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Pathogenicity, ultra-structural growth and development of green muscardine fungus, *Metarhizium anisopliae* (Metschnikoff) Sorokin (Ascomycota: Hypocreales) on maize fall armyworm, *Spodoptera frugiperda* (J. E. Smith) (Lepidoptera: Noctuidae)

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Abstract

Background: The fall armyworm (FAW), *Spodoptera frugiperda* (J. E. Smith) (Lepidoptera: Noctuidae) is a devastating pest of maize, difficult to manage using a single pest management tactic. The entomopathogenic fungus (EPF), *Metarhizium anisopliae* being an eco-friendly component, could be an effective tool for managing this notorious pest owing to its preferred habitat, principally the whorl region.

Results: *Metarhizium anisopliae* (TNAU-MA-GDU) isolated from FAW was evaluated against 2nd instar larvae of *S. frugiperda* reared under laboratory conditions. The pathogenicity and infection process were also studied through scanning electron microscope (SEM). The LC_{50} value for *M. anisopliae* isolate TNAU-MA-GDU was recorded as 5.8×10^4 spores ml^{-1} against the second instar larvae of *S. frugiperda.* SEM observations on the infection process revealed the adherence of the conidia on the head, cuticular ornamentations of the thorax, sensory seta, and legs at 24 h post-infection (hpi). Formation of germ tube and appressoria was observed from 48 hpi with subsequent hyphal development at 72 hpi. Complete networking of mycelium and conidiogenesis observed after 168 hpi.

Conclusion: An understanding of fungal–host interactions at the ultramicroscopic levels could reform present tactics for developing hyper-virulent EPF strains.

Keywords: *Spodoptera frugiperda*, *Metarhizium anisopliae*, Bioassay, Scanning electron microscope (SEM)

Background

The fall armyworm (FAW), *Spodoptera frugiperda* (J.E. Smith) is an economically important pest of maize native to tropical and subtropical regions of the world (Wiseman et al. 1966). This migratory noctuid pest was first arrived on the African continent in late 2016 and it spread over to 44 countries (Cock et al. 2017). Following its invasion in Africa, its incidence has also been recorded in India at Karnataka in 2018 (Sharanabasappa et al. 2018). It is known to damage more than 353 cultivated plant species including maize, rice, sorghum, cotton and various vegetable crops but the greatest damage is being reported in maize (Bateman et al. 2018). This key pest attacks the crop at an early stage, resulting in yield losses ranging from 45 to 100% (Blanco et al. 2014).

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The larvae make shot holes on leaves, followed by elongated irregular holes, and damage to growing tassels and cobs (Day et al. 2017). Management of this pest is a great challenge because they reproduce fast and in large numbers, can migrate greater distances, and hence prolonged applications of conventional insecticides proved less effective (Westbrook et al. 2019) and resulted in the development of resistance in FAW to at least 29 insecticidal active components with six different mode-of-action categories (Gutiérrez-Moreno et al. 2019).

Pest management through biological control has immense potential in view of the adverse effects of insecticides. Entomopathogens such as viruses, fungi, protozoa, bacteria, and nematodes were found to be vulnerable to FAW (Molina-Ochoa et al. 2003). Entomopathogens have also proven to be an effective control strategy in many situations of severe pest outbreak (de Faria and Wraight 2007). Among the bioagents, entomopathogenic fungi (EPFs) play a vital role owing to their broad host range, pathogenicity and ability to control insect pests (Akutse et al. 2019). The potential of EPF like *Metarhizium rileyi* (Farlow) Samson, *Beauveria bassiana* (Balsamo) Vuillemin, and *Metarhizium anisopliae* (Metschnikoff) Sorokin against FAW under field conditions has been reported by Herlinda et al., (2021).

EPFs are known for the production of cuticle degrading enzymes and secondary metabolites (Vey et al. 2001 and Wang et al. 2021). Scanning electron microscope (SEM) studies provide more insight on the trophic interactions between EPFs and host insect. However, information on the infection process of EPF on FAW through ultramicroscopy are very meagre. The present investigation attempted to evaluate the efficacy and pathogenicity of the EPFs through ultra-microscopy and altered metabolomes during infection to *S. frugiperda* larvae.

Methods

Insect culture

FAW was mass cultured at the Department of Agricultural Entomology, Centre for Plant Protection Studies, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India. The initial culture of FAW was established from the field collected larva from Coimbatore, Tamil Nadu and maintained on semi-synthetic diet developed by CIMMYT (Tefera et al. 2010) until pupation. The pupae were collected and placed in cages ($45 \times 45 \times 45$ cm) containing maize seedlings for oviposition process by the emerging adults. Ten per cent honey solution fortified with multivitamin was provided as adult food. The egg masses were collected daily and the neonates were reared in plastic rearing containers ($16 \times 10 \times 5$ cm) with maize leaves grown under hydroponics. Second -to-sixth instars were maintained in individual containers ($3 \times 2.5 \times 3.5$ cm) with diet to avoid cannibalism. Thus, the larvae of *S. frugiperda* were reared for generations under controlled conditions (28 ± 2 °C, 65 ± 10% RH and 12:12 h (L:D) photoperiods) for further studies.

Source of fungal isolate

The fungal infected cadavers of FAW collected from the maize fields in Gedimedu village, Coimbatore district, during survey in 2019 were surface sterilized with 70% ethanol (30 s), 0.5% sodium hypochlorite (60 s), washed three times with sterile distilled water and air dried on sterile tissue paper under aseptic conditions. Surface-sterilized insect cadaver was plated in Potato Dextrose Agar (PDA) medium and incubated at 28 ± 2 °C in BOD incubator (Goettel and Inglis 1997). The isolated culture was further sub-cultured for purification and maintained at 25 ± 2 °C in an incubator.

