Studies on Selection of Entomopathogenic Fungi with High Virulence and Field Persistence for Controlling Common Cutworm, Spodoptera litura

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DEDICATION

This is dedicated to my beloved wife and our children, Sofanit, Sidona and Kaleb; for their unwavering love, support and everlasting encouragement keep me strong and determined, which finally comes to meaningful accomplishment.

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Abbreviations and Acronyms

µL: Micro liter

ARSEF: Agricultural research service entomopathogenic fungi

Bt: *Bacillus thuringiensis*

CFUs: Colony forming units

cm: Centimeter

DPI: Days post inoculation

EPF: Entomopathogenic fungi

LC_{50}: Lethal concentration 50

LT_{50}: Lethal time 50

mL: Milliliter

NPV: Nucleopolyhedrovirus

PDA: Potato dextrose agar

ppm: Parts per million

RF: Rain fall

SSYA: Sabouraud sucrose yeast extract agar

UV: Ultraviolet

General Introduction

Distribution and economic importance of *Spodoptera litura*

Common cutworm, (*Spodoptera litura*) is a destructive and polyphagous pest attacking hundreds of plant species. It is a cosmopolitan pest in tropical and subtropical regions of the world widely distributed in Asia and Oceania regions (Noma et al., 2010). In different location it has named as cotton armyworm, oriental leaf worm, Asian armyworm, tabacco cutworm and rice cutworm. The pest possesses a complete life cycle in four developmental stages viz, egg, larvae, pupa, and adult. Eggs are laid in mass up to 890.50 ± 16.26/individual female. Hatched eggs give up to 6^{th} stage larval instars and pupate in soil (Ramaiah and Maheswari, 2018).

It is one of the economically important pests in field crops, vegetable, fruit, and ornamental crops etc. (Noma et al., 2010). Its economic losses ranged from 25.8 to 100 percent based on crop stage and field infestation level. It was reported as a major pest of tobacco, cotton, rice, maize, soybean, and groundnut (Natikar and Balikai, 2015). Further its incidence on citrus crops was also reported (Ullah et al., 2016). Various past studies revealed that the pest caused heavy damage on soybean varieties (Fattah et al., 2018, Fattah et al. 2020). The previous studies showed every addition of one larva per plant resulted in 3.92–8.58% and 0.65–0.80% of leaf damage and seed yield losses, respectively. Relationship between pest population and leaf damage intensity was found approximately 93.58–96.10%. Huge damage potential of the pest was seen in vegetable crops as well (cabbage, cucumber, tomato, and sweet pepper) (Vashisth et al., 2012; Sahu et al., 2020).

Population buildup of *S. litura* has been affected by various environmental factors (abiotic and biotic). Studies detailed that temperature, relative humidity (RH), rain fall, sunshine hours, crop type and date of sowing are determinant factors that affect incidence and population buildup. Temperature ranges of 26.0℃ to 35.1℃, RH of 62–89%, zero rainfall, photoperiod of 64.6 h/week, resulted with maximum population counts (Selvaraj et al., 2010), while 38℃ was proved as lethal temperature to *S. litura* (Fand et al., 2015). Other scientific reports included that low rainfall weeks followed by higher rainfall favored for larval gregarious phase (Patel et al. 2020).

Host preferences are also considered other means of restriction factors for infestation levels of *S. litura*. Crops like Chinese cabbage was more preferred for egg oviposition and higher longevity of larval survival. Finding added that more females than males were found among emerged adults and that male adults lived 1–2 d longer than females (Xue et al., 2010). According to Abdullah et al. (2019), cabbage was suitable for fecundity and larval survival, however female life span was found higher than male on all the diets provided. In addition, sesbania and rapeseed were reported as potential breeding sites of *S. litura* (Tuan et al., 2014).

Efficiency of biopesticides against insect pests

Biopesticides are natural products of plants, animals, and microorganisms (bacteria, fungi, viruses, and protozoans) used to combat pests. In the current sustainable agricultural practices, the use of biological agents such as EPF and other microorganisms get advances in pest management strategies. These modern pest control weapons emphasized the avoidance of persistent and hazardous effects of synthetic (conventional) pesticides for human safety, environmental protection, and animal welfare. Some species of EPF with narrow host range can regulate pest population without affecting non-target organisms (Digvijay et al., 2017). They are advantageous for pest management in diversified agroecosystems. Recent advances have been done on the use of EPF controlling not only agriculturally economic pests but also disease-causing insect vectors like mosquitoes. Studies reported that combined use of conventional insecticides and entomopathogenic microorganisms was considered as the best solution of pest resurgence and synthetic pesticide resistance **(**Samuels et al., 2016). However, the potential occurrence and persistence of those bioagents could be adversely affected by many biotic and abiotic factors. Some of the underlined determinant factors include fungal characters (host range, latency, spore density, dispersal, and compatibility), insect factors (behavioral, morphological, and physiological characters), and environmental ones (temperature, sunlight, humidity, and rainfall) (Qayyum et al., 2021). These are critical factors which hinders the developments of EPF as mycopesticides.

Entomopathogenic organisms are known for checking insect population buildup. However, their usage still lower as compared to conventional pesticides. Some of the possible reasons for this are virulence of species and lifecycle of the target pests. Data with *S. litura* NPV (SltMNPV), showed potential at early instars of the larvae with peak mortality at LC_{50} values

of 1.92×10^3 to 3.64×10^3 occlusion bodies/mL with LT_{50} values of 69.30 to 72.80 h (Ahmad et al., 2018). Apart standalone applications, combined use of biopesticides with chemical insecticides with high compatibility promote insect population reduction. For example, combined use of NPV with Flubendiamide enhanced the effectiveness in controlling *S. litura* larvae, pupae, and adults but was safer to natural enemies and beneficial arthropods (Maqsood et al., 2017). In addition, the pest was reported susceptible to combined sub-lethal dose rates (2×10^3, 4.5×10^3, and 6×10^3 polyhedral inclusion bodies/larva) of NPV and Spinosad (0.01 ppm) (Ahmad et al., 2020).

EPF formulated as mycopesticides have been used for many years and played a significant role. They are capable to destroy various insect species. They infect and cause death of pest insects at different stages. Fungal species of *Beauveria bassiana* sensu lato and *Metarhizium anisopliae* sensu lato were reported for significant infection against field and storage insect pests. These fungal species are commonly recovered from the cadavers of red flour beetle (*Tribolium castaneum*), rice weevil (*Sitophilus oryzae*), lesser grain borer (*Rhyzopertha dominica*), rusty grain beetle (*Cryptolestes ferrugineus*) and cowpea weevil (*Callosobruchus maculatus*) (Wakil et al., 2014).

Unlike NPV and *Bt*, EPF were reported with multiple infection pathways on the target pests. Invasion through the complex exoskeleton structure of insect cuticle by chemical and physical penetration using germ tube and appressoria is a unique character among the other entomopathogenic organisms (Mora et al., 2017). Oral route fungal infection was also observed with *B. bassiana* as those fungi shared genes with other non-fungal pathogens that infect orally, such as *Bt* (Mannino et al., 2019).

Besides, the secondary metabolite of EPF induce a significant role for insect death. The fungi have strong selection pressure for traits to destruct insect immune system. Suppressor metabolites such as cordycepin compound from *Cordyceps* sp. (Woolley et al., 2020) and inhibition of phenoloxidase enzyme with *Beauveria* sp. (Gurmeet and Sanehdeep, 2013) are potential in insect immune responses.

Thermotolerance, abundance and field persistence of EPF

Fungal strains show varying degree of vulnerability to high temperatures, UV radiation and other environmental factors. According to Alfiky (2022), *M. anisopliae* strains followed by *B.*

bassiana strains were heat tolerant at 45℃ at 180 min exposure time while *C. javanica* was very susceptible and responded with the least percentage of conidial germination. Despite its vulnerability to variable temperature and UV light, *C. javanica* (wf GA17) was observed causing widespread epizootics among insect pests (whiteflies). The strain responded as high virulent as other EPF species at temperature ranges of 15–30℃, however, at extremely lower and higher temperatures (35 and 40℃), the strain had found inferior to *M. brunneum* F52 and *B. bassiana* GHA (Wu et al., 2020).

Abundance of EPF in soil varies depending on various factors despite their presence widespread in forest and agricultural fields. They promote pest control naturally unlike natural enemies which are very sensitive and hindered by pesticides application and conventional practices. Organic fields are significantly abundant with EPF than conventional fields. Further regression analysis revealed organic fertilizers and silt percentages were found positively correlated with EPF abundance. While concentration level of N_2 and tillage correlated negatively and no substantial effect on EPF due to pesticide applications (Clifton et al., 2015).

According to Moraga et al. (2007), soils with lower organic matter and higher clay and pH, predominated with *B. bassiana* than *M. anisopliae* irrespective of habitat type. Therefore, these were considered as predictive variables for EFP occurrence in soil. Contrary findings by Tkaczuk et al. (2014) stated that higher CFUs of *B. bassiana* were detected from organic fields, and *Isaria fumosorosea* from conventional soils. On the other hand, increased periods of exposure to strong UV light in open environment induced in reduction of viability and virulence of EPF strains (Wu et al., 2020).

Persistence of EPF strains in fields is highly influenced by the inherent capacity of fungal strain and other environmental factors. Studies indicated that *B. bassiana* persisted higher as compared to *M. anisopliae* on soyabean leaves (Souza et al., 2022). Its efficacy against *Helicoverpa armigera* decreased with increasing time of application. The efficacies were 86–30% for *B. bassiana* and 78–4.2% for *M. anisopliae*. The above statement disagreed with Swiergiel et al. (2016), which reported that *M. brunneum* F52 persisted on soil better than *B. bassiana* GHA. Field study detailed those soils of no-till system cultivation exhibited higher occurrence of EPF than tilled soils, however, no density difference of fungal species was observed on leaves (Gomez et al., 2001). In addition, dispersal of fungal strains was affected

by formulation type. For example, Guinossi et al. (2012) reported that spatial dispersal of *B. bassiana* was significantly higher when applied with oil than as kaolin dust formulation.

Ecological roles of EPF

In the present day, development of resistance to chemical insecticides and their possible residue in harvested crops are major concerns in insect pest management. Therefore, intensive investigation on microbial pathogens was done to develop ecofriendly pest management and overcome undesirable effects of chemicals on non-targeted organisms (Kachhawa, 2017). It is considered as one of the best alternatives to synthetic pesticides because some entomopathogenic microorganisms have high virulence and specificity to their natural host insect species. Its development initiates modernization of agriculture. Recently numerous biochemical products got released and dominate the market (Samada and Tambunan, 2020). However, in less developed countries, its implementation is currently limited due to lack of skilled manpower, high costs, and inadequate infrastructure (Ivase et al., 2017). This pushes to heavy dependency on utilization of synthetic pesticides that pose unacceptable risks.

Chemical pesticides and fertilizer cause numerous side effects in human health including tingling, muscle pain, skin disease, tremor, stress, sleep disorder, dizziness, memory, and cardiac problems. So, moving toward biopesticides such as EPF provides growers with valuable tools in pest management because its active and inert ingredients are generally considered safe and supportive to sustainable agro-ecosystem (Rana et al., 2019). Its demand is increasing day by day globally despite its instability in nature (Nath, 2020). Recent discoveries added that many of EPF play additional roles in nature as endophytes and possibly promote plant growth in rhizosphere (Vega et al., 2009).

Problems and Objectives of the study

The study of EPF as biopesticides has dated for several years. In recent studies they are considered as modern pest control weapons for the avoidance of persistent and hazardous effects of conventional pesticides for human safety, environmental protection, and animal welfare. However, they are highly varied not only in virulence but vulnerability to environmental abiotic factors including temperature and UV radiations. On top of this the inherent capacity of the fungal strain (Yu et al., 2020; Kim et al., 2011), fungal conidia formulation type (Oliveira et al., 2018), and geographic location of isolates (Rangel et al., 2005) relatively influence their tolerance potential. Currently, some EPF strains of *Metarhizium* and *Beauveria* reported with considerable persistence in field application while *Cordyceps* strains were highly sensitive to variable temperature (Wu et al., 2020; Alfiky, 2022).

EPF have a unique character among the other entomopathogenic organisms and cause interruption of the physiological process of treated insects by cuticular penetrations (Mora et al., 2017). EPF possibility infect insect through oral route (Mannino et al., 2019). Nevertheless, their infectivity varied with the life stages of the host insect, concentration of inoculum, and time elapsed post inoculations. The younger the larvae found with high susceptibility to fungal treatment application (Asi et al., 2013; Idress et al., 2022), while virulence for older larvae, pupae and soil-dwelling insects are relatively lower. There are limited reports available on virulence to 6^{th} instar larvae and pupae of *S. litura* since fully grown larvae and pupation under the soil make them resistant and inaccessible for control. Hence their implementation in agricultural fields is limited.

Selection of virulent fungal strains from different sources of isolation and geographic location against older larvae and pupae of insect pests could be crucial for development of mycopesticides. In addition to this, abiotic factors adversely affect the potential of occurrence, persistence, and epizootics are considered as determinant factors. Thus, understanding the relationship between *in vitro* heat tolerance and field persistence of fungal strains could be an advancement to discover the potential usability of *in vitro* thermotolerance assay for selection of field persistent fungal strains.

Therefore, this study aimed to evaluate the inherent virulence capacity of 16 fungal strains of three genera viz *Beauveria*, *Metarhizium* and *Cordyceps* against last instar larvae and pupae

of *S. litura* under laboratory condition. The second objective of the current study is to assess *in vitro* heat tolerance of 32 fungal strains at 45℃ for 2 h and 4 h heat exposure and to demonstrate performance synchronicity of selected five fungal strains for heat tolerance and field persistence on soils and cucumber leaves in hot and cold seasons.

CHAPTER I

Virulence of entomopathogenic fungi against last instar larvae and pupae of common cutworm, *Spodoptera litura* (Lepidoptera: Noctuidae)

Abstract

Virulence of soil and insect source strains of EPF was assessed against the sixth instar larvae and pupae of *S. litura*. It is one of the most notorious pests with significant yield losses to more than one hundred plant species all over the world. Bioassay treatments done with 16 EPF strains of three genera (*Beauveria*, *Cordyceps* and *Metarhizium* sp.) under laboratory conditions. Inoculation of conidial suspension at 1×10^8 conidia/mL resulted utmost cumulative mortality 100% with *M. rileyi* Nr4, *M. pingshaense* MS1, *M. brunneum* ARSEF 3294 and *M. pingshaense* ARSEF 8736, which were significantly different to *B. bassiana* TBY-L1, *B. bassiana* OMNS 150429-3 and *B. bassiana* GHA. While a maximum pupal mortality 100% with *B. brongniartii* TNO6 and 96.7% with *C. javanica* Czy-LP were achieved at 10 DPI, which was significantly higher than other fungal strains. LT_{50} values signified that *M. pingshaense* MS1 and *M. brunneum* F709 recorded the most virulent strains for larvae while *B. brongniartii* TNO6 was observed with the least LT_{50} value (5.27 days) for pupae followed by *C. javanica* Czy-LP and *M. pingshaense* MS2. In addition, large percent of deformed adults registered with *B. bassiana* TNO12 and *M. rileyi* Nr4. Current finding suggested that *B. brongniartii* TNO6, *C. javanica* Czy-LP, *M. brunneum* F709 and *M. pingshaense* MS2 were virulent EPF strains to both developmental stages of *S. litura* and could be developed as mycoinsecticides for integrated pest management programs.

Keywords: Biocontrol, Metarhizium, Beauveria, Cordyceps, Spodoptera litura, Mortality, Virulence, Deformed adult.

