fungus

The study was conducted at the ICAR-Central Institute for Cotton Research (CICR), Regional station, Coimbatore, Tamil Nadu, India during 2010-2011. The fungal isolate utilized in this experiment were obtained from ICAR-National Bureau of Agriculturally Important Insects, Bangalore, India. The fungal isolate was maintained on Saboured Dextrose Agar Yeast (SDAY) medium for 10 days at 25±1ºC in darkness before being used to inoculate on the *P. marginatus* adults. Fungal inoculum was prepared from conidia harvested from ten days old cultures by scraping the surface of culture plates and flooded with distilled water containing 0.05% Tween 80® as surfactant. A Neubauer haemocytometer was used to estimate the conidial concentration and the resulting suspension was standardized to 1x 10^8 conidia ml^{-1}.

Inoculation of *Paracoccus marginatus* with *Metarhizium anisopliae*

A sample of hundred mealybug adults which were reared under laboratory condition were surface sterilized with 0.1% sodium hypochlorite solution, inoculated by spraying conidial suspension with a Sigma® hand atomizer and released on cotton leaves with stalks wrapped at the bottom with wet cotton and filter paper moistened with sterile distilled water and covered in aluminium foil. In the untreated check, mealybugs were sprayed with 0.05 per cent Tween 80® only. After inoculation, the cotton leaves containing insects were incubated at 24 ±1ºC and at high relative humidity (100%) in petridishes (90mm dia.) with a photoperiod of 14:10 hr (L:D).

Histopathological studies

In order to study the histopathology of *M. anisopliae* on *P. marginatus,* ten adults of *P. marginatus* were transferred from the treated leaves at successive intervals *i.e*. 24, 48, 72, 96, 120, 144 and 168 hours after inoculation. These adults were then fixed in aqueous Bouin's solution (Saturated picric acid 75ml, Formaldehyde 40%-20ml and Glacial acetic acid-5ml) at room temperature for 24 hours after which they were rinsed three times with distilled water to remove the fixative. The insects were dehydrated in a graded series of ethanol, cleared in methyl benzoate containing 2% celloidin for three days followed by benzene three times for 10 min each. Infiltration was done by immersing the samples in two changes of molten paraffin wax for 30 min each and subsequently left overnight in the oven (60°C). Embedding of the infected adult in molten paraffin wax was accomplished when a solid bottom layer had formed in the paper mould (1.5 cm^3). The embedded insects were left at room temperature for four days before preparing sections. The processed insects were sectioned to 10µm using a rotary microtome (Reichert-Jung model 2030), spread on glass slides, evenly smeared with Mayer's albumin solution and then left to dry (40°C) overnight. Embedding materials from the sections were extracted with xylene, dehydrated in absolute ethanol and air-dried then attached to the aluminium stub and observed under a light microscope at 100x magnification as per the methodology of Askary *et al.,* (1998).