The fungus was identified morphologically based on conidial and mycelial characters and was supported by rDNA- ITS studies. The culture isolated was designated as TNAU-MA-GDU and deposited in the Institute repository. DNA of the isolated fungus was extracted from 50 mg of mycelial cells in 2 per cent CTAB (Cetyl trimethyl ammonium bromide) buffer as per the procedure described by Wu et al. (2001). PCR amplification of ITS1-5.8S-ITS2 and 18S regions of fungal isolate was done using universal primers viz., ITS1 (5'-TCCGTAGGT GAACCTGCGG-3') as a forward primer and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') as a reverse primer. PCR amplifications were performed in a total volume of 40 µl which included 20 µl of master mix (smart prime), 8 µl of nuclease free water, 4 µl of forward primer, 4 µl of reverse primer and 4 µl of genomic DNA (50 ng). The PCR process involved 40 cycles of 95 °C for 10 min, 60 °C for 1 min, and 72 °C for 1 min. PCR amplifications were determined by gel electrophoresis using 1.5 per cent agarose gel with ethidium bromide and the amplified PCR products were sanger sequenced by Eurofins Genomics (Bangalore, India) and the sequences thus obtained were edited and assigned in the MEGA ver. 7 and compared to GenBank nucleotide database using BlastN.

Preparation of spore suspension

For laboratory bioassay, the fungal isolate was cultured in Petri dishes (9 cm dia.) containing PDA medium and incubated at 28 ± 2 °C for 10–14 days. After complete sporulation, spores were scrapped from the plates and suspended in 10 ml sterile distilled water containing 0.05 per cent Tween 80®. The spore suspension was vortexed for homogenization and the spore count was assessed using improved Neubauer haemocytometer as suggested by Lomer and Lomer (1996). From the stock solution,

further dilutions were made to prepare required spore concentrations for further studies.

Bioassay

Spore concentrations of *M. anisopliae* TNAU-MA-GDU were prepared at different spore loads ranging from 1×10^3 spores to 1×10^8 spores ml^{-1}. Newly molted 2nd instar FAW larvae placed in culture boxes ($16 \times 10 \times 5$ cm) with diet were topically sprayed with different spore suspension using glass atomizer. Larvae treated with 0.05% Tween 80® served as untreated control. The experiment was performed in completely randomized design with seven treatments replicated three times. Ten number of second instar were released for each replication and were incubated at room temperature of 28 ± 2 °C. Mortality of larvae was recorded up to 9 days. Dead larvae were collected aseptically and kept in humid chamber and observed for mycosis. Larvae showing mycelial growth were surface sterilized in 70% ethanol followed by three rinses in distilled water and transferred to petri plates lined with wet sterilized filter paper to allow fungal growth on the surface of cadaver. Mortality caused by *M. anisopliae* was confirmed by microscopic observation of spores on the surface of the cadavers. Percentage mortality was corrected using Abbotts (1925) formula. Linear relationship between mortality and concentrations was worked out using Probit analysis (Finney 1971) to estimate LC_{50} with 95% confidence limits. Concentration mortality responses were analyzed through SPSS Statistics Data Editor Ver.21, IBM software.

Scanning electron microscope analysis

Second instar FAW larvae were topically sprayed with spore suspension of 1×10^8 spores ml^{-1} of *M. anisopliae* with 0.05% Tween 80® using glass atomizer and placed on culture boxes ($16 \times 10 \times 5$ cm) with diet. Larva treated with sterile distilled water containing 0.05% Tween 80® served as untreated control. Samples were collected at 24, 48, 72, 96, 120 and 168 hpi and processed for observations under SEM. Infected larvae were fixed with 4% glutaraldehyde in 0.05 M phosphate buffer (pH 7.3), then rinsed three times with phosphate buffer. The samples were dehydrated at room temperature using an ethanol series of 30, 50, and 70% with a 30 min delay between each stage. Critical point drying was accomplished by immersing samples in 100% ethanol for 45 min (Guerri-Agullo et al. 2010). The dried samples were then placed on pin stubs in various orientations with double-sided tape. Specimens were sputter coated with gold and observed under the FEI Quanta 250 (Netherlands) having an Everhart Thornley Detector with tungsten as an electron source.

Results

Isolation, pathogenicity and characterization of entomopathogenic fungi

The fungus isolated from the FAW cadaver collected from Gedimedu village of Coimbatore district showed white coloration during the vegetative state followed by green coloration at the sporulation stage, *i.e.*, 5 days after post-incubation. Microscopic observation on the morphological character revealed branched, septate mycelium with green, broadly ellipsoid conidia. The PCR amplification of the ITS1-5.8S-ITS2 region revealed the product size of 500 bp and the sequences comparison with BLAST confirmed the fungal isolate as *M. anisopliae.* The sequences thus obtained were submitted in NCBI nucleotide database with the accession number MZ749658.

Pathogenicity studies on *M. anisopliae* TNAU-MA-GDU against 2nd instar larvae of *S. frugiperda* showed a significant mortality with different spore concentrations (Fig. 1). Mortality was initiated after 3 days post-infection (DPI) and it ranged from 10.00 to 46.67% at tested spore concentrations. Similarly, at 7 DPI, mortality ranged from 18.87 to 86.67% at the tested doses of 1×10^3–1×10^8 spores ml^{-1}. The highest mortality of 90% was obtained at 1×10^8 spores ml^{-1} concentration and the lowest mortality (10%) at 1×10^3spores ml^{-1}.*M. anisopliae* TNAU-MA-GDU recorded the LC_{50} value of 5.8×10^4 spores ml^{-1} against 2nd instar of *S. frugiperda* larva (Table 1).