1.1 Introduction

S. litura (Lepidoptera order: Noctuidae family) is a serious agricultural pest worldwide, attacking more than 389 species of industrial crops in both tropical and subtropical areas (Lin et al., 2019). In the Asia-pacific region, it is reported from more than 100 plant species; *Gossypium hirsutum, Ricinus communis, Brassica oleracea* are major host plants (Ahmad et al., 2013). The larvae cause damage on different stages of the host plants and adults are free living. Its economic loss ranges from 25.8–100% depending on the stage of the crop and its level of infestation in the field, where an average larval population of 0.1 larvae/head is capable enough to cause 2 % of plant infestation on cabbage crop (Sahu et al., 2020). Post research studies added that leaf consumption per larvae on tomato, sweet pepper and cucumber reached 4.45 g, 2.89 g and 5.08 g, respectively (Vashisth et al., 2012). It is also threatened in soybean fields and reduced yield up to 90.2% to 96.4% and 94.8 to 96.4 for vegetative and generative phases (Fattah et al., 2020). Furtherly the pest was reported in citrus plant infestation like orange's cultivars (Feutrell's Early) and Seedless Kinnow (Ullah et al., 2016).

Agricultural sectors were dependent on various insecticides such as organophosphates, carbamates, and pyrethroids to control the pest, however, those chemical pesticides posed unacceptable risks to human health, natural enemies, and environment contaminations (Mohamed and Mohamed, 2015; Neylon et al., 2022). On top of that, the pest has developed resistance to insecticides. Nevertheless, less resistance was reported for selective pyrethroids and new chemical insecticides like emamectin benzoate (Tong et al., 2013; Saleem et al., 2016; Ahmad et al., 2022). Studies on implementation of biological agents (microbials) has been done for years. Recent research findings exposed that entomopathogenic effect of Bt was greatly influenced by the host plant (Narvekar et al., 2018); though, the toxins Cry1A.105 and Cry2Ab2 were detrimental for larvae to adult stage of the pest (Pratiwi et al., 2016). In pest management, SpltMNPV were also studied as outstanding microbes. It caused high mortality on 4^{th} and 5^{th} larvae of *S. litura* and inhibited adult emergence (Monobrullah and Shankar, 2008). Further synergetic virulence of NPV was enhanced than applied alone. Integrated application of SpltMNPV with Azadirachtin, chlorantraniliprole, and emamectin benzoate (Proclaim® 19EC) exhibited higher larvae mortality and reduction in pupation and adult emergence (Nathan and Kalaivani, 2005; Nathan and Kalaivani, 2006; Yasin et al., 2020; Sarwar et al., 2021). According to Maqsood et al. (2017), NPV effectiveness get

reduced with age of treated larvae and was inefficient to older larvae and pupae. The need of extra chemical combinations made it incapable in managing *S. litura* pupae and last instar larvae.

Apart from NPV and *Bt*, EPF have gained distinct advances in agriculture due to their potential in pest management and are considered as efficient substitutes of chemical insecticides. EPF cause hindrances in the growth and development of insect pests in ecofriendly manner. They interrupt the normal physiological process by reducing relative consumption rates and ultimately cause death of the insect pest (Ullah et al., 2019). Those organisms invade insects through cuticular penetration (Mora et al., 2017), which is a unique character among the other entomopathogenic organisms such as *Bt* and viruses. In addition to that, an EPF *B. bassiana* had the possibility to infect insect pests through oral route because of shared genes with other non-fungal pathogens that infect orally, such as *Bt* (Mannino et al., 2019). EPF varied in response against the life stages of *S. litura.* The report by Herlinda et al. (2020) showed that *B. bassiana* and *M. anisoplia* had a positive impact on second instar larvae of *S. litura* with a mortality of 86.67% each at conidia suspension of 1×10^6 conidia/mL. An isolate of *B. bassiana* (BbR2) caused 83.33% mortality (Dhar et al., 2019). *M. lepidiotae* (NCHU-9), *M. pinghaense* (NCHU-11, NCHU-64), and *M. anisopliae* (NCHU-69 and 95) caused 100% mortality at 6 DPI (Chang et al., 2021). Inoculation of *B. bassiana* BNBCRC caused 80% mortality on third instar larvae at 1×10^8 conidia/mL (Petlamul and Prasertsan, 2012).

Extensive study of virulence of EPF has been done with different life stages of *S. litura*, however, lesser infectivity has been reported for pupae applications (Asi et al., 2013; Idress et al., 2022). This could be due to the hard shell of the pupa and lifecycle of the pest, where pupation took place inside the soil at depth of 5 to 6 cm (Deepak et al., 2020). Some research findings reported that soil drenching of *M. anisopliae* (ARSEF 7487) at 10^8 conidia/g reduced adult emergence by 81.3% from treated pupae (Anand et al., 2009). Nevertheless, it is a fact that virulence of entomopathogenic organisms is directly correlated with susceptibility of life stage of the host insect, concentration of inoculum, and time elapsed post treatments. Studies on the relationship stated that younger larvae exhibit more susceptibility to fungal treatments (Asi et al., 2013; Idress et al., 2022).

Application of EPF added more attentions and intensive investigation for biological control of insect pests, however, their usage is limited to specific life stage, age, and habitat of the

target pest. As for *S. litura*, limited reports are available on virulence against 6th instar larvae and pupae since fully grown larvae and pupation under the soil made them resistant and inaccessible for biocontrol. Hence laboratory bioassay was conducted to evaluate virulence of EPF strains against last instar larvae and pupae with the aim to develop sound biocontrol strategy in managing *S. litura*.

1.2 Materials and Methods

Rearing and preservation of *S. litura* for bioassay

The insect for virulence assay was provided by ISHIHARA SANGYO KAISHA, LTD. (Osaka, Japan). Newly emerging adults were kept in a rearing box covered with glass for oviposition. It was maintained at a temperature: 25 ± 3℃, photoperiod: 14L:10D). The laid egg masses were harvested and transferred to sterilized petri plates for larval emergence and kept in insect rearing room for 3 d under ambient temperature for egg hatching; from which larvae were collected and fed with artificial diet up to third instars larvae. Immature larval instars (4^{th}–6^{th}) for experiment purposes were provided with fresh cabbage leaf every other day, while some matured larvae continued with artificial diet and pupate in a separate plastic container (17 × 25 × 8 cm). Emerging adults were fed with 10% honey solution for egg laying for the next generation.

Preservation and maintenance of EPF cultures

EPF strains of *Beauveria* sp. *Cordyceps* sp. and *Metarhizium* sp. were selected from different source of isolations and geographic origins (Table 1.1). MS1, MS2, MS3, and MS5 were isolated from soil by the author. GHA was isolated from a commercial mycoinsecticide "Botanigurd" (Arysta LifeScience Corp., Tokyo, Japan). The F strains were provided from fungal culture collection of Forest Entomology Division at Forestry Forest Product Research Institute, Tsukuba, Ibaraki, Japan) and currently deposited as NBRC strains in Biotechnology Center at National Institute of Technology and Evaluation (Kisarazu, Chiba, Japan). The ARSEF strains were provided from USDA-ARS Entomopathogenic Fungal Culture Collection (Ithaca, NY, United States). The other isolates were from the culture collection of Laboratory of Insect Pathology and Microbial Control, Kyushu University. Strains were cultured in PDA (potato dextrose broth 24 g/L, agar 15 g/L, chloramphenicol 0.3 g/L) and SSYA (sucrose 20 g/L, peptone 10 g/L, yeast extract 10 g/L, agar 15 g/L, chloramphenicol 0.3 g/L). Fungal cultures were incubated at 25℃ for 15 d before use in bioassay.

Preparations of fungal conidia suspension

Harvesting of fungal conidia was made by scraping the surface of 14–15 d old culture using a sterile spatula. It was transferred to 50 mL plastic tube and added with 0.05% Tween-80

solution. The mixture was shaken and thoroughly mixed with a vortex mixer for one to two minutes. The conidial suspension was filtered through sterile cotton to remove hyphal debris. The conidial concentration of each strain was determined by using a hemocytometer. There after the desired conidial suspension was produced at 1×10^8 conidia/mL by serial dilution for bioassay test.

Virulence assay

The last instar larvae and pupae (3 or 4 d after the last molting) were inoculated by dipping in a 10 mL suspension in a 50 mL centrifuge tube for 10 s, dried on a paper towel, and transferred to a plastic container (12.5 × 12.5 × 4.5 cm, 5 larvae and 10 pupae/container) lined with a paper towel moistened with RO water. The containers had a slight ventilation through small gaps in the lid. Ten milliliters of 0.05% Tween 80 were used for the control experiment (mock inoculation). The containers were placed in a large plastic box (32.5 × 25 × 44 cm) with a loose lid and kept at 25 ± 1℃ and 14L:10D photoperiod in an incubator (MIR-554-PJ, PHC Corp., Tokyo, Japan). Humidity in the small container in the same condition (without insects) reached 100%RH within 1 h after closing the container and was kept constant for 10 days, as measured with Illuminance UV Recorder TR-74Ui-S (T&D Corp., Nagano, Japan). The inoculated larvae were fed fresh cabbage leaves and transferred to new plastic containers every 48 h. The individuals were inspected every other day until the 10th day after inoculation, and cadavers were transferred to other containers each time they occurred. Survival of pupae was confirmed by picking up pupae with a sterilized tweezer and checking movements in abdomen. The number of cadavers with or without mycelia and sporulation was recorded every other day. The frequencies of deformations found in emerged adults in the pupal assays were also recorded on the final day. Ten larvae or pupae were used for each replicate. The test was repeated three times using an independently prepared fresh inoculum.

Statistical analysis

For mortality, mycosis proportion (frequency of cadavers with growing fungus observed on the surface) and affected proportion (frequency of dead or deformed individuals) at 10 DPI, arcsine transformation was conducted prior to multiple comparisons. Then, means of three trials for different fungal strains were compared using Tukey's honestly significant difference

test (Tukey's HSD test) at a significance level of 5% with R4.2.2 (function "glht" in the R package "multcomp").

Correlation on virulence of the fungal strains against both larvae and pupae of the target pests was evaluated through the LT_{50}. The LT_{50} was calculated by "dose.p" function (glm, $p = 0.5$) from the R package "MASS" with default parameters on a binomial model fitting the mortality to DPI, with the "glm" function from the R package "stats".

1.3 Results

Virulence of EPF to *S. litura* larvae and pupae

The last instar larvae and pupae of *S. litura* showed relative susceptibility to all applied EPF strains in the lab bioassays. Cumulative mortality of the last instar larvae ranged from 56.7% to 100%, which was significantly higher than the control (Figure 1.1, Tukey's HSD test, $p < 0.05$). Larval mortality for *M. pingshaense* MS1, *M. rileyi* Nr4, *M. pingshaense* ARSEF 8736, and *M. brunneum* ARSEF 3294 reached 100% at 10 DPI, which was significantly higher than that for *B. bassiana* GHA (Tukey's HSD test, $p < 0.05$). The commercial fungal strain, *B. bassiana* GHA, was recorded with a minimum percent of larval mortality among the assayed strains (Figure 1.1).

There was considerable variation in the response of fungus-treated pupae. Among them, the *B. brongniartii* TNO6 and *C. javanica* Czy-LP had the highest pupal mortality (Figure 1.2). The two strains were significantly higher in terms of pupal mortality than *B. bassiana* OMNS150429-2, *B. bassiana* TNO12, *M. pingshaense* ARSEF 8736, and *M. rileyi* Nr4 (Tukey's HSD test, $p < 0.05$). The minimum percent of pupal mortality at 10 DPI was observed with 16.7% of *M. rileyi* Nr4 (Figure 1.2). *M. brunneum* F709 and *M. pingshaense* MS2 had considerable virulence (≥90% mortality) against both stages, while the other strains with higher virulence against larvae remained less virulent to pupae and *vice versa*.

Expression of fungal mycosis on cadaver (larvae and pupae of *S. litura*)

Development of fungal mycelium was observed for all fungal strains in larval assay; however, it was restricted for some strains in pupal assay (Figure 1.1, 1.2 and 1.3). As indicated in Figure 1.2, *M. pingshaense* MS5, *M. rileyi* Nr4, *M. pingshaense* ARSEF 8736, *B. bassiana* OMNS 150429-2, and *B. bassiana* TNO12 were not observed with apparent fungal mycelial growth or conidia productions on pupal cadaver. The development was gradual during the post inoculation period. Exposure at 2 d to 6 d to all the treatments applications showed equal performance. However, strains were significantly different at 8 and 10 DPI, where *M. rileyi* Nr4 was found with the highest larval mycosis proportion (96.67%) and showed significant difference from the least 6 strains (the 5 *B. bassiana* strains and *M. pingshaense* MS5) at 10 DPI (Figure 1.1, Tukey's HSD test, $p < 0.05$).

On top of that, the two strains, *B. brongniartii* TNO6 and *C. javanica* Czy-LP, resulted in the highest mycosis proportion in pupae at 10 DPI (86.67% and 96.67%, respectively), which were significantly higher than those of the least 9 strains (Fig. 1.2, Tukey's HSD test, $p < 0.05$).

Deformed adults and survival rate of treated pupae

Results on adult emergence from pupae elucidated that >90% survival rates from control found different as compared to fungal treated pupae. Strains of *B. brongniartii* TNO6 and *C. javanica* Czy-LP showed the highest reduction of adult emergence (0% and 3.3%, respectively), followed by *M. brunneum* F709 (8.33%) and *M. pingshaense* MS2 (10%). Nevertheless, higher survival rate of treated pupae was seen with *M. rileyi* Nr4 (83.3%), *B. bassiana* OMNS150429-2 (66.7%), *B. bassiana* TNO12 (56.67%), and *M. pingshaense* ARSEF 8736 (56.67%).

In the pupal assay, malformation of wings and abdomen was observed for emerged adults from pupae treated with fungi, but not for adults in the mock inoculation (Figure 1.4). In the comparison of affected proportion (frequency of dead or malformed individuals), the proportions for *B. brongniartii* TNO6, *M. brunneum* F709, *M. pingshaense* F2865, and *M. pingshaense* MS2 reached 100%, which was significantly different from those for the least 3 strains (*B. bassiana* OMNS150429-2, *B. bassiana* GHA, *M. rileyi* Nr4) (Fig. 1.2, Tukey's HSD test, $p < 0.05$). Besides, variations were observed for proportion of malformed adults. *B. bassiana* TNO12 and *M. rileyi* Nr4 yielded utmost 36.7% deformed adults each, which was the highest among the applied treatments. Zero percent of deformed adults recorded from fungal strains of *B. brongniartii* TNO6 and *C. javanica* Czy-LP and the control (Figure 1.2).

The LT_{50} values of larvae and pupae treated with EPF

The LT_{50} values of larval and pupae coordinated in X and Y axis, respectively (Figure 1.5). It showed fungal strains of *B. brongniartii* TNO6, *C. javanica* Czy-LP, *M. brunneum* F709, *M. pingshaense* MS2 and *M. pingshaense* F2685 had strong virulence against both life stages of *S. litura* among the tested strains.

1.4 Discussion

Bioassay

Experiments handled to determine potential virulence of selected fungi strains for last instar larvae and pupae of *S. litura*. Entomopathogenic viruses and bacteria require high technology of production and their infectious nature greatly influenced by host plant like *Bt* (Narvekar et al., 2018). On top of that, the lesser infectivity of SpltMNPV to mature larvae (Tran and Chaudhari, 2002; Ahmad et al., 2018) and reduced inhibiting nature for adult emergence (Monobrullah and Shankar, 2008) made fungal strains an appealing option to overcome the devastating nature of *S. litura*. It's easier and cheaper mass production techniques and virulence capacity are also strengthened for intensive studies.

The current research finding demonstrated that selected fungal strains were capable to infect last instar larvae and pupae of *S. litura* at fungal conidial suspension 1×10^8 conidia/mL. *M. rileyi* Nr4, *M. pingshaense* MS1, *M. pingshaense* ARSEF 8736 and *M. brunneum* ARSEF 3294 were more infectious and resulted utmost mean cumulative mortality (100%) among the 16 fungal strains for last instar larvae, while results inversed with pupae mortality. Maximum pupae average mortality was observed with 90% of *M. pingshaense* MS2, 83.3% of *M. pingshaense* MS5, 87.5% of *M. pingshaense* F2685 and 91.67% of *M. brunneum* F709 which was quite higher as compared to the scores with *M. rileyi* Nr4, *M. pingshaense* ARSEF 8736 and *M. brunneum* ARSEF 3294. Anand et al. (2009) reported that *Metarhizium* strains such as *M. anisopliae* caused maximum pupae mortality up to 85.8%. Other studies explicated that *M. rileyi* causes larval mortality up to 63.33% to 2nd instar larvae at 1×10^8 conidia/mL (Grewal et al., 2021) and reached 85% at 1×10^7 spores mL^{-1} to 3rd instar larvae of *S. litura* (Saheb et al., 2021).