RESULTS AND DISCUSSION

Conidial adhesion, germination and sequence of cuticular penetration

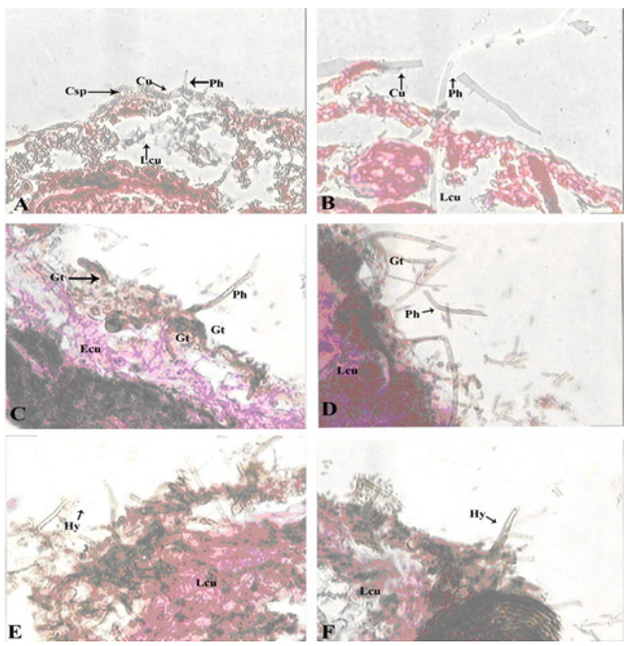

Fig. 1. Infection process of *Metarhizium anisopliae* on the cuticle of *Paracoccus marginatus.* A) Conidiophores (Csp) adhering to the host cuticle (Cu) and lysis of endo cuticle (Lcu) at 24 to 48 hours after inoculation. B) A penetrant hypahe (Ph) entering in to the cuticle (Cu) and Lysis of the endocuticle (Lcu) of mealybug at 48 to 72 hours after inoculation. C) Germ tube (Gt) formation and Lysis of the endocuticle (Lcu) at 48 to 72 hours after inoculation. D, E & F) Colonisation, Lysis of the endocuticle (Lcu) and Hyphae (Hy) developed.

The histopathology of *M. anisopliae* in *P. marginatus* was studied, in comparison with healthy insect. The conidial adhesion and germination process of *M. anisopliae* occurred within 24 hours after inoculation (Fig. 1A). Penetration of hyphae through the insect cuticle was first observed within 48 hours to 72 hours after inoculation. The hyphae penetrated directly into the epicuticle and reached

the endocuticle (Fig. 1B). Some germ tubes were grew on the surface without penetrating the cuticle, but most of the hyphae penetrated the cuticle close to the conidium. It occurred with or without appressorium formation. Lysis of the endocuticle occurred while the penetrant hyphae invaded into the epidermis (Fig. 1A and 1B). Penetration by the germ tubes was observed at 48 to 72 hours after inoculation (Fig. 1 C) on different regions of the cuticle. Intersegmental penetration was not restricted to any particular site on the insect but, especially common at the thinner intersegmental areas of the body wall. Areas surrounding the point of entry were darkened after penetration indicating lysis presumably due to enzymatic action (Fig. 1D).

The direct enzymatic activity could be playing the initial role prior to fungal invasion followed by mechanical pressure as was first suggested by Zacharuk (1971) in elaterid larva. The lipolytic enzyme, chitinase, could dissolve the chitin layer of the insect cuticle and thus might opened the way for penetration (Samsinakova *et al.,* 1971). The detection of endocuticular cell lysis due to enzymatic action prior to the invasion of hyphae into the epidermis was described by Mohamed *et al*., (1978). Infection by *B. bassiana* has been shown to require direct penetration into insect host integument by growing hyphae, apparently facilitated by both mechanical and enzymatic activity. Several studies have shown that a spectrum of enzymatic activities on the insect cuticle occurred in conidial preparations of *B. bassiana* (St Leger *et al*., 1986; 1987). The disappearance of the wax layer beneath appressoria of *M. anisopliae* on the wire worm cuticle indicates enzymatic activity (Zacharuk, 1981). Potentially, the primary function of many of the enzymes associated with conidia, including those of *B. bassiana*, is to hydrolyze the epicuticular wax layer and provide nutrients required for germ tube formation.

Colonization of *Metarhizium anisopliae* in internal tissues of *Paracoccus marginatus*