Ultramicroscopic changes in S. frugiperda larva during M. anisopliae infection

SEM images of healthy second instar *S. frugiperda* larvae (Fig. 2) were compared to SEM images of *M. anisopliae* infected *S. frugiperda* larvae and presented in Figs. 3, 4, 5, 6, 7, 8. Signs of infection by *M. anisopliae* were expressed in the larvae of *S. frugiperda* between 72 and 168 hpi. Adherence of conidia in different body parts including head and thorax (Fig. 3a), stemmata (Fig. 3b), around seta, cuticular ornamentations (Fig. 3c) and prolegs (Fig. 3d) were observed at 24 hpi. The thoracic cuticular ornamentations and setal structures of *S. frugiperda* aided in the conidial attachment effectively and crotchets of prolegs with claws were found to hold abundant conidia. Conidia that had been adhered began to germinate and form the germ tube and appressoria for penetrating the cuticle at 48 hpi (Fig. 4). Numerous conidia with germ tubes or appressoria were seen in head around setal structures (Fig. 4a), around stemmata (Fig. 4b), cuticular surfaces in thoracic regions (Fig. 4c), setal region (Fig. 4d), thoracic legs (Fig. 4e) and also around spiracular openings (Fig. 4f). *M. anisopliae* produced appressoria from germ tubes at 48 hpi with prominent appressorial structures around stemmata, cuticular

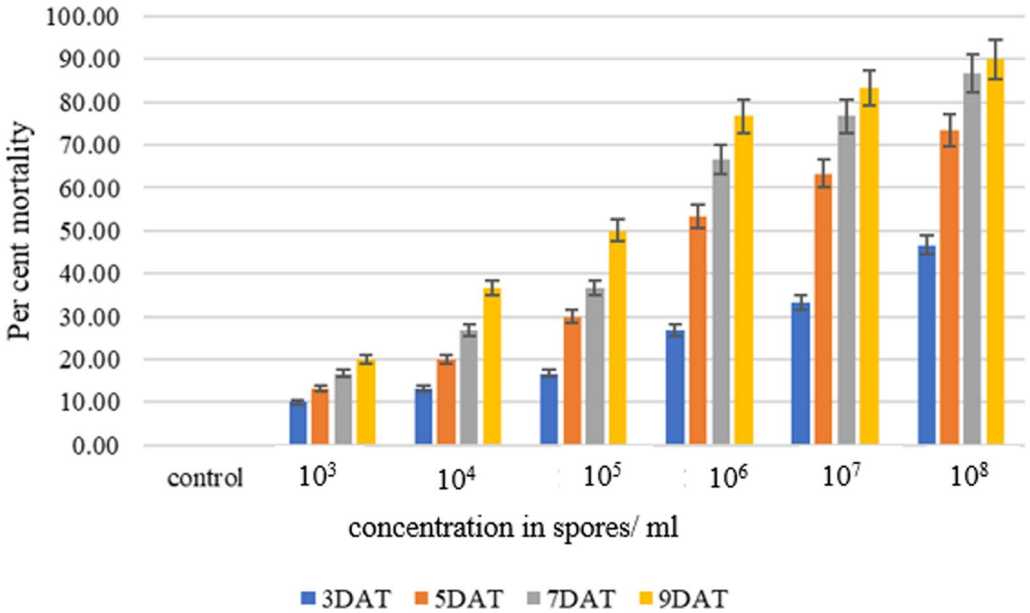

Fig. 1 Concentration mortality response of *M. anisopliae* treated with different spore concentrations of *Metarhizium anisopliae* (TNAU-MA-GDU)

region in thorax, thoracic legs and spiracles for possible penetrations.

At 72 hpi, head regions and labrum showed thin hyphal development (Fig. 5a, b) and the spiracular regions were enclosed with abundant conidial germlings and hyphal growth (Fig. 5c). Well-differentiated deep penetration regions were clearly visible near stemmata in head region and thoracic cuticle (Fig. 5d, e), similarly, intercalary appressorium developed from hyphae were also observed around setal region at 72 hpi (Fig. 5f). Multiple points of penetration on stemmata, setal sockets and cuticular ornamentations were predominantly seen at 72 hpi. Extensive hyphal development covering head region (Fig. 6a, b) and stemmata (Fig. 6c) with deep hyphal penetration in setal socket was also observed at 96 hpi (Fig. 6d).

Extrusion of hyphae from larval body parts viz., setal alveoli, thoracic legs, abdominal prolegs and spiracular regions started from 120 hpi (Fig. 7a–d). The openings of spiracles were plugged with mycelia as seen in (Fig. 7d). Since, spiracles and setal alveoli were natural openings on larval surface; the extrusion of hypha was more in these regions. Extensive mycelial networking and sporulation began at 168 hpi. Head region and entire larval surfaces were completely colonized with mycelial networks and numerous spores at 168 hpi (Fig. 8). The major phases of infection viz., adhesion, germ tube and appressoria formation, penetration, colonization, extrusion and conidiogenesis are shown in Fig. 9.