Furthermore, laboratory bioassay resulted higher average larval and pupal mortality with *C. javanica* Czy-LP and *B. brongniartii* TNO6 which were virulent enough to both life stages of *S. litura*. The comparative virulence of *B. brongniartii* TNO6 with the reference strain *B. bassiana* GHA was significantly higher in cumulative mortality to larvae and pupae of *S. litura*. Various research papers publicized *B. bassiana* GHA was relatively effective at lower doses to house fly larvae (*Musca domestica* L.) (Anderson et al., 2011) and apple sawfly (*Hoplocampa testudinea*) (Swiergiel et al., 2016). It was also reported that GHA caused less

mortality but high interruption of metamorphosis in lepidopteran insect pest such as *S. exigua* (Pacheco et al., 2021).

In general, the selected EPF strains used for the bioassay were comparatively infective to larvae than pupae. This is due to the higher tolerance of pupae to fungal applications. Therefore, the current results have in line with previous research findings (Asi et al., 2013). In addition, bioassays experiments revealed deformed adults from pupae of *S. litura* treated with EPF. Strains of *B. bassiana* TNO12 and *M. rileyi* Nr4 showed maximum malformed adult emergence 36.67% each. Although no more detailed information is available with previous research studies, malformation effects of *Metarhizium* strains to *S. frugiperda* were reported (Herlinda et al., 2020).

1.5 Conclusions

The comparisons of cumulative mean mortalities and LT_{50} values provide tangible evidence to understand fungal strains varied in virulence to last instar larvae and pupae of *S. litura*. *M. rileyi* Nr4, *M. pingshaense* MS1, *M. pingshaense* ARSEF 8736 and *M. brunneum* ARSEF 3294 were infectious to last instar larvae; nevertheless, they found with the least virulence to pupae of *S. litura*. While the least LT_{50} was recorded with *M. pingshaense* MS1 followed by *M. brunneum* F709 for larvae and strains of *B. brongniartii* TNO6, *C. javanica* Czy-LP and *M. pingshaense* MS2 against pupal stage. Those strains were seen with parallel virulence potential to both life stages of *S. litura.* Pupae treated with EPF produce abnormalities, adults with short and wrinkled wings and deformed abdomen. *B. bassiana* TNO12 and *M. rileyi* Nr4 showed maximum malformed adults during the bioassay experiments. Therefore, bioassay findings on virulence capacity of selected strains against 6^{th} instar larvae and pupae of *S. litura* proved that *B. brongniartii* TNO6, *M. brunneum* F709, *C. javanica* Czy-LP and *M. pingshaense* MS2 were composite strains and could be intensively investigated and developed as potential mycopesticides in integrated pest management programs against *S. litura*.

Table 1.1. EPF strains used for bioassay

Fungal species	Strain	Source of isolation	Location
B. bassiana	TBY-L1	Lepidoptera (Larvae)	Fukuoka, Japan
	OMNS150429-2	Lepidoptera (Larvae)	Ibaraki, Japan
	OMNS150429-3	Coleoptera (Adult)	Ibaraki, Japan
	TNO12	Lepidoptera (Larvae)	Iwate, Japan
	GHA	Botanigurd (commercial mycoinsecticides)	_
B. brongniartii	TNO6	Coleoptera (Adult)	Iwate, Japan
C. javanica	Czy-LP	Lepidoptera (Pupae)	Fukuoka, Japan
M. brunneum	ARSEF 3294	Lepidoptera: Noctuidae, *S. frugiperda* (Larvae)	Colima, Mexico
	F709 (NBRC 112631)	Coleoptera: Scarabaeidae	Hokkaido, Japan
M. pingshaense	MS1	Soil	Fukuoka, Japan
	MS2	Soil	Fukuoka, Japan
	MS3	Soil	Fukuoka, Japan
	MS5	Soil	Fukuoka, Japan
	ARSEF 8736	Lepidoptera: Noctuidae, *Spodoptera* sp. (Larvae)	Cameron Highlands, Malaysia
	F2685 (NBRC 112657)	Hymenoptera:Vespidae	Ibaraki, Japan
M. rileyi	Nr4	Lepidoptera: Noctuidae (Larvae)	Fukuoka, Japan

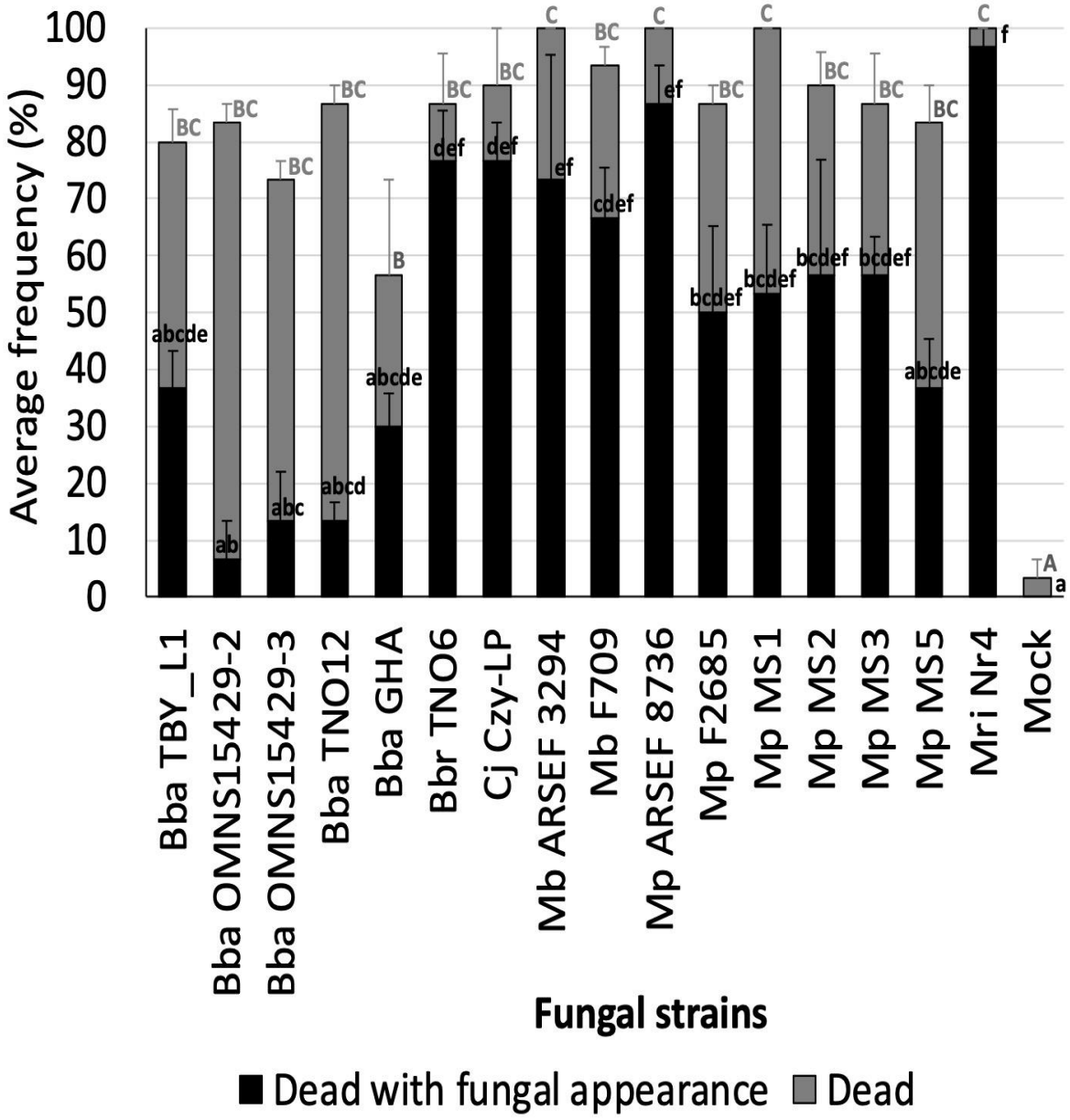

Figure 1.1. Mortality and mycosis proportions of *S. litura* (last instar larvae) at 10 DPI of 16 EPF strains. Error bars represent standard errors for cumulative frequencies (n=3). The cumulative bars with the same letters are not significantly different (Tukey's HSD test, $p>0.05$). Scientific names for the EPFs are abbreviated as follows: *B. bassiana*, Bba; *B. brongniartii*, Bbr; *C. javanica*, Cj; *M. brunneum*, Mb; *M. pingshaense*, Mp; *M. rileyi*, Mri.

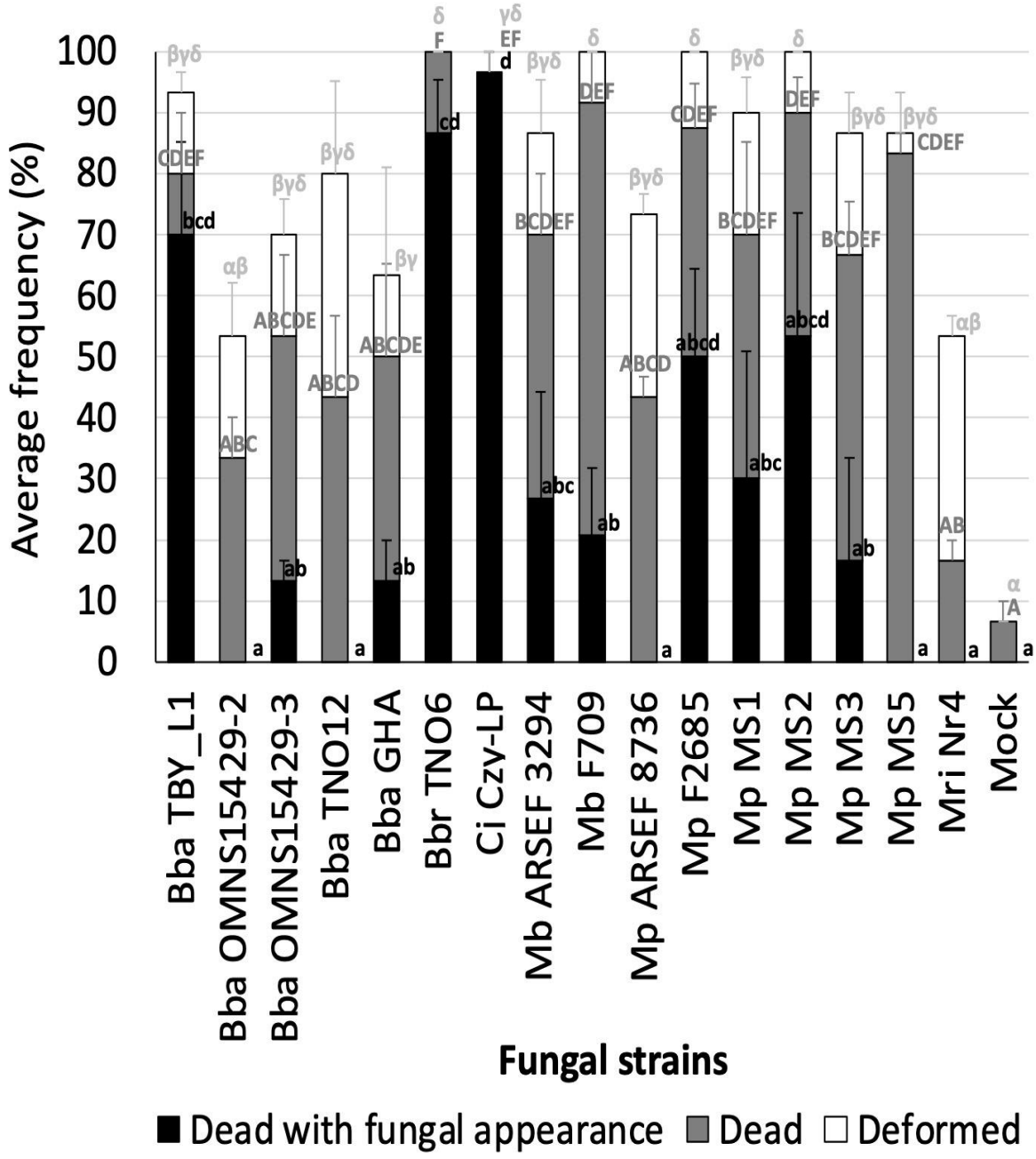

Figure 1.2. Mortality, mycosis, and deformed proportions of *S. litura* (pupae) at 10 DPI of 16 EPF strains. Error bars represent standard errors for cumulative frequencies (n=3). The cumulative bars with the same letters are not significantly different (Tukey's HSD test, p>0.05). Scientific names for the EPFs are abbreviated as follows: *B. bassiana*, Bba; *B. brongniartii*, Bbr; *C. javanica*, Cj; *M. brunneum*, Mb; *M. pingshaense*, Mp; *M. rileyi*, Mri.

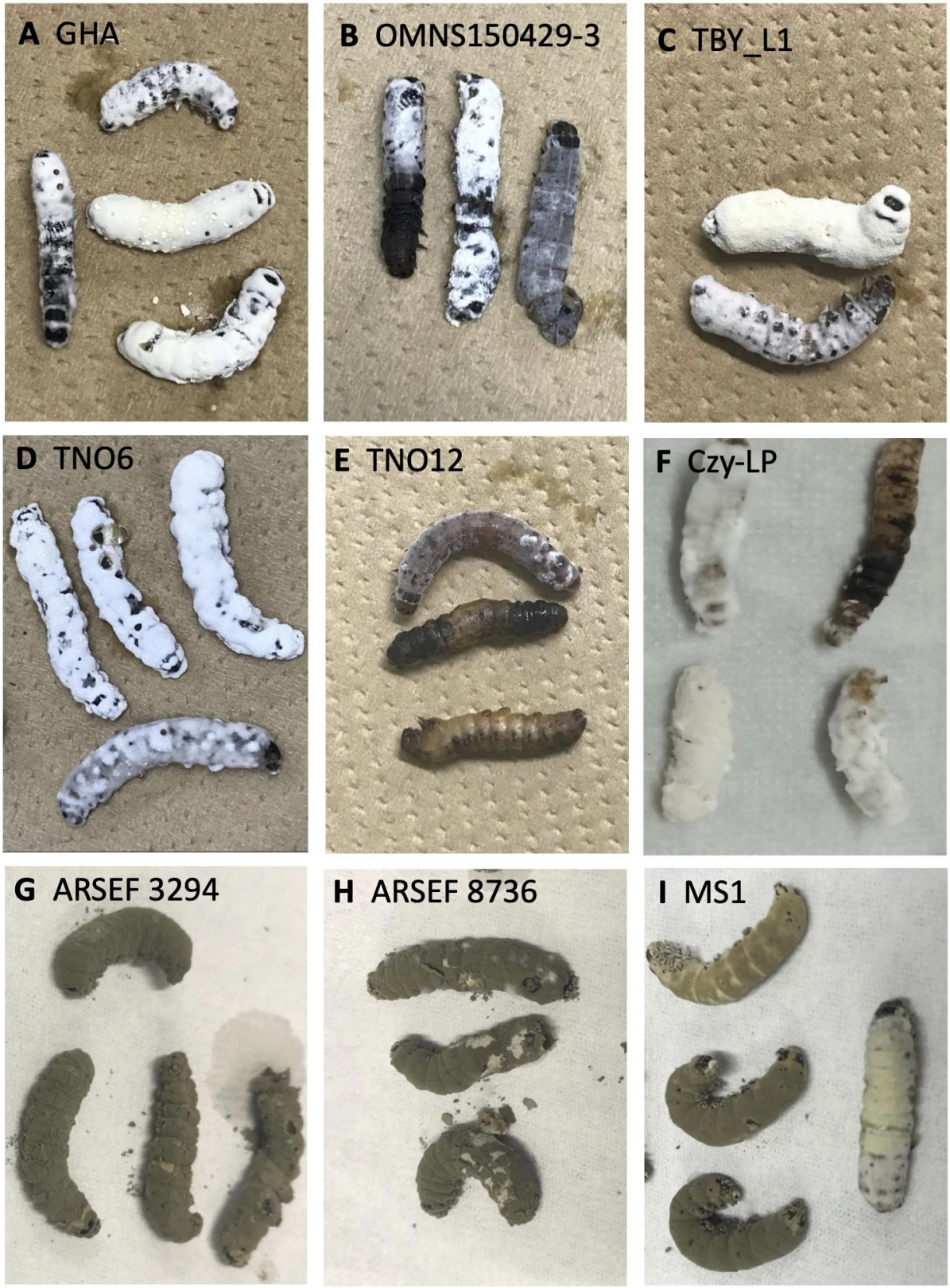

Figure 1.3. Fungal mycosis observed on the larvae (A-M) and pupae (N, O) of *S. litura* inoculated with EPF.