By 120 to 144 hours after inoculation, there was considerable increase in abundance of hyphae, which penetrated the epidermis. *M. anisopliae* hyphae extensively colonized on the insect cuticle prior to and concomitant with host penetration and infection. Invasion and colonization of hyphal bodies into the haemocoel was observed at 72 to 120 hours after inoculation (Fig. 1D, 1E & 1F). At 120 hours after inoculation, *M. anisopliae* colonised the entire haemocoel of *P. marginatus*. Most insects (approximately 80 per cent) died between 96 hours and 120 hours after inoculation. Hyphal penetration into the fat bodies, alimentary canal and tracheal systems started at 144 hours after inoculation and complete invasion occurred 168 hours after inoculation (Fig. 2A, 2B & 2C). By this time, hyphal invasion occurred in all the internal tissues. During the colonization, the hyphae penetrated and invaded into the internal organs of *P. marginatus* both by mechanical and enzymatic action. Due to the enzymatic action of *M. anisopliae,* most of the visceral organs were disintegrated (Fig. 2D, 2E & 2F). At this stage, the insects became moribund and dead.

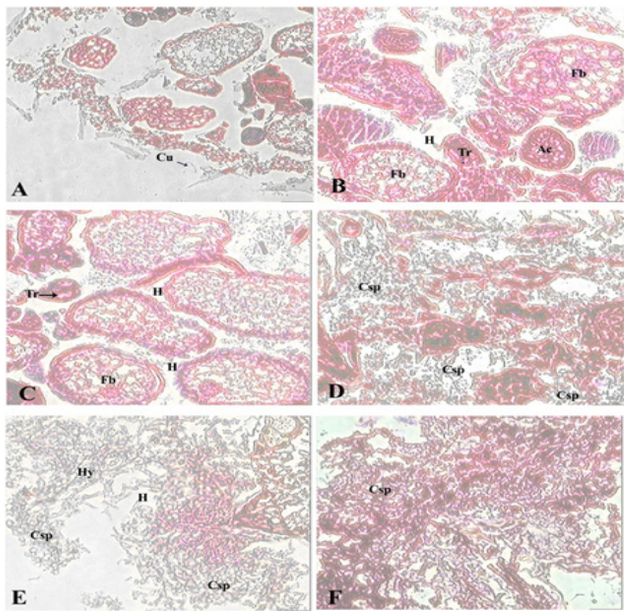

Fig. 2. Infection process of *Metarhizium anisopliae* in the internal tissues of *Paracoccus marginatus* at 144 to 168 hours after inoculation. A) Disintegrated cuticle (Cu) due to enzymatic action of *M. anisopliae.* B & C) The *P. marginatus* haemocoel (H) completely invaded by *M. anisopliae*, most vital organs such as fat bodies (Fb), alimentary canal (Ac) and tracheal (Tr) systems invaded by conidia and hyphae at 144 hours. D, E & F) Disintegrated internal organs in the haemocoel (H) due to enzymatic action of *M. anisopliae* at 168 hours after inoculation.

Though the cuticular chitin serves as barrier for fungal penetration, *M. anisopliae* produce an active chitinase enzyme to degrade the chitin on the cuticle surface. As a result, the cuticle becomes thin and fragile. At this moment, the penetrating hyphae invaded into the insect body and established vegetative mycelial growth. Towards the end of pathogenesis, no evidence of cuticular structure could be seen because of disintegration of cuticular membrane and the muscular tissue surrounding the hyphae was under lysis. (Fig 2D, 2E & 2F). In agreement with earlier observations by Schreiter *et al.* (1994) but atypical of other entomophagous hyphomycete fungi, *M. anisopliae* hyphae extensively colonized the insect cuticle prior to and concomitant with host penetration and infection. At this stage of infection, the fungus produced hyphae that ramified at the host surface and sporulated.

Re-emergence of hyphae from infected *Paracoccus marginatus* cadavers

At the moribund stage, all the internal organs had extensively disintegrated and hyphae started re-emerging towards the cuticle at 168 hours after inoculation (Fig. 3A to 3D). Conidiogenesis initiated between 96 to and 120 hours after inoculation, but the intensity was high between 120 and 144 hours and reached the peak between 144 and 168 hours after inoculation. Initial points of re-emergence were noticed through the membranous intersegmental region. Sporulation started soon thereafter. The hyphae emerged from the cuticle of the dead adult and grew all over the surface forming a green mycelial mat. Newly formed conidia were visible on conidiophores at 172 hours after inoculation. Subsequently, cadavers were completely covered by greenish conidia.