Discussion

The FAW, nowadays, is the most devastating pest of maize and cause negative impact on food security due to its voracious feeding behavior (Harrison et al. 2019). Chemicals used for the management of fall armyworm are broad spectrum in nature causing adverse effects both on natural enemies and on FAW (Lewis et al. 2016). EPFs play a major role in ecofriendly management of this pest owing to its preferred habitat, principally the whorl region. In the present investigation, studies were carried out on the isolation, characterization, pathogenicity of *M. anisopliae* (TNAU-MA-GDU) against FAW, SEM photography and metabolome analysis of *M. anisopliae* infected and healthy FAW larvae. Results of the pathogenicity studies revealed the highest mortality (90%) at the concentration of 1×10^8 spores ml^{-1} and the lowest

Table 1 Concentration mortality response of *Spodoptera frugiperda* to *Metarhizium anisopliae* (TNAU-MA-GDU)

Isolate name	LC_{50} (spores ml)	Heterogenicity	Regression equation	Fiducial limit (spores ml^{-1})
Metarhizium anisopliae (TNAU-MA-GDU)	5.8×10^4	0.9807	$y = 0.4362x + 2.8998$	1.36×10^4 to 2.49×10^5

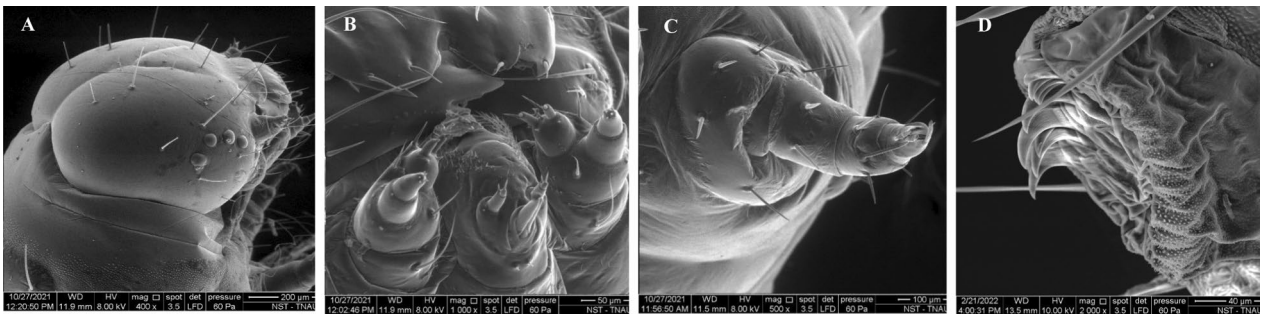

Fig. 2 SEM images of healthy second instar of *Spodoptera frugiperda*. **a**. Dorsal view of head region. **b**. Ventral view of head region showing mouthparts. **c**. Thoracic leg with seta. **d**. Full view of abdominal prolegs with crochets (arrowheads)

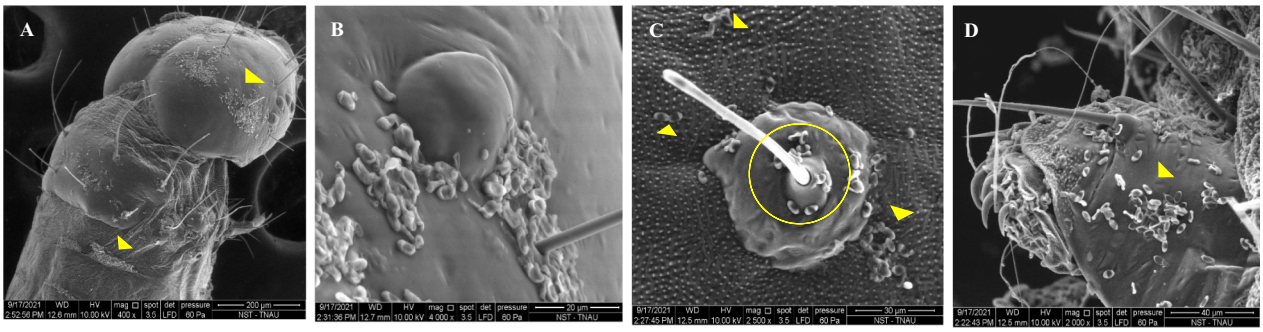

Fig. 3 SEM images of second instar of *Spodoptera frugiperda* infected with *Metarhizium anisopliae* (24 hpi). **a**. Adherence of conidia on head and thoracic region (arrowheads). **b**. Close-up view of conidial clusters on peripheral region of dorsal ocelli. **c**. Spores around setal alveoli (circle), conidia adhered to cuticular ornamentations (arrowhead). **d**. Conidia attached to base of prolegs (arrow head)

mortality (10%) at the concentration of 1×10^3 spores ml^{-1} with a LC_{50} value of 5.8×10^4 spores ml^{-1} against 2nd instar FAW larvae. The efficacy of *M. anisopliae* against FAW was also reported by Gutiérrez-Cárdenas et al. (2019) reported that the CP-MA 1 strain of *M. anisopliae* could cause 72.5% mortality at a spore concentration of 54×10^4 spores ml^{-1}. Akutse et al. (2019) also observed that 1×10^8 conidia ml^{-1} of *M. anisopliae* strain ICIPE 41 could cause the highest mortality of 96.5% in FAW neonates.