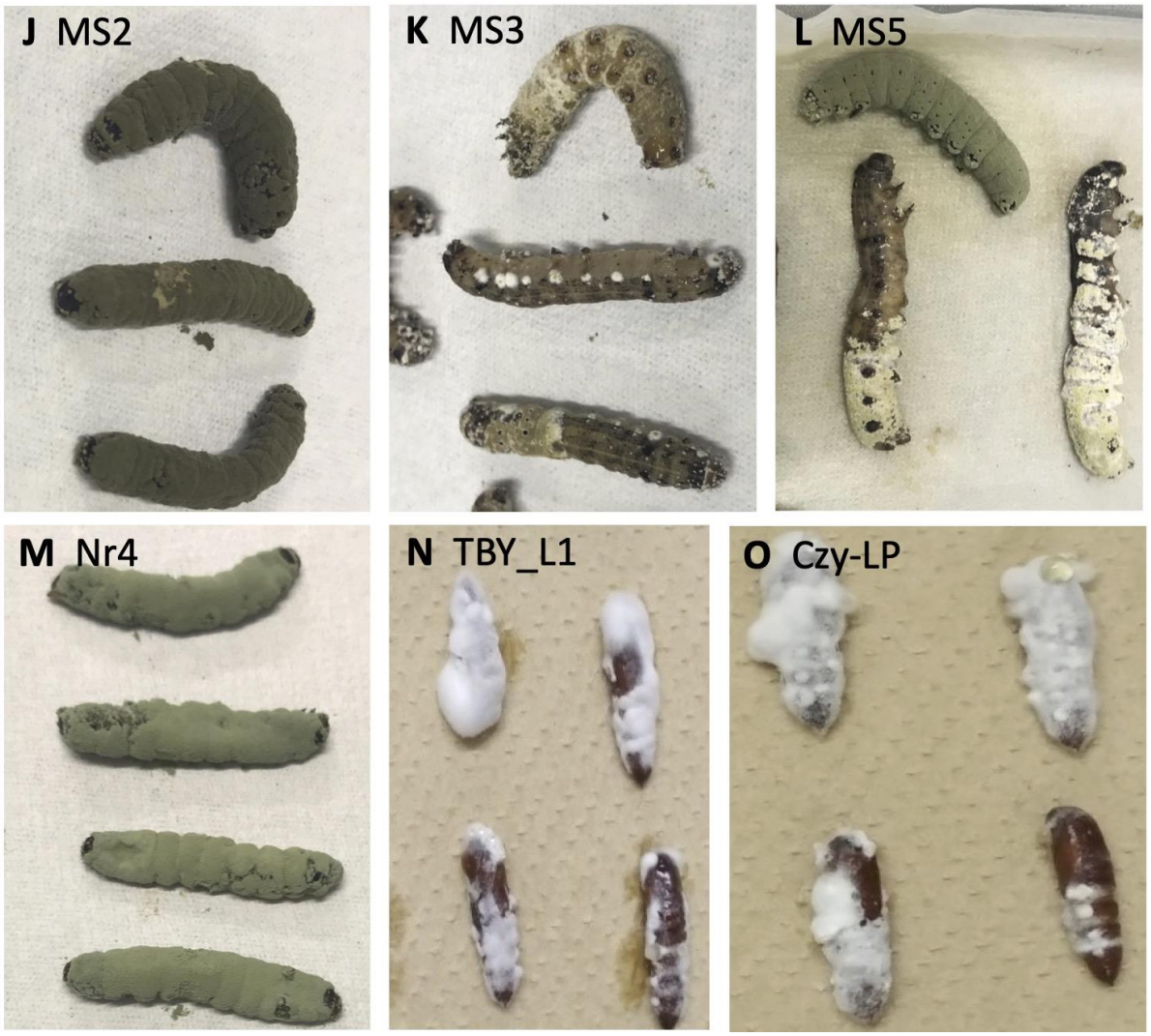

Figure 1.3. (Continued).

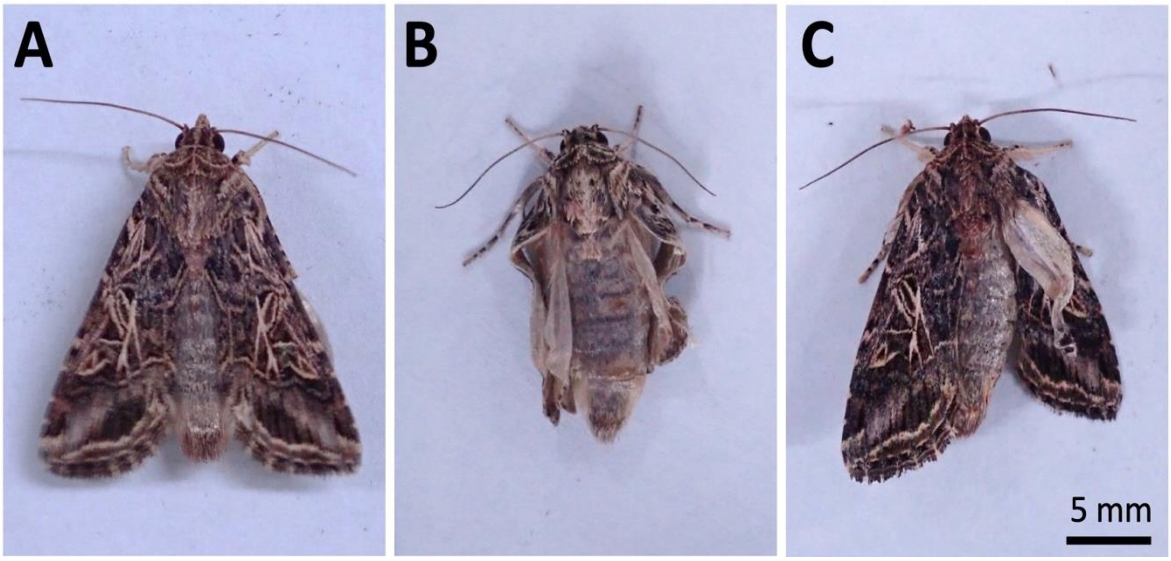

Figure 1.4. Sublethal effect on adults by inoculation of *M. rileyi* (Nr4) on pupae of *S. litura*. (A) An adult with no symptom (mock inoculation). (B) An adult with severely wrinkled wings (treated wirth Nr4 at its pupal stage). (C) An adult with a slightly wrinkled wings (treated wirth Nr4 at its pupal stage).

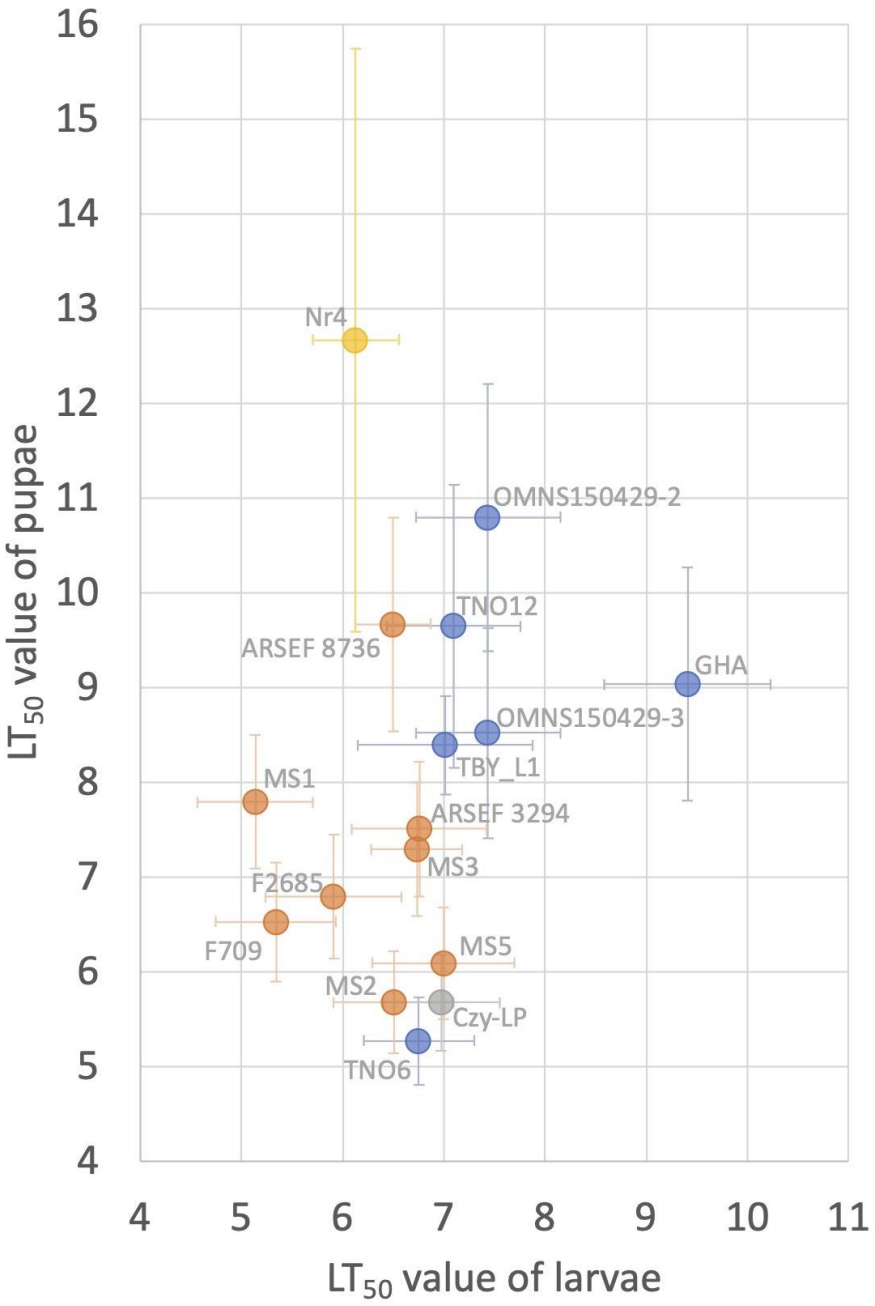

Figure 1.5. Virulence (LT_{50} value) of 16 EPF strains against last instar larvae and pupae of *S. litura*. Error bars indicate 95% confidential interval. Plot colors represent fungal species or genera: *B. bassiana* or *B. brongniartii,* blue; *C. javanica*, grey; *M. brunneum* or *M. pingshaense*, orange; *M. rileyi,* yellow.

CHAPTER II

In vitro heat tolerance assessment of entomopathogenic fungal strains

Abstract

Entomopathogenic *Beauveria* sp. and *Metarhizium* sp. are widely used biological agents to control agricultural insect pests. However, their heat sensitivity limits survival under field conditions and virulence after applications. In the current study, 32 EPF represented three genera viz *Beauveria,* C*ordyceps* and *Metarhizium,* were assayed for thermotolerance. It was found that fungal strains behaved differently at 45℃ for 2 h and 4 h heat exposure with survivals ranging from 0 to 100%. Fungal strains including *C. javanica* Czy-LP, *C. fumosorosea* BPS2, *B. brongniartii* TNO6, and *M. pingshaense* MS3 were among the least performers and significantly lower in survival.

Key words: Beauveria, Metarhizium, Cordyceps, Heat tolerance, CFUs.

2.1 Introduction

EPF are components of biological weapons in integrated pest management programs. They are applied in various agro-climatic zones to manage wide host of arthropod agricultural pests. However, evaluations on its virulence capacity and its fitness to withstand given environmental conditions are primary steps for future use in control of pest population. Abiotic factors such as temperature, sunlight, humidity, rainfall have detrimental effect and restricts the use of EPF (Qayyum et al., 2021). Hence fungal strains belonging to different species, such as *Cordyceps*, *Beauveria*, and *Metarhizium*, behave differently regarding abiotic factors. Most *Metarhizium* isolates tolerates a temperature exposure of 40℃ with relative germination capacity of 90% and above at 12 h post exposure. However, viability deteriorated with increasing temperature and time of exposure. At temperatures of 45℃ for 8 h and 12 h exposure, *M. anisopliae* isolates viz., ARSEF 324 and ARSEF 3609 had a germination capacity of 91.6 and 79.4%, and 90 and 47.1%, respectively (Rangel et al., 2005).

Heat tolerance test for selections of field persistence of *Metarhizium* sp. showed a germination percentage of 83.6–97.4 at 40℃ exposures, however, its relative germination capacity dropped significantly to 15.0–69.3% at 42℃ (Mesquita et al., 2020). Other reports clarified that germination viability of *Metarhizium* isolates (NCHU-9, 69, and 95) and *B. australis* (NCHU-113) get diminished up to 0.4–5.2% and 0.4–2%, respectively, after exposure for 90 and 120 min at 45℃ (Chang et al., 2021). Variability in thermotolerance among *Beauveria* isolates including standard isolates (GHA and ARSEF 252) exhibited higher heat tolerance with thermal death point of 46℃ for 6 h and relatively cold active. On top of that, it was demonstrated that exposure to high temperatures slowed down germination speed of fungal conidia (Fernandes et al., 2008), and higher performance of *B. australis* NCHU-113 was observed over the tested *Metarhizium* isolates (Chang et al., 2021).

Recent studies revealed that relative virulence and heat tolerance capacity of fungal isolates affected by substrates of conidia production. Yu et al. (2020) demonstrated significant relationship between conidial thermo-tolerance and fungal media. Italian millet was found suitable for production of thermo-tolerant conidia compared to sorghum and other grains. Higher thermotolerance and germination rate at 50°C for 8 h exposure was recorded for conidia of *B. bassiana* isolates produced on Italian millet as compared to ones of *M.*

anisopliae isolates. It was reviewed that millet media was potential for production of highly thermotolerant stains of *B. bassiana* (GHA and ERL1170) and *M. anisopliae* (ERL1171 and ERL1540) than 1/4 SDAY and whey permeate (Kim et al., 2011). On the other hand, emulsifiable oil-based formulations protect fungal conidia from adverse effects of high-water temperature and probably enhance viability and temperature tolerance of EPF isolates (Oliveira et al., 2018).

Suitable and effective usage of EPF in integrated pest management triggers for the selection of thermotolerant fungal strains. Fungal strains with wide range of heat tolerance may have great capacity to be launched and implemented in biological control strategies. Therefore, *in vitro* thermotolerance assay of 32 fungal strains were done for selection of tolerant strains for use in field persistence assay. Strains were collected from different locations (areas with different temperature ranges) and sources (soil and arthropod hosts). Comparisons among the fungal strains explored variable response for heat tolerance.