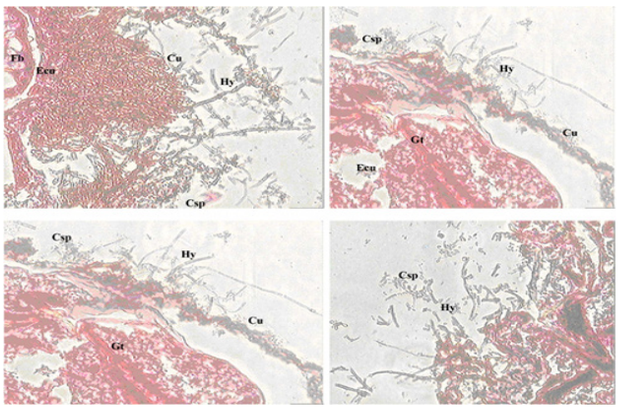

Fig. 3. Re-emergence of hyphae (Hy) and conidiophores (Csp) of *Metarhizium anisopliae* from the disintegrated tissues of *Paracoccus marginatus* at 168 hours after inoculation Fb-Fat bodies, Ecu- cuticle, Gt – Germ tube.

Hyphal bodies were multiplied by the budding of pre-existent cells in accordance with Madelin (1963). This could be achieved through enzymatic and mechanical forces involved in fungus re-emergence from host cadavers (Goettel *et al.,* 1989). At the end phase of fungus metamorphosis, a series of conidiophores re-emerged from inner tissues leaving wide hollow space in haemocoel. The extensive hollowness made in the body may be due to exhaustion of the nutrient constituents and rapid disintegration of internal tissues by enzymatic and mechanical action of *M. anisopliae*.

External symptoms of mycosis

In general, insects began to show symptoms of mycosis 48 hours after inoculation. The fungus infected adult became sluggish and failed to respond to external stimuli within 96 hours of inoculation. They also stopped feeding and there was change in body color from white to brown. As infection progressed, there was gradual loss of functional movement and insect became moribund. Mortality started 96 hours after inoculation and by 144 hours, all the insects were dead as against none in the untreated check. A compact mass of mycelia was observed on the cadavers followed by the emergence of conidiophores at 72 to 120 hours after inoculation (Fig. 4). The cadavers became soft and supple. The mycelia of the fungus emerged through the intersegmental membrane and covered the entire body, masking the identity of insect.

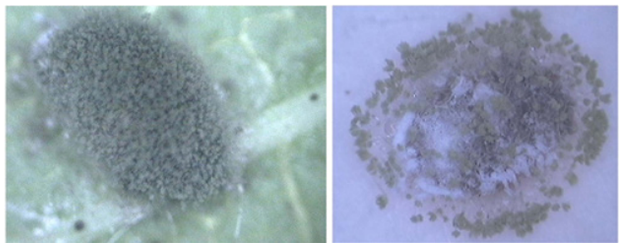

Fig. 4. Compact mass of mycelium and conidiophores of *Metarhizium anisopliae* was observed on the cadavers of *Paracoccus marginatus* at 72 to 120 hours after inoculation.

Histological evidence shows that the complete developmental cycle of *M. anisopliae* took about 172 to 196 hours to disintegrate and kill the *P. marginatus* from the day of inoculation. The possible mechanism of pathogenicity of *M. anisopliae* on *P. marginatus* could involve invasion of penetrating hyphae through cuticle, both enzymatic and mechanical action and reach haemocoel tissues and or organs leading to physiological malfunctioning of vital organs. It would probably result in fatality by the entomopathogen during mycosis or alteration of haemocoel pH might be the possible causes of death. An important component of future studies should be devoted to the role of phylogenetic and ecological constraints in the expression of host range by entomopathogenic fungi.

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