Light microscopy and SEM studies may provide further information on fungal development inside the insect. The infection process of *M. anisopliae* through SEM was earlier reported by several researchers against different insect pests excluding FAW. Investigations carried out on the ultra-microscopical studies with the FAW specific virulent isolate indicated that the 2nd instar larvae inoculated with *M. anisopliae* (TNAU-MA-GDU) showed adherence of conidia on head, thorax, legs and seta alveoli during the initial phases of infection. The lipophilic nature of the cuticular structures helps in the adherence of hydrophobic *M. anisopliae* conidia all over body surfaces especially more conidia around hairs or seta (Boucias et al. 1988). However, the germination of conidia was further determined based on the presence of cuticular inhibitory compounds such as phenols or quinones and antibiosis mechanism by host (Lord and Howard 2004) and under favorable conditions, the conidia germinate and differentiates into germ tube and appressorium for penetration into epicuticle (Leger et al. 1989). *M. anisopliae* produces only one germ tube from each conidium as reported by Schabel (1978). Similarly, in the present study, it was also observed that the *M. anisopliae* conidia germinated and produced only one germ tube with appressoria at 48 hpi on the infected larvae. Also, appressoria or penetration peg were formed in developing mycelia at intercalary position. These features suggest that penetration may occur directly from conidia with appressoria or from developing hyphae. These results are consistent with the findings of Nithya et al. (2021) who found that the fungi could produce hypha directly from the conidia when conditions are unfavorable for penetration process. Further, the fungus penetrates the host cuticle through the production of cuticle degrading enzymes

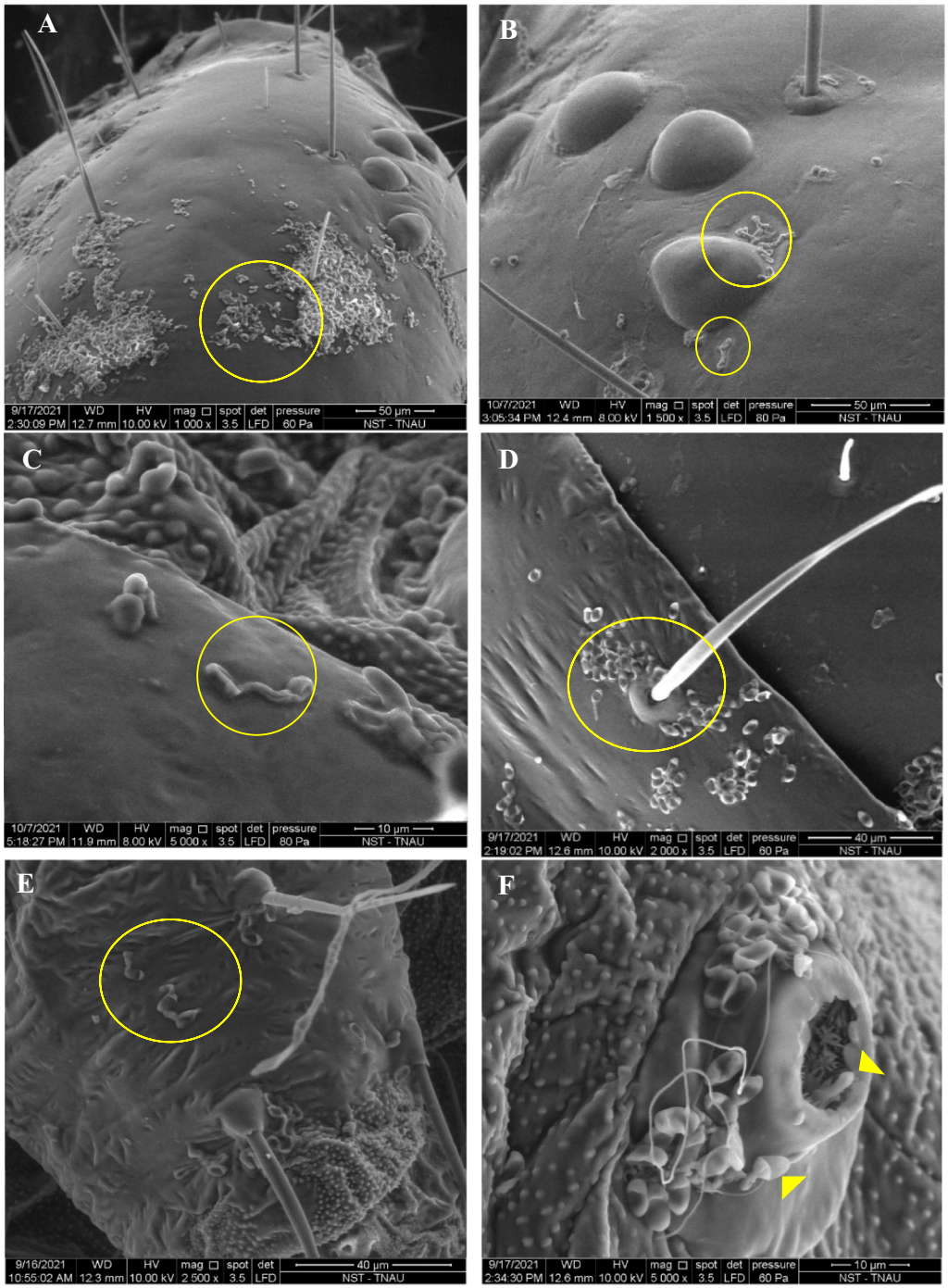

Fig. 4 SEM images of second instar of *Spodoptera frugiperda* infected with *Metarhizium anisopliae* (48 hpi). **a**. Head region showing germinating conidial clusters (circle). **b**. Close-up of germinating conidia with germ tube around stemmata (arrowhead). **c**. Conidia with germination tube in thoracic region (circle). **d**. Conidial germination near the base of setae (circle). **e**. Base of prolegs having conidia with germ tube and appressoria (circle). **f**. Conidial germlings of *M. anisopliae* with germtube around spiracular region (arrowheads)

or by mechanical pressure exerted by developing hyphae and enters the haemocoel of insects as blastospores (Schrank and Vainstein 2010).