2.2 Materials and Methods

Preservation and maintenance of EPF cultures

Thirty-two fungal strains (2, 15, and 15 strains from *Cordyceps*, *Beauveria,* and *Metarhizium*, respectively) from different isolation sources and geographic origins were used in this study (Table 2.1). Sixteen of the 32 strains were those used in Chapter I. Among the new 16 strains, BS3 and BPS2 were isolated from soil by the author. The sources of the F strains were presented in Chapter I. The other strains were from the laboratory culture collection. Strains were cultured on PDA (potato dextrose broth 24 g, agar 15 g, chloramphenicol 0.3 g per 1 L RO water), SSYA (sucrose 20 g, peptone 10 g, yeast extract 10 g, agar 15 g, chloramphenicol 0.3 g per 1 L RO water), and oatmeal (oats flour 60 g, agar 15 g, chloramphenicol 0.3 g per 1 L RO water) at 25℃ for about 15 days before use in heat tolerance test.

Preparations of fungal conidia suspension

Fungal conidia harvested by scraping the surface of 14–15 days old fungal culture using stainless steel spatula and kept in 1.5 mL plastic tube with sterile aqueous solution of 0.05% Tween-80. The mixture got shaken and thoroughly mixed with magnetic shaker for one to two minutes. Fungal hyphal debris in the suspension were removed by a cotton equipped syringe to get purified conidial suspension. Concentration of conidia of the suspension was determined by using a hemocytometer. Serial dilution was done to produce conidial suspension of 2×10^3 conidia/mL.

In vitro heat tolerance assessment

Evaluation of selected EPF strains for heat tolerance was done by inoculating 100 µL of 2×10^3 conidia/mL on PDA media. Conidial suspension of each fungal strain applied with 6 PDA plates, of which two plates for each heat exposure period 2 h, 4 h and 0 h (control). Inoculated plates were kept in an incubator at a temperature of 45℃ for 2 h and 4 h heat exposure while control plates placed in controlled room at temperature of 25 ± 1℃. The heat assay was carried out in a total of 3 replications. Data on CFUs recorded consecutively at 3, 5 and 7 DPI.

$$\text{Relative CFUs (survival rate)} = CFUsp_1 / CFUsp_0$$

Where $CFUsp_1$ is CFUs recovered 2 h and 4 h exposure at 45℃. $CFUsp_0$ is CFUs from control plates incubated at 25℃).

Statistical analysis

Means of relative CFUs from 3 replications for different fungal strains were compared using Tukey's honestly significant difference test (Tukey's HSD test) at a significance level of 5% with R4.2.2 (function "glht" in the R package "multcomp").

2.3 Results

Varied responses to the heat exposure were observed in relative CFUs counts of fungal strains. In the 2 h heat exposure assay, most assayed strains were recovered up to 85%, except with few fungal strains resulted in lower counts of CFUs. Based on the average scores, *B. bassiana* OMNS150429-2, *B. brongniartii* TNO6, *C. fumosorosea* BPS2, *C. javanica* Czy-LP, and *M. pingshaense* MS3 were seen with lower relative CFUs counts (Figure 2.1). The overview of average scores indicated that higher thermotolerance was observed from *Metarhizium* strains than *Beauveria* and *Cordyceps* strains, with the least relative CFUs counts from *C. fumosorosea* BPS2 (0.1%).

The results also signified that 4 h heat exposure caused serious distortion in CFUs for most fungal strains, although the reaction to the heat exposure was different with regard to the applied fungal strains (Figure 2.2). Only few *Metarhizium* strains crossed 90%, such as *M. pingshaense* F1234, *M. pingshaense* F2685 and *M. pingshaense* MP34. On the while, *Beauveria* and *Cordyceps* strains never touched the ceiling point. The top 9 strains exceeded 70% relative CFUs, of which 7 strains were *Metarhizium* spp. and the remaining 2 were *B. bassiana* (B10 and B11). The bottommost 0% relative CFUs count was recorded for *C. javanica* Czy-LP, *C. fumosorosea* BPS2, *B. brongniartii* TNO6, and *M. pingshaense* MS3.

2.4 Discussion

Laboratory based heat tolerance assay was conducted to evaluate thermotolerance capacity of three fungal genera (*Beauveria, Metarhizium and Cordyceps*). EPF are temperature sensitive organisms and respond great variability. Its efficiency under field conditions also highly influenced by environmental factors (temperature). The present study was designed to demonstrate the relative germination potential of 32 fungal strains at 45℃ in 2 h and 4 h heat exposure. Significant variation was observed among different fungal species. Strains of same fungal species also exhibited disparities in performance of *in vitro* heat tolerance. Outcomes clarified that CFUs count of fungal strains get distorted with the lengthy periods of heat exposure and selected temperature level (45℃).

Higher relative CFUs count was observed at 2 h exposure with majority of fungal strains hit moderate to high relative conidia germinations at 85–100% except lower results recorded from *Beauveria* strains TBY-L1, OMNS 150429-2, TNO6, and *Cordyceps* BPS2, Czy-LP. However, heat exposure up to 4 h showed violent germination reduction and lethal death point for strains of *B. bassiana* OMNS 150429-2, *B. bassiana* TBY-L1, *B. brongniartii* TNO6, *C. fumosorosea* BPS2, *C. javanica* Czy-LP, and *M. pingshaense* MS3.

Among the assayed fungal strains, *Metarhizium* strains demonstrate dominance in heat tolerance in a prolonged exposure. Current results agreed with Rangel et al. (2005) which showed that *M. anisopliae* tolerates a temperature exposure of 40℃. Viability of EPF strains deteriorated with increasing temperature and time of exposure (Mesquita et al., 2020). Chang et al. (2021) revealed that a diminished fungal conidia germination of *Metarhizium* and *Beauveria* isolates was seen after exposure for 90 and 120 min at 45℃. In contrary to the above statements, *Beauveria* isolates (GHA and ARSEF 252) recorded higher cold activity and heat tolerance with exposure at 46℃ for 6 h (Fernandes et al., 2008).

Present study results added that higher heat sensitivity observed with *Cordyceps* strains. This agreed with the reports of Alfiky (2022) that *C. javanica* was susceptible compared to *M. anisopliae* and *B. bassiana* strains at 45℃ heat exposure. Apart from the reduction in relative CFUs of fungal conidia, extended period of high temperature exposure caused delay in germination speed of fungal conidia. It was in confirmation with the findings of Fernandes et al. (2008).

2.5 **Conclusions**

Evaluations on tolerance assay of fungal strains showed substantial variation in the ability to withstand high temperatures. *B. bassiana* B11, and *M. pingshaense* F1234, F2685, MP34**,** and *M. brunneum* F3239 counted with maximum (>80%) average relative CFUs at 4 h heat exposure. Whereas strains of *B. brongniartii* TNO6, *C. fumosorosea* BPS2, and *C. javanica* Czy-LP identified among the least performers at 45℃.

Table 2.1. EPF strains used for *in vitro* heat tolerance assay

Fungi Species	Strains	Isolation source	Geographic location
B. bassiana	TBY-L1	Lepidoptera (Larvae)	Fukuoka, Japan
	OMNS150429-2	Lepidoptera (Larvae)	Ibaraki, Japan
	OMNS150429-3	Coleoptera (Adults)	Ibaraki, Japan
	TNO12	Lepidoptera (Larvae)	Iwate, Japan
	GHA	Botanigurd (commercial)	_
	B3	Coleoptera: Scarabaeidae	Fukuoka, Japan
	B6	Hymenoptera:Vespidae	Fukuoka, Japan
	B7	Homoptera: Pentatomidae	Fukuoka, Japan
	B9	Coleoptera: Cerambycidae	Fukuoka, Japan
	B10	Homoptera: Plataspidae	Kagoshima, Japan
	B11	Hymenoptera	Fukuoka, Japan
	OMNS180915-30	Homoptera: Largidae	Mie, Japan
	OMNS190810-22	Homoptera: Cicadidae	Miyazaki, Japan
	BS3	Soil	Fukuoka, Japan
B. brongniartii	TNO6	Coleoptera (Adults)	Iwate, Japan
C. fumosorosea	BPS2	Soil	Fukuoka, Japan
C. javanica	Czy-LP (MAFF 244759)	Lepidoptera (Pupae)	Fukuoka, Japan
M. brunneum	ARSEF 3294	Lepidoptera: Noctuidae, *S. frugiperda* (Larvae)	Colima, Mexico
	F395 (NBRC 112628)	Coleoptera: Scarabaeidae	Nagano, Japan
	F709 (NBRC 112631)	Coleoptera: Scarabaeidae	Hokkaido, Japan
	F3239 (NBRC	Homoptera: Cydnidae	Fukuoka, Japan

	112635)		
	OMNS170126-1	Homoptera: Cydnidae	Saga, Japan
M. pingshaense	MS1	Soil	Fukuoka, Japan
	MS2	Soil	Fukuoka, Japan
	MS3	Soil	Fukuoka, Japan
	MS5	Soil	Fukuoka, Japan
	ARSEF 8736	Lepidoptera: Noctuidae, *Spodoptera* sp. Larvae	Cameron Highlands, Malaysia
	ITO2G10-10-9	Soil	Fukuoka, Japan
	F1234 (NBRC 112649)	Coleoptera: Scarabaeidae	Ibaraki, Japan
	F2685 (NBRC 112657)	Hymenoptera:Vespidae	Ibaraki, Japan
	MP34	Soil	Fukuoka, Japan
M. rileyi	Nr4	Lepidoptera: Noctuidae (Larvae)	Fukuoka, Japan

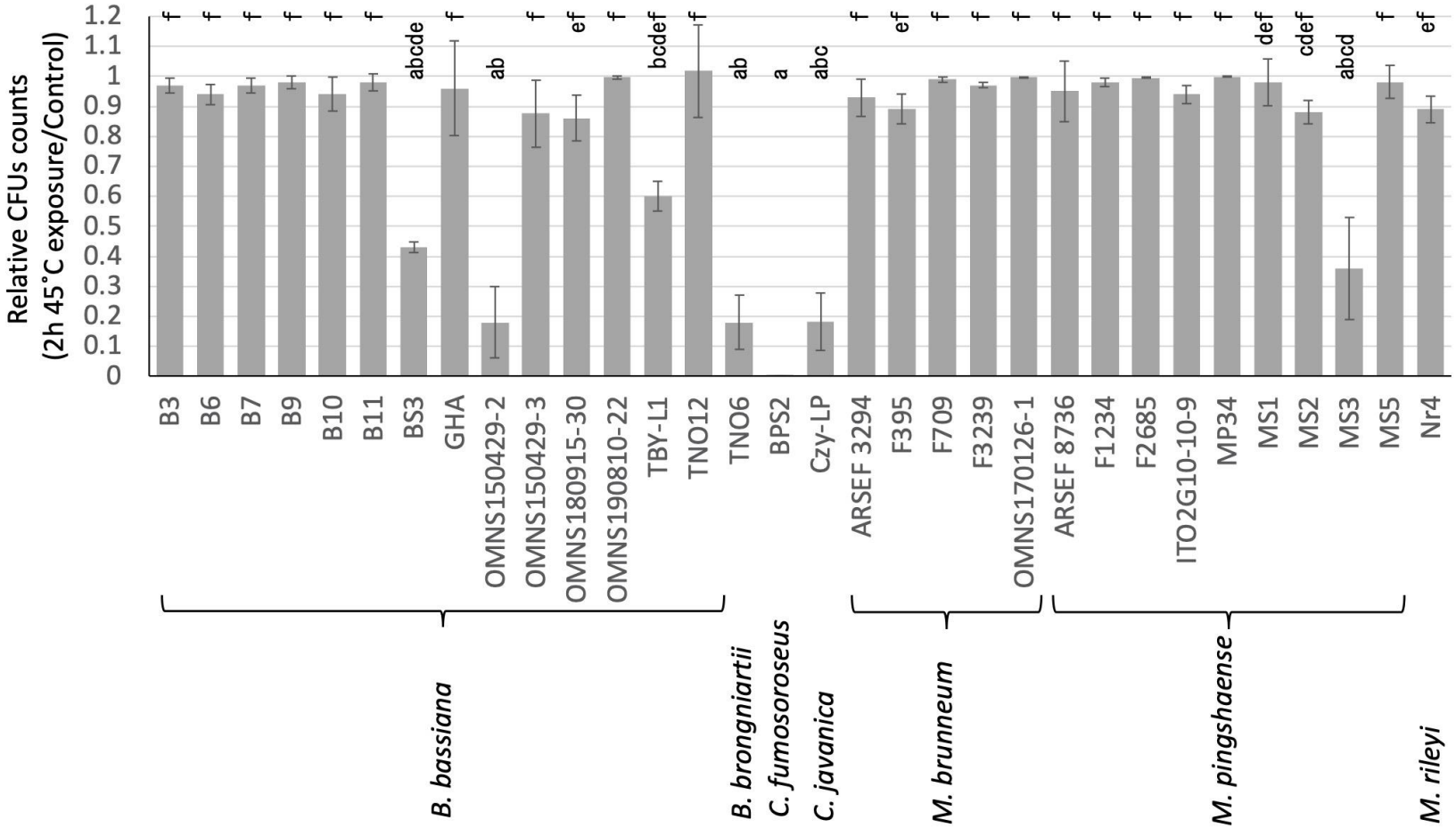

Figure 2.1. Comparisons of heat tolerance (conidia exposed to 45℃ for 2 h) of 32 EPF strains. Bars represent means of relative CFUs counts (n=3) and standard errors. The bars with the same letters are not significantly different (Tukey' s HSD test, $p>0.05$).

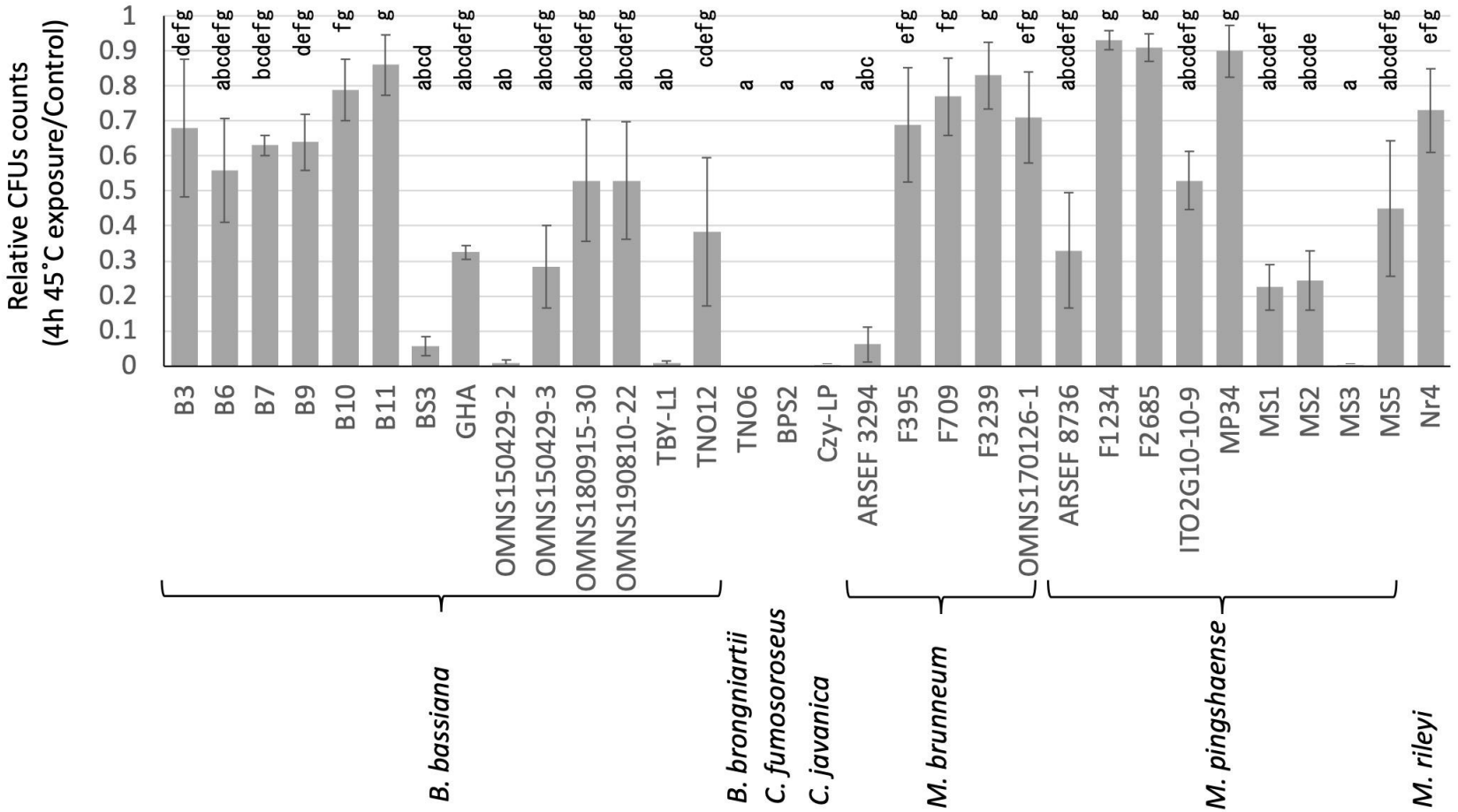

Figure 2.2. Comparisons of heat tolerance (conidia exposed to 45℃ for 4 h) of 32 EPF strains. Bars represent means of relative CFUs counts (n=3) and standard errors. The bars with the same letters are not significantly different (Tukey' s HSD test, $p > 0.05$).