SEM images also revealed mycelial networking on the infected cadavers of *S. frugiperda* at later stages of infection (96 hpi). Generally, fungal penetration and

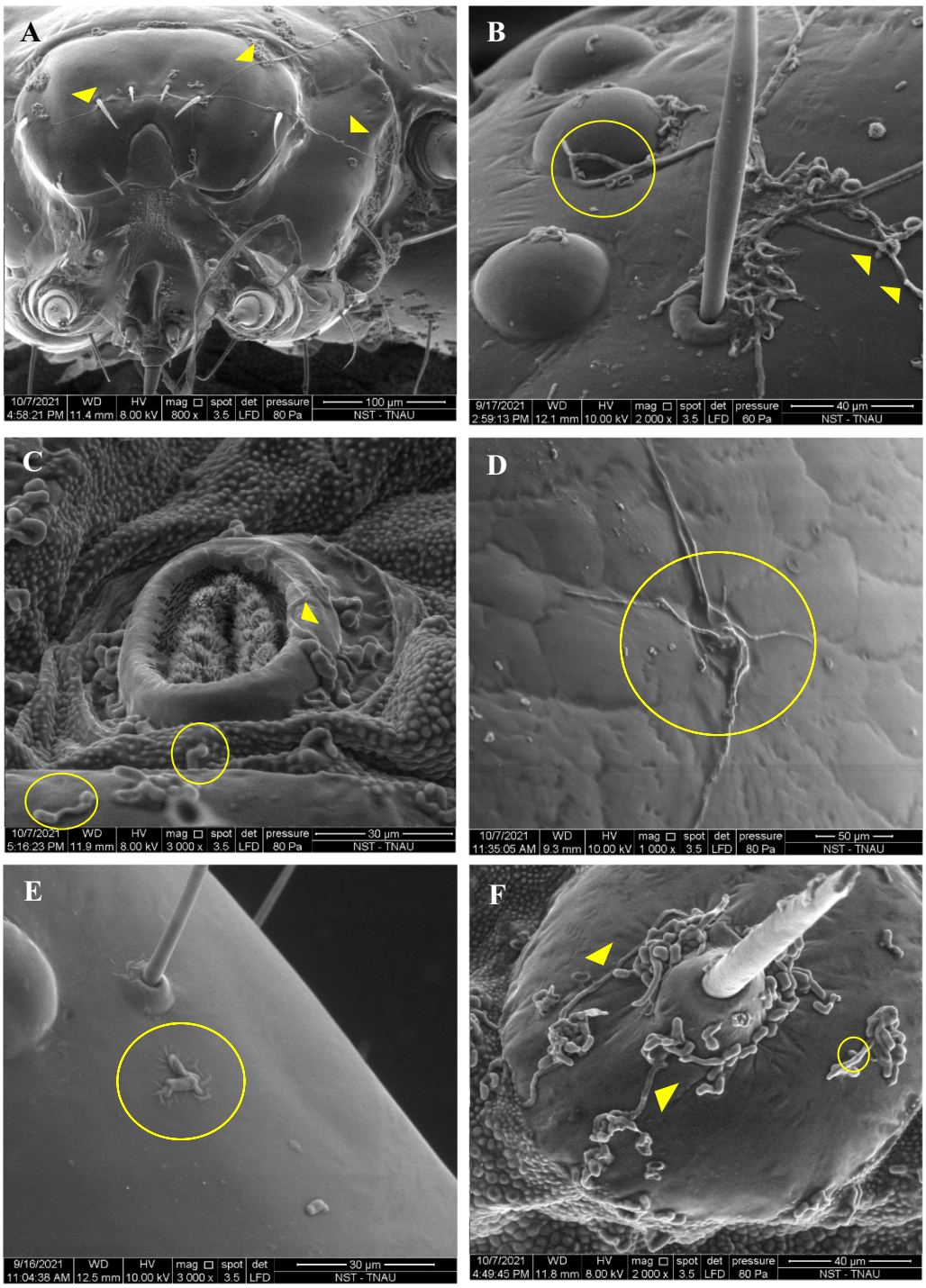

Fig. 5 SEM images of second instar of *Spodoptera frugiperda* infected with *Metarhizium anisopliae* (72 hpi). **a**. Labrum with initial hyphal development (arrowhead). **b**. Hyphae with appressorium (arrow heads) around stemmata region (circles). **c**. hyphal growth in spiracular region (arrowhead), germlings of conidia with germ tube and appressorium (circle). **d**. Hypha penetrating the cuticle (circle) e. penetration of hypha from conidia in head region (circle). **f**. Close-up of developing hyphal clusters around setal region (arrowheads), Hyphae with intercalary appressorium (circle)