CHAPTER III

Evaluation of entomopathogenic fungal strains for field persistence and its relationship to *in vitro* heat tolerance

Abstract

EPF are naturally safe and eco-friendly biological agents. Their potential of host specificity and ease of handling made them appealing options to substitute synthetic pesticides in pest control programs. However, they are highly delicate and unstable under field conditions. Therefore, the current experiment was held to search out persistent fungal strains by defining the relationship between *in vitro* heat tolerance and field persistence for five selected fungal strains. Current results on assay for persistence on leaf and soil revealed that *M. pingshaense* F2685, *M. pingshaense* MS2, and *M. brunneum* F709 exhibited maximum relative CFUs counts (survival rate) in hot seasons from sampled soils and leaves and in cold season from soil samples. Whereas relative CFUs of *B. brongniartii* TNO6 found significantly higher in cold weather leaf treatment application as compared to hot season and found as persistent as other fungal strains while higher deterioration of fungal conidia seen with *M. pingshaense* MS2. In the current study, strains of *B. brongniartii* TNO6 and *Cordyceps javanica* Czy-LP were relatively vulnerable in field condition with utmost CFUs reduction and least survival rates. Further the relationship of heat tolerance and field persistence was strong linear positive correlations, which elucidated that *in vitro* heat test could be used in selection of field persistent fungal strains for hot season applications.

Keywords: Entomopathogenic fungi, Heat tolerance, Field persistence, CFUs, Survival rates.

3.1 Introduction

EPF are one pillar of biopesticides used in managing various agricultural pests. Its potential of diseases causing, ease of production and host specificity made them best substitutes of synthetic pesticides in integrated pest management programs. However, they are highly vulnerable to environmental abiotic factors including temperature, UV radiations and relative humidity etc. Hence field evaluations on performance are necessary tasks to understand their potential under field conditions. Tolerance capacity also relatively influenced by several factors such as inherent capacity of the fungal strain, culture media (Yu et al., 2020; Kim et al., 2011), fungal conidia formulation type (Oliveira et al., 2018), and geographic location of isolates (Rangel et al., 2005).

In outdoor treatments and laboratory studies, they have shown with varying degree of persistence to heat, cold and UV radiations and reported *M. anisopliae* were more potential to survive in agro-ecosystems as resistant to UV radiation with shortest LT_{50} (10.51 days) compared to *B. bassiana* (18.09 days) (Herlinda et al., 2019). It added that field establishment and abundance of *M. brunneum* strains found with greater fungal inoculum and interfere in survival nature of wireworms in soil (Reinbacher et al., 2021). According to Kim et al. (2020), incorporation of *M. anisopliae* (JEF-314) for controlling soil dwelling insects respond positive results in larval mortality 66.39 ± 12.22% after 15 days of treatment application with 3 g millet grains cultured fungus added to 50 g nursery bed soil. Further proved the efficiency of *B. bassiana* (ZGNKY-5 strain) against fire ant (*Solenopsis invicta)* at three dosages of 500 mL, 750 mL, and 1,000 mL per nest through injection control technology methods under field conditions (Li et al., 2016) and its persistence capacity seen higher gave a way as potential bio-control agents for groundnut insect pests (Sahayaraj and Namachivayam, 2011). On the other hand, research exposed the virulence and field persistence of EPF strains degraded with lengthy time and reduced its efficiency as well against insect pest (*S. litura)* in glasshouse pot and field experiments. CFUs of fungi treatments declined by 93% and 99% within 180 days and 360 days under pot and field trials respectively. Fungal strain of *M. anisopliae* (Ma09) found longest persistence as compared to *B. bassiana*. In addition, abundancy of fungal strains conidial density greatly varied with in the soil layers, where middle layer (10–15 cm) of the treated soils found with higher conidia density than upper soil layer (Yang et al., 2019).

The effectiveness and field persistence of EPF strains is heavily restricted by numerous environmental factors and its vulnerable nature limits their usage in integrated pest management programs under field conditions. This of course pushes agricultural growers to use synthetic insecticides in diverse agro-ecosystems to protect devastating pests, that leads to increase a concern on environmental pollution and human health risks. It is obviously understood that EPF strains with wide range of heat tolerance have great capacity to be launched and implemented in biological control strategies. Therefore, leaf and soil persistence assay of five fungal strains (1 strain from *Beauveria* sp., 3 strains from *Metarhizium* spp., and 1 strain from *Cordyceps* sp.) carried out under field conditions. Selected fungal strains were having varying degree of *in vitro* heat tolerance and sources of isolation. The experiment was done to evaluate the synchrony between *in vitro* thermotolerance and field persistence of strains and to determine the capacity and field stability of strains in soil and cucumber leaf under hot and cold seasons.

3.2. Materials and Methods

Preservation and maintenance of EPF cultures

Fungal cultures used during the study were composed of three fungal species viz. *Beauveria* sp., *Cordyceps* sp. and *Metarhizium* sp. with different source of isolations and geographic origins (Table 3.1). Selected strains were priorly assayed *in vitro* heat tolerance under laboratory conditions and found with varying degree of thermotolerance. Fungal strains were cultured in PDA (potato dextrose broth 24 g, agar 15 g, chloramphenicol 0.3 g per l L RO water), SSYA (sucrose 20 g, peptone 10 g, yeast extract 10 g, agar 15 g, chloramphenicol 0.3 g per 1 L RO water) and oatmeal agar (oats flour 60 g, agar 15 g, chloramphenicol 0.3 g per 1 L RO water). Fungal cultures were incubated at 25℃ for 14-21 days before use for field persistence assay.

Preparations of fungal conidia suspension

Harvesting of fungal conidia was made by scraping the surface of 14–21 days old fungal culture added to 200 mL and 150 mL bottle containing sterile aqueous solution of 0.05% Tween-80 per fungal strain for soil and leaf persistence assay, respectively. The mixture was shaken and thoroughly mixed with magnetic shaker for one to two minutes and hyphal debris removed using funnels equipped with gauze to get purified conidial suspension. The conidial concentration of each strain was determined by dropping 10 µL of diluted conidia suspension on hemocytometer and direct count using compound microscope with a magnification rate of 200×. A desired volume of 200 mL and 150 mL fungal conidia suspension was prepared at the concentrations of 1×10^8 and 2×10^7 conidia/mL for soil and leaf persistence assays, respectively.

Persistence assessment of fungal strains from soil

Experiment on persistence assay of EPF on soil was conducted in a greenhouse at Ito campus, Kyushu university (Motooka, Fukuoka, Japan). Trial with soil persistence assay of five fungal strains conducted in hot (30th June 2022–28th July 2022) and cold (23rd November 2022–21st December 2022) for a period of 28 days. Temperature, humidity, strength of UV, and brightness in the filed during the period were continuously recorded with a datalogger (Illuminance UV Recorder TR-74Ui-S, T&D Corp., Nagano, Japan) (Table 3.2). It was done in four replications (total of 20 pots), where pots filled with garden soil and properly labeled.

Inoculations of freshly prepared fungi conidial suspension was made by evenly injecting 50 mL suspension at concentrations 1×10^8 conidia/mL per pot using 5,000 µL micro pipettes. Immediately at the day of inoculation, soil samples were collected from each replication (pot) with 2 cm diameter pork borer and samples composed in plastic bottle and weighed. Each sample spot area was about 3.14 cm^2 and total of eight sampling spots/replication (pot) (area = 25.12cm^2) (Figure 3.1). Sampling materials were disinfected with 70% ethanol, kitchen bleach and rinse with RO water for every strain application. Plastic bottle with composite soil sample weighed and added with 135 mL 0.05% Tween80 solutions thoroughly shaken for about 10 seconds from which 30 mL of soil suspension transferred to 50 mL tube and got vortexed in 1 minute. Further dilutions made by adding 800 µL of soil suspension to 540 µL of 0.05% Tween 80% solutions in 1.5 mL tube, out of which serial dilutions of 10 folds 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} were prepared. Conidial inoculation was done with 10^{-3} and 10^{-4} dilutions on selective PDA plates (PDA added with 0.03% chloramphenicol and 0.1% cycloheximide), where 100 µL suspension form dilutions of 10^{-3} and 10^{-4} replicated in two petri plates each for all replications and kept at 25℃. CFUs was checked and recorded to evaluate persistence of fungal strains during hot and cold seasons. For further studies fungal treated pot soils were irrigated two times a week and soil sampling repeated at 28 DPI to determine the variation on CFUs count, survival rates, and overall performance of fungal strains in soil within the given duration.

$$\text{Relative CFUs counts} = \text{CFUs } t_1/\text{CFUs } t_0$$

Where CFUs t_1 represented the CFUs recovered at 28 DPI. CFUs t_0 represented the CFUs recovered immediately after application of conidial suspension per cm^2 soil area and per gram soil sample.

Persistence assessments on cucumber leaf

Experiment on persistence assay of EPF on leaves was conducted in a greenhouse at Ito campus, Kyushu university (Motooka, Fukuoka, Japan). Evaluation of EPF strains for leaf persistence carried out in 3 pots per fungal strain (15 pots for the five strains). Treatment applied for a period 7 days in hot (8^{th} July–15^{th} July 2022) and cold seasons (12^{th} November–19^{th} November 2022). Freshly prepared conidial suspension 50 mL at concentration of 2×10^7 conidia/mL per pot or plant sprayed uniformly. Cucumber leaves sampled on day of inoculation and 7 DPI where a total of four leaves/plant marked with four dots in the size of 4 cm × 4 cm. On the day of inoculation, a total of six leaves (two leaves per pot) per fungal

strain were sampled and considered as replications. Leaf suspensions were prepared by deicing sampled leaves, added with 25 mL of 0.05% Tween-80 in 50 mL tube, and got vortexed for 1 min. The leaf pieces were transferred to a mortar, grinded with a pestle, and suspended in 25 mL of 0.05% Tween-80. Further, 600 µL of each vortexed and grinded leaf suspensions were mixed in a 1.5 mL tube and 10^2 and 10^3 times dilutions were made. The original and diluted suspensions of each sampled leaves were inoculated (100 µL/plate, two plates/leaf) on selective PDA plates and kept at 25℃ for CFUs counts. Leaf sampling was repeated at 7 DPI to determine the variation on potential capacity of CFUs and relative survival rates of the assayed fungal strains. Whereby the stability of assayed fungal strains in hot and cold seasons under field conditions got evaluated.

Statistical analysis

Means of CFUs and relative CFUs of the 6 replications (leaf persistence assay) and 4 replications (soil persistence assay) were compared by Tukey's honestly significant difference test at a significance level of 5% with R4.2.2 (command "Tukey HSD" in default package sets).

3.3 Results

Persistence potential of fungi stains on cucumber leaf

In the persistence assay on leaves in hot season, maximum average of CFUs obtained from *Metarhizium* strains compared to *Beauveria* and *Cordyceps* strains ranging 2.7×10^4–1.2×10^5 CFUs/cm^2 leaf and 5.7×10^3–5.99×10^4 CFUs/cm^2 leaf on day of inoculation and 7 days of exposure, respectively (Figure 3.2). Where *M. brunneum* F709 followed by *M. pingshaense* (F2685 and MS2) recorded highest CFUs per cm^2 leaf area on day of inoculation (0 DPI). However, the rank among the *Metarhizium* strains was changed at 7 DPI. Utmost CFUs count was recorded for *M. pingshaense* MS2 and *M. brunneum* F709. Nevertheless, all these *Metarhizium* strains remained at par and were higher in CFUs count at 7 DPI than *C. javanica* Czy-LP and *B. brongniartii* TNO6 (Figure 3.2). Further those strains found with maximum relative survival rate (0.698, 0.481) (Figure 3.3). The result also added that *M. pingshaense* MS2 showed significant difference to strains of *C. javanica* Czy-LP, which was the most susceptible and showed least performance of leaf persistence with relative survival rates (0.27) (Figure 3.3, Tukey's HSD test, $p < 0.05$).

In the persistence assay on leaves in cold season, trials generated that though bit higher CFUs count on day of inoculation and exhibited difference among the strains (Figure 3.2). However, a sharp reduction in CFUs count of all fungi strains was seen at 7 DPI. As for the survival rate (relative CFUs) during the 7 days, *M. brunneum* F709 was observed with the highest value however not significantly different from the least strain of *M. pingshaense* MS2 (Figure 3.3). *M. pingshaense* MS2 remained very delicate in cold weather and performed as weak as *C. javanica* Czy-LP. Current overview of the five fungi stains were not suitably to withstand cold weather under field conditions. It looked detrimental with maximum CFUs reduction (81.94–93.64%). Nevertheless, some fungal strains like *M. brunneum* F709 possess moderate leaf persistence in both seasons. The current research through higher deterioration of fungal conidia during cold weather resulted in least relative survival rates of assayed fungal strains as compared to hot weather.

Persistence potential of fungal strains in soil

In the persistence assay on soil in hot season, *M. pingshaense* (F2685 and MS2) remained higher in CFUs recovery at 28 DPI (Figure 3.4). Both strains attained utmost relative CFUs (0.69, 0.50), which were by far highly persistent among the assayed fungal strains in hot

seasons (Figure 3.5). The current field trial added that *B. brongniartii* TNO6 and *C. javanica* Czy-LP had significantly lower in relative CFUs (0.006 and 0.0087, respectively) (Figure 3.5, Tukey's HSD test, $p < 0.05$).

Trials under cold weather conditions had also adverse effect on persistence capacity of fungal strains in soil. However, strains response showed variation; *M. pingshaense* MS2, *M. brunneum* F709 and *M. pingshaense* F2685 got the uppermost mean CFUs count per cm^2 soils (Figure 3.4). Among all the fungal treatments, *M. pingshaense* MS2 showed higher in CFUs count in both sampling dates. Whereas maximum survival rates recorded for *M. pingshaense* F2685, *M. brunneum* F709 and *M. pingshaense* MS2 (0.967, 0.862, and 0.802, respectively), and significantly different from the rest fungal strains (Figure 3.5, Tukey's HSD test, $p < 0.05$). The minimum relative survival (0.133) was observed from *C. javanica* Czy-LP. Furtherly the study showed *B. brongniartii* TNO6 was averagely persistent in soil during cold weather compared to hot conditions (Figure 3.5). Eventually, the field persistence assay explicated that *Metarhizium* strains were adequately persisted in soil and could be suitable to implement in hot and cold seasons.

Performance relationship in field and *in vitro* heat tolerance of EPF strains

Correlation analysis between the two parameters (field persistence and heat tolerance) was executed to prove the relationship in endurance of fungal strains under hot seasons field conditions with respect to *in vitro* heat tolerance. Current experiment confirmed that there was strong positive linear correlation between the parameters of heat tolerance (2 h and 4 h exposure to 45℃) and persistence (survival rate) on leaf and soil (Table 3.3).

3.4 Discussion

Persistence assay of EPF strains carried out in hot and cold season with the objective to demonstrate the relationship of *in vitro* thermotolerance for selection of field persistent fungal strains. EPF are components of biopesticides, easy for mass production, naturally safe and regulating pest population with high host selectivity (Digvijay et al., 2017). On top of that, they are potential enough in management of vector insects and possess high capacity of compatibility with synthetic pesticides to combat chemical resistance and pest resurgence **(**Samuels et al., 2016). Apart pest control abilities, they play roles in promoting plant growth as endophytes (Vega et al., 2009). However, their stability and implementation in agricultural fields get limited through various reasons. The biotic and abiotic factors adversely affect its potential occurrence, persistence, and epizootics. Fungal characters, insect biology, and environments are some of the underlined determinant factors (Qayyum et al., 2021). Therefore, it is timely to find out the relationship between heat tolerance and field persistence to discover the potential usability of thermotolerance assay for selection of field persistent EPF strains. Current study results evidenced that fungal strains behaved differently and exhibited varying degrees of persistence in leaf and soil assays under hot and cold field condition. Whereby strains of *Metarhizium* species recorded with highest endurance among the assayed strains of fungal species. *M. pingshaense* MS2 and *M. brunneum* F709 found with maximum mean CFUs and survival rate (0.698 and 0.481) per cm^2 leaf area respectively after 7 days exposure in hot season. Similar reports by Swiergiel et al. (2016), explained that *M. brunneum* strains were persistent compared to *B. bassiana*, however, contrary results emphasized higher persistence capacity of *B. bassiana* than *M. anisopliae* on soyabean leaves in mild temperatures (Souza et al., 2022). The current result also added that strains of *M. pingshaense* F2685 and MS2 scored higher survival rate on soil during hot season. All the *Metarhizium* strains (F2685, MS2, and F709) showed significantly higher performance under cold condition for soil assay. However, reversed relative survival of all assayed fungal strains were seen in cold weather experiments, where CFUs significantly lowered in leaf assay. Further results revealed that strains of *B. brongniartii* TNO6 and *C. javanica* Czy-LP are susceptible scored minimum mean survival rates (relative CFUs count) at 7 DPI and 28 DPI for leaf and soil persistence in hot and cold field conditions. However, *B. brongniartii* TNO6 observed with moderate capacity of persistence on soil in cold weather trails, which was considerably higher compared to hot season records.

Moreover, results proved that *Metarhizium* strains were tolerant for both *in vitro* heating at 45℃ and field condition compared to *Beauveria* and *Cordyceps* strains. It agreed with Alfiky (2022), which reported *C. javanica* was very susceptible for high temperatures and scored least germination percentage. According to Wu et al. (2020), *C. javanica* GA17 was highly vulnerable to variable temperature and UV light and inferior to *M. brunneum* F52 and *B. bassiana* GHA. The relationship between the two parameters (heat tolerance and field persistence) confirmed with strong positive correlation coefficient values.

3.5 Conclusions

Executed results on mean relative CFUs count (considered as survival rates) excavated that EPF strains showed variation in field persistence, of which *M. pingshaense* (F2685 and MS2) and *M. brunneum* F709 adequately persisted in field as well heat tolerance assays compared to *B. brongniartii* TNO6 and *C. javanica* Czy-LP. However, those strains were highly deteriorated in cold weather leaf assays. Unlike, their relative survival was substantially higher in soil assays. Hence, strains of *B. brongniartii* TNO6 and *C. javanica* Czy-LP were evidenced very delicate and susceptible which were uncomfortable applications in hot and cold seasons. Eventually fungal strains showed a strong positive correlation between heat tolerance and overall field persistence in hot season. It recommended that heat tolerance assay could be used to select field persistent fungal strains.

Table 3.1. EPF strains used in the filed persistence test

Species	Strains	Isolation source	Location	Heat tolerance
B. brongniartii	TNO6	Coleoptera (Adult)	Tono, Iwate, Japan	Susceptible
C. javanica	Czy-LP (MAFF 244759)	Lepidoptera (Pupae)	Chikuzen-yamate, Fukuoka, Japan	Susceptible
M. pingshaense	MS2	Soil	Ito campus, Fukuoka, Japan	Tolerant
M. pingshaense	F2685 (NBRC 112657)	Hymenoptera: Vespidae	Ibaraki, Japan	Tolerant
M. brunneum	F709 (NBRC 112631)	Coleoptera: Scarabaeidae	Hokkaido, Japan	Tolerant

Table 3.2. Weather data during field persistence assays

Parameter		Leaf persistence assay		Soil persistence assay	
		Hot/Summer season	**Cold/Fall season**	**Hot/Summer season**	**Cold/Fall season**
Soil Temperature (°C)	Day high	32.66	19.3	35.69	20.91
	Day low	29.27	15.18	28.02	4.21
	Mean	31.04	17.18	31.67	12.35
Brightness (klx)	Day high	28.75	12.60	28.75	12.70
	Day low	12.57	3.76	90.06	0.13
	Mean	19.75	8.69	20.08	5.66
UV Radiation (mW/cm^2)	Day high	0.459	0.186	0.465	0.94
	Day low	0.214	0.06	0.162	0.000042
	Mean	0.327	0.142	0.343	0.0897
Air Temperature (°C)	Day high	32.25	16.71	32.89	19.34
	Day low	28.12	13.63	28.12	3.18
	Mean	30.24	15.39	30.18	10.61
Humidity (%)	Day high	58.48	64.96	66.5	72.42
	Day low	49.21	54.21	47.63	33.88
	Mean	53.38	58.18	54.39	51.42

Table 3.3. Correlation coefficient values between field persistence and *in vitro* thermotolerance of the five EPF strains.

	Persistence on leaves	**Persistence on soils**	**Heat tolerance (45 °C , 2 h)**	**Heat tolerance (45 °C , 4 h)**
Persistence on leaves	1			
Persistence on soils	0.83	1		
Heat tolerance (45 °C, 2 h)	**0.95***	**0.88***	1	
Heat tolerance (45 °C, 4 h)	**0.68***	**0.69***	0.87	1

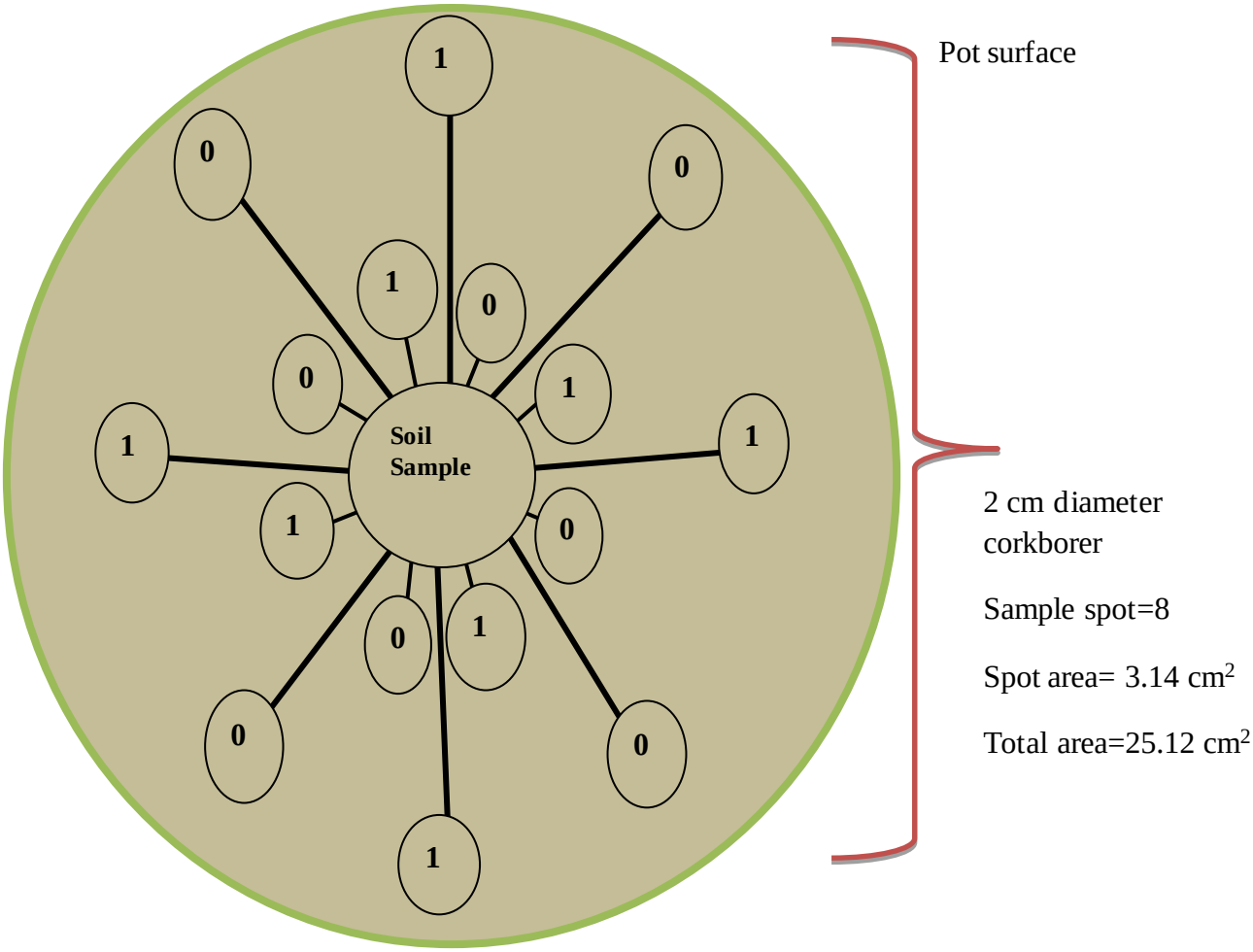

Figure 3.1. Schematic representation of pot soil sampling, (0) on day of inoculation and (1) 28 DPI.

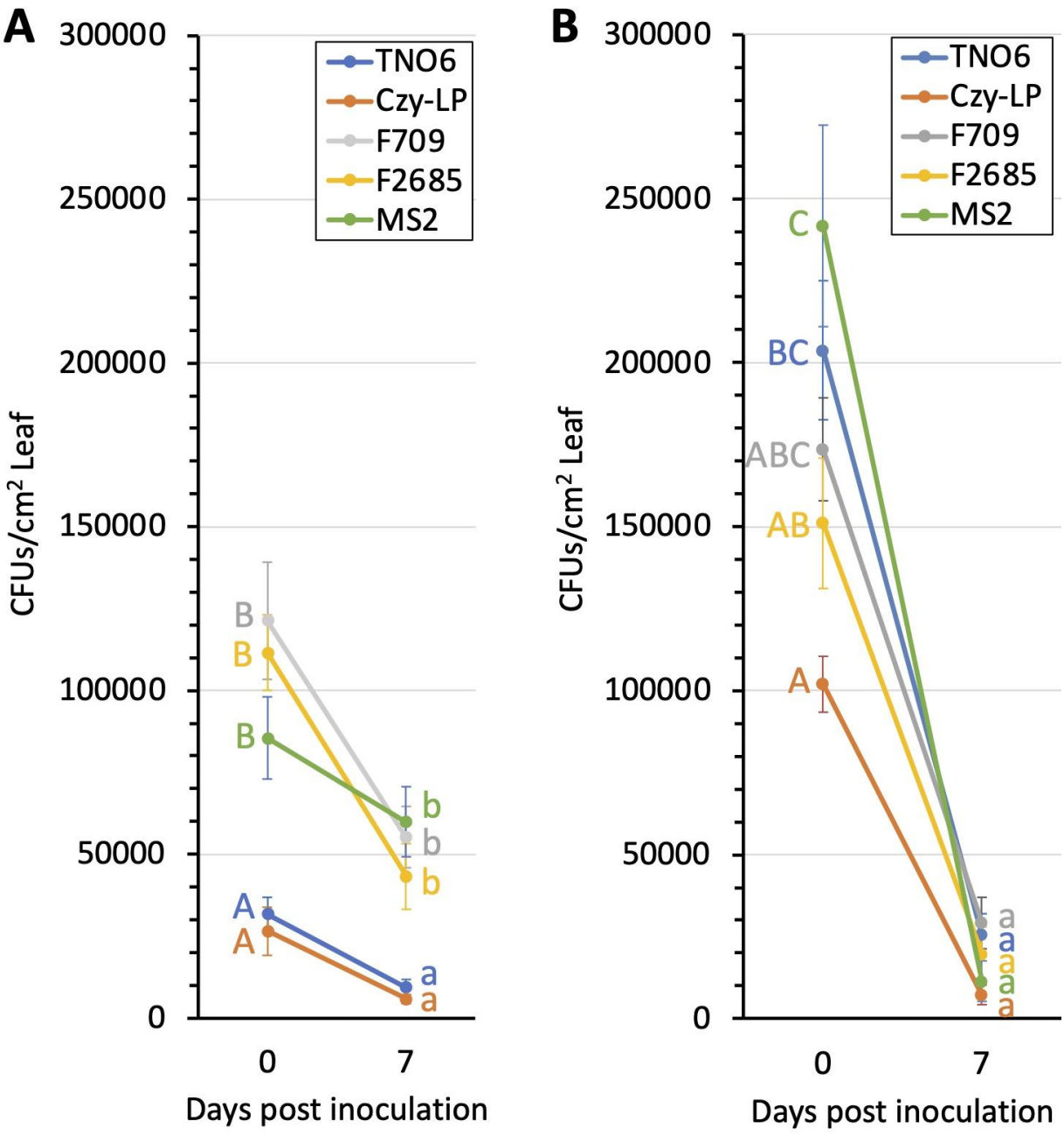

Figure 3.2. Mean CFUs count of fungal strains per cm^2 leaf area in hot (A) and cold (B) season. The plots represented mean (n=6) and standard errors. Letters indicate significant difference among fungal strains. Means with same letters showed no significant difference (Tukey's HSD, α=0.05).

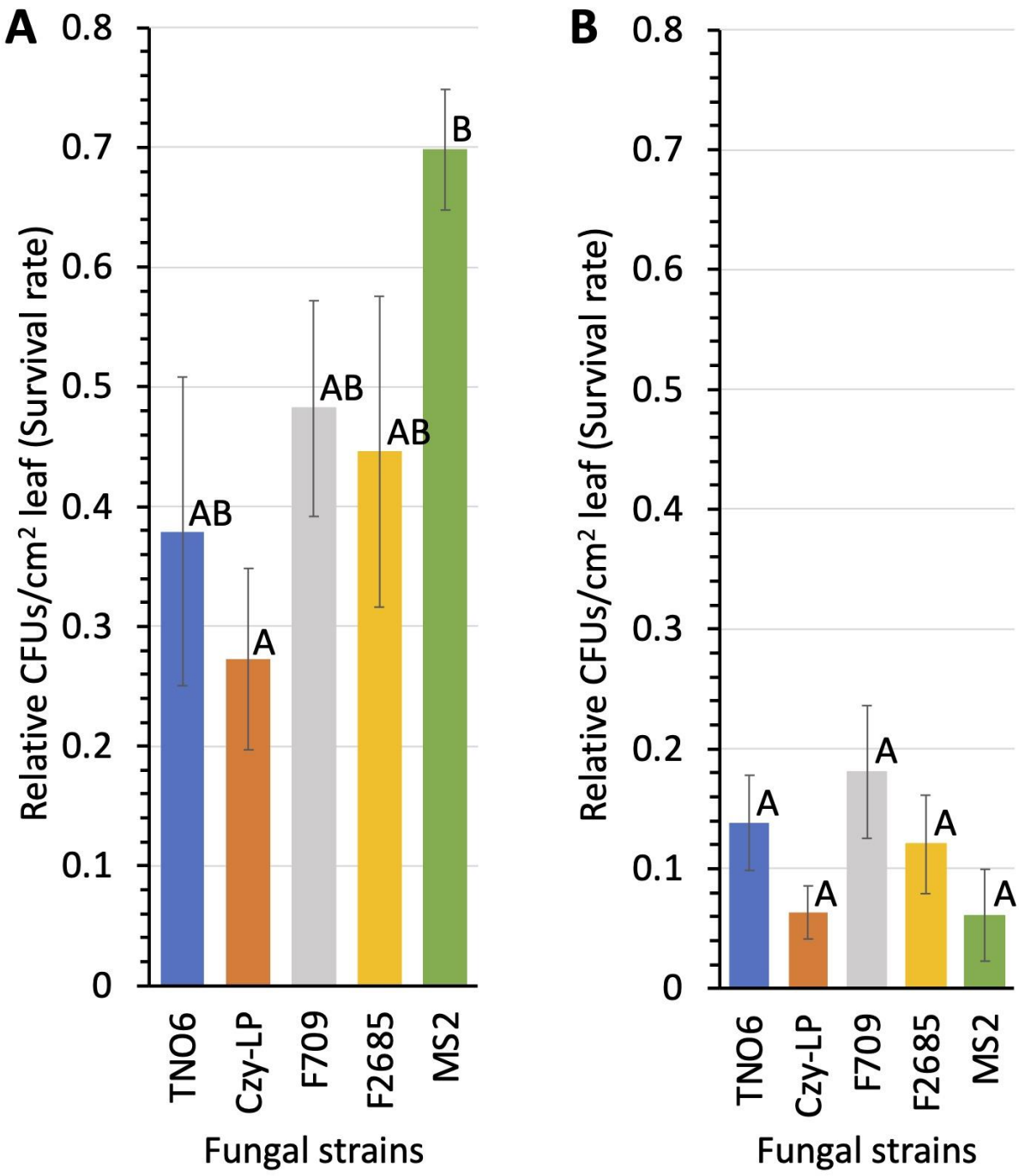

Figure 3.3. Relative survival rate of EPF strains in leaf persistence assay in hot season (A) and cold season (B). Bars represented mean and standard errors (n=6). Letters indicate level of significance difference among fungal strains. Bars with same letters showed no significant difference (Tukey's HSD, α=0.05).