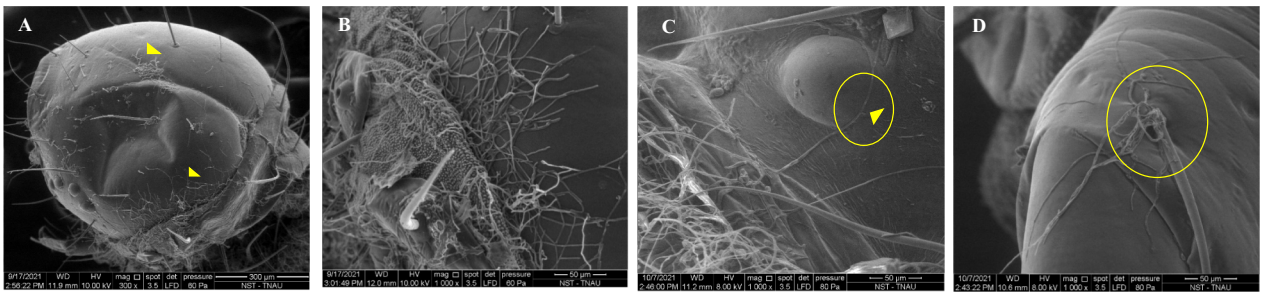

Fig. 6 SEM images of second instar of *Spodoptera frugiperda* infected with *Metarhizium anisopliae* (96 hpi). **a**. Extensive hyphal development in head region (arrowheads). **b**. Close up of hyphal penetration site in head region (circle). **c**. Stemmata region with penetrating hypha (circle), appressorium (arrowhead). **d**. Penetration of hypha through seta socket in thoracic leg (circle)

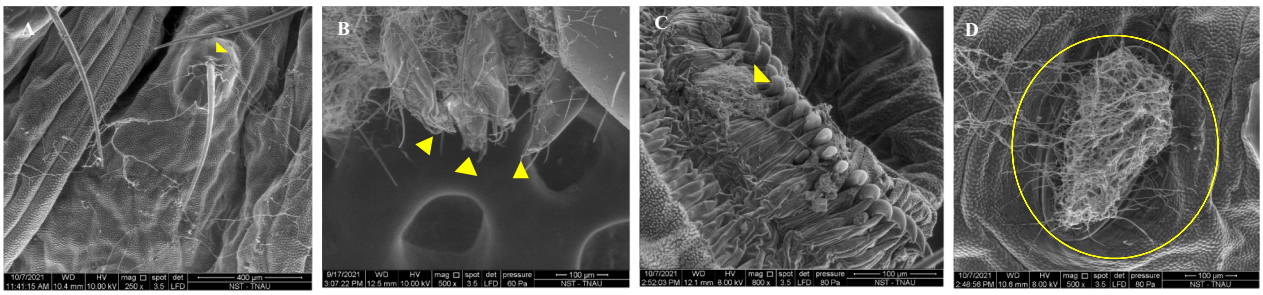

Fig. 7 SEM images of second instar of *Spodoptera frugiperda* infected with *Metarhizium anisopliae* (120 hpi). **a**. Extrusion of mycelium from setal socket (arrowhead). **b**. Mycelia growth over thoracic legs (arrowheads). **c**. Anal prolegs with hyphal clusters (arrowhead). **d**. Close-up of abdominal spiracular region with hyphal clusters (circle)

emergence occur in the less sclerotized regions. Most germ tubes penetrate at the setal base because the setal base cuticle is softer than other regions, allowing fungi to use fewer enzymes in the degradation process. This corroborates with the observations made at 96 hpi where in the penetration of germ tube was observed at setal base. After utilizing host nutrients, hypha extrudes through less sclerotized body parts of larva viz., spiracles, intersegmental membrane and setal alveoli. Likewise, extrusion of mycelial networks was observed in the spiracular region. These results are strengthened by the findings of Neves and Alves (2004) who reported that host colonization of *M. anisopliae* occurred between 72 and 120 hpi with hyphal extrusion from weaker areas of insect like setal base, legs and intersegmental membranes. The SEM images taken at 120 hpi displayed dense growth of hyphae covering entire body surface and it sporulated at 168 hpi. These asexual spores (conidia) are the infective propagules that aids in dissemination of fungus (Butt et al. 2016).

Overall, the infection process of *M. anisopliae* on host insects is complex process involving the adherence of conidia by hydrophobic interaction, conidial germination and germ tube production, formation of appressoria, penetration, host colonization, hyphal extrusion and conidiogenesis (Aw and Hue 2017) which was evident from the present investigation.

Conclusion

SEM and metabolite studies of EPF infection help in better understanding of interaction between host and the insect, both externally and internally on host insect so as to decipher the analysis of metabolites during the different days of infection process. A better understanding of the trophic relationships between EPF and its host through SEM and metabolome studies aids in successful application of this biocontrol agent against significant insect pests. SEM study confirmed the adhesion, germination, penetration, hyphal development and sporulation process of *M. anisopliae* on *S. frugiperda.* The study concluded that *M. anisopliae* (TNAU-MA-GDU) is found promising against the maize fall armyworm. Also, it's essential to formulate this isolate with an appropriate carrier and test its toxicity, persistence, and safety to natural enemies in the maize ecosystem.