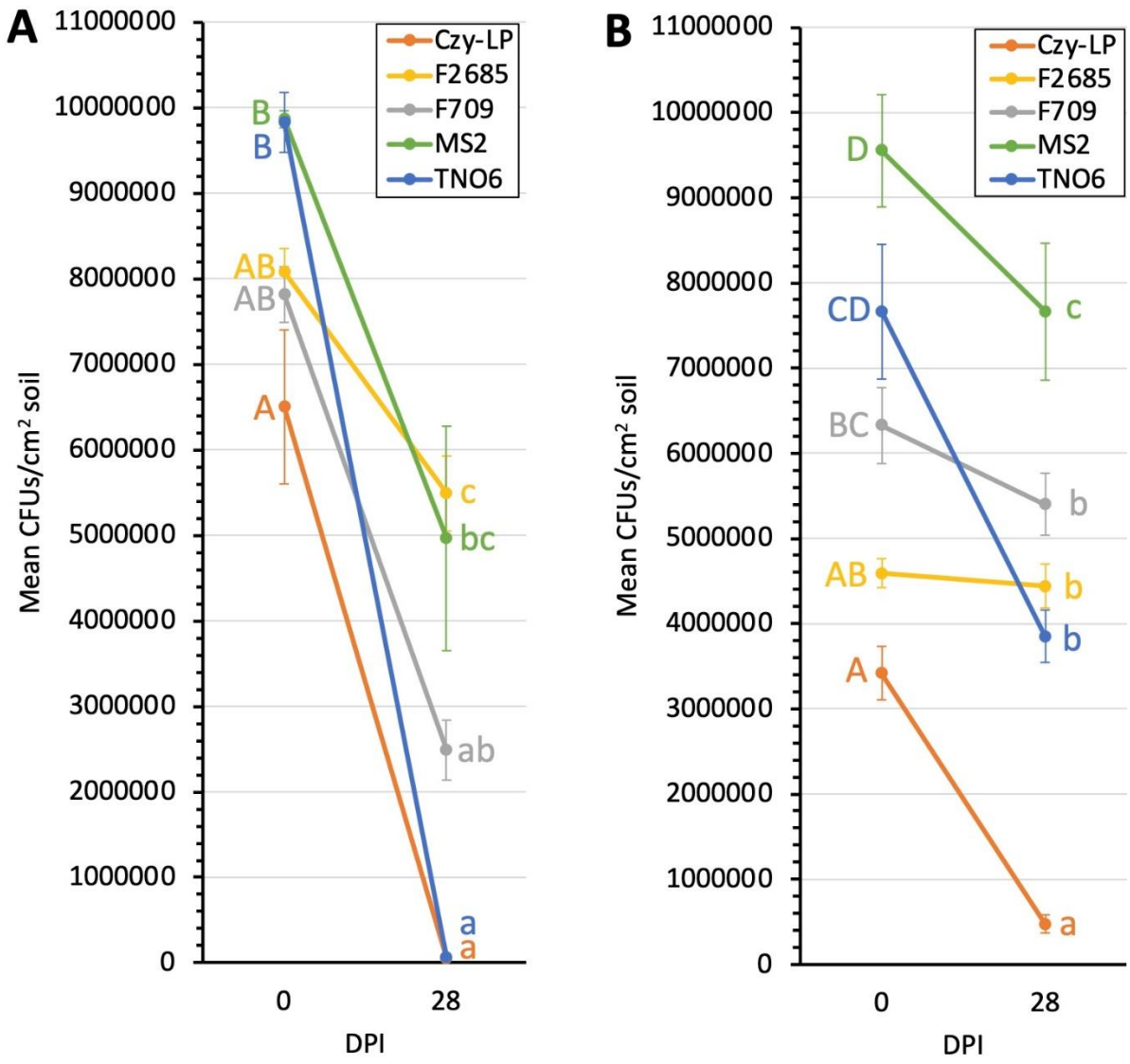

Figure 3.4. Average CFUs count per cm^2 soil area in hot (A) and cold (B) season at day of inoculation (0 and 28 DPI. Results represented mean and standard errors (n=4). Letters indicate level of statistical difference among the treatments (Tukey's HSD, α=0.05).

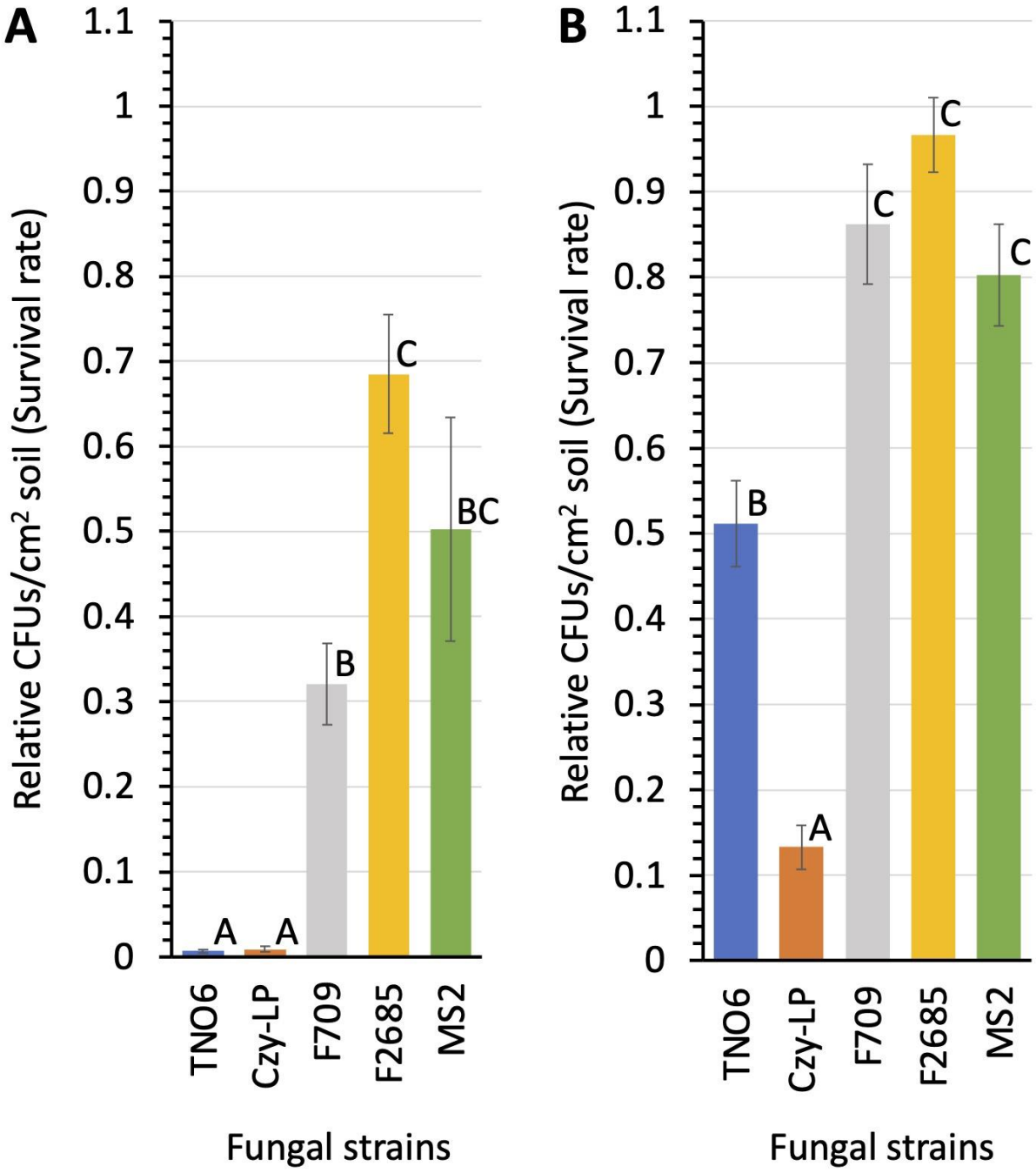

Figure 3.5. Survival rate (relative CFUs per cm^2 soil area) in hot (A) and cold (B) season. Bars represented mean and standard errors (n=4). Bars with same letters showed treatments not statistically significant different (Tukey's HSD, α=0.05).

General discussion

EPF have been formulated as mycopesticides for many years and played a significant role in managing various insect species and orders. EPF belonging to Ascomycetes, such as *B. bassiana* sensu lato and *M. anisopliae* sensu lato, were reported to infect field and storage insect pests and cause death of pests of different stages (Wakil et al., 2014). They were reported to have multiple infection pathways on the target pests and to produce secondary metabolites that play a significant role in insect death (Woolley et al., 2020). In addition to this, their ease of mass-production and application and high host selectivity make EPF preferable comparing to other entomopathogenic organisms (Digvijay et al., 2017).

However, variability in virulence of the fungal strains against the target insect pests is substantially influenced by the age, life stage, and habitat. Hence bioassay experiments conducted to determine the potential virulence of selected fungal strains of different source of isolations for last instar larvae and pupae of *S. litura*. The laboratory bioassay results elucidated that all fungal strains showed varying degree to cause the potential infection for last instar larvae and pupae. Mortality percentage reached up to 100% with *M. rileyi* Nr4, *M. pingshaense* MS1, *M. pingshaense* ARSEF 8736, and *M. brunneum* ARSEF 3294 for last instar larvae, nevertheless they caused minimum infection on treated pupae. While higher average larval and pupal mortality was recorded for *C. javanica* (Czy-LP) and *B. brongniartii* (TNO6). Both strains virulent enough to both life stages of the target pest.

Despite their ability to infect both larvae and pupae, the selected EPF strains were comparatively efficient to larvae than pupae. This could be due to the higher tolerance of pupae to fungal applications. Therefore, the current results have in line with previous research finding by Asi et al. (2013). Even though no more supporting information on adult deformation for *S. litura,* this study discovered malformed adults from fungal treated pupae where *B. bassiana* (TNO12) and *M. rileyi* (Nr4) recorded with maximum percentage.

Apart from the inherent virulence capacity of fungal strains, environmental stability of EPF is critical for pest control under field conditions. The abiotic factors adversely affect its potential occurrence, persistence, and epizootics (Qayyum et al., 2021). These are some of the underlined determinant factors which keep away the abundant usage of fungal strains as compared to conventional pesticides for field pest control. Therefore, it is timely to find out

the relationship between heat tolerance and field persistence to discover the potential usability of *in vitro* thermotolerance assay for selection of field persistent fungal strains.

This research included *in vitro* heat tolerance screening of 32 fungal strains as temperature is one of the abiotic factors greatly influence field application of fungal strains. It also involved evaluation of five selected fungal strains for field persistence on soil and cucumber leaf in hot and cold seasons. The discoveries of the assays exposed their sensitivity to temperatures and significant variation among the fungal genera and species. Higher relative CFUs count was observed at 2 h heat exposure with majority of fungal strains. However, exposure at 45℃ for 4 h caused remarkable reduction in relative CFUs. It was observed as a lethal death point for *B. bassiana* (OMNS 150429-2), *B. brongniartii* (TNO6), *C. fumosorosea* (BPS2), *C. javanica* (Czy-LP) and *M. pingshaense* (MS3). *Metarhizium* strains showed dominance at the lengthy period of heat exposure while *Cordyceps* strains were the most heat sensitive during the assay. This agreed with the reports of Alfiky (2022), which reported that *C. javanica* was susceptible at 45℃ heat exposure.

Regarding the relative CFUs recovered from soil and leaf in persistence assays, *Metarhizium* strains found with the highest persistence among the assayed fungal strains under field conditions. Higher relative CFUs were recorded for *M. pingshaense* MS2 and *M. brunneum* F709 per cm^2 leaf area and *M. pingshaense* F2685 and MS2 per cm^2 soil area during hot season at 7 DPI and 28 DPI, respectively for leaf and soil assays. Results revealed all the *Metarhizium* strains (F2685, MS2, and F709) significantly higher in performance in cold season soil assay. However, relative CFUs distorted in cold weather leaf assay. Further the current study disclosed that fungal strain *C. javanica* (Czy-LP) was susceptible and scored the least mean survival rates (relative CFUs count) at 7 DPI and 28 DPI for leaf and soil persistence in hot and cold field conditions.

The performance of fungal strains for *in vitro* heat tolerance and field persistence proved that *Metarhizium* strains were tolerant compared to *Beauveria* and *Cordyceps* strains. The relationship between the two parameters confirmed with strong positive correlation coefficients at 2 h and 4 h heating with relative survival of CFUs form leaf and soil persistence assays in hot season.

Thesis Summary

The current research was laboratory and field-based experiments with three genera of entomopathogenic fungi *Beauveria, Metarhizium* and *Cordyceps* of which different geographic locations and source of isolations. Entomopathogenic fungal strains belonging to those genera are among the known components of biological weapons in integrated pest management programs. They are naturally safe and eco-friendly biological agents. Their potential of host specificity and ease of handling make them options to substitute synthetic pesticides in pest control. The general outlines of the research was organized in three sections, (1) laboratory based bioassay with 16 entomopathogenic fungal strains against the last instar larvae and pupae of *S. litura*, (2) screening of 32 fungal strains for *in vitro* heat tolerance at 45℃ in 2 h and 4 h exposure period, (3) field persistence assay of selected fungal strains (5 strains) with variable performance of thermotolerance under laboratory for field persistence from pot soils and cucumber leaves in hot and cold season.

The efficiency of fungal strains for controlling agricultural, household, vector insects and dairy farm pests were proved through various post research studies, However, they are sensitive and unstable under field condition. Evaluations on its fitness to withstand a given environmental conditions such as temperature, UV radiations and relative humidity, are important to select strains with better biocontrol efficacy in fields, because these factors have detrimental effect and restricts the use of entomopathogenic fungi in field. Hence screening of the fungal strains done to check the synchrony in potential of fungal strains for *in vitro* heat tolerance and field persistence. All assayed fungal strains come up with certain degree of heat tolerance, where significant variation observed among fungal genera and strains of same species.

Results of *in vitro* heat tolerance assay clarified that colony forming unit counts of fungal strains were seen with the potential tolerance ranging from 0-100% and fungal conidia get distorted with the lengthy periods of heat exposure at the selected temperature level