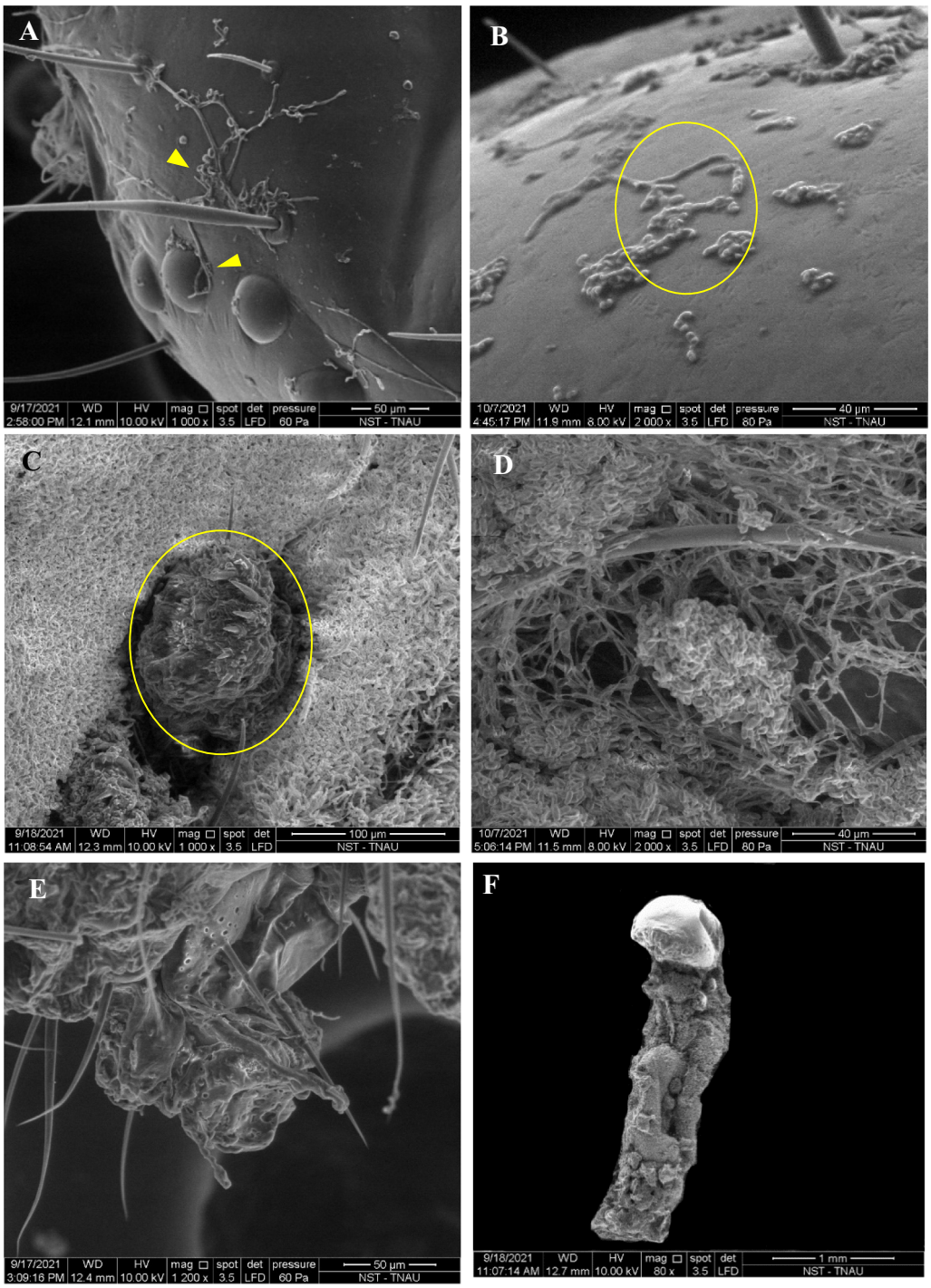

Fig. 8 SEM images of second instar of *Spodoptera frugiperda* infected with *Metarhizium anisopliae* (168 hpi). **a**. Close-up of seta socket and stemmata with hypha and conidia (arrowheads). **b**. Extruding hypha with conidia in head region (circle). **c**. Extensive growth of hypha around prolegs (circle). **d**. Close-up of mycelial threads with conidia in abdominal region. **e**. Thoracic leg completely covered by fungal mycelial network. **f**. Full view of mummified cadaver

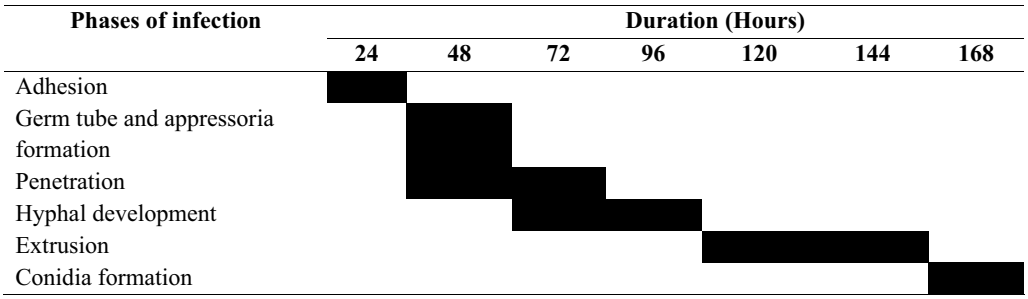

Fig. 9 Stages of infection by *Metarhizium anisopliae* (TNAU-MA-GDU) at different tested periods after treatment

Abbreviations
FAW: Fall armyworm; EPF: Entomopathogenic fungi; SEM: Scanning electron microscope; hpi: Hours post-infection; PDA: Potato dextrose agar.

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Author contributions
This work was carried out in collaboration among all authors. SJ, NS, MM, US, DU provided the technical guidance. GK carried out the research experiments and produced the manuscript. Authors SJ and NS revised the manuscript. All authors read and approved the final manuscript.

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Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
All the authors give their consent to publish the submitted manuscript as "Original paper" in EJBPC.

Competing interests
All the authors declared that they have no competing interests. The manuscript is approved by all the co-authors and I assure that the submitted manuscript or any part of it has not been under consideration or published elsewhere.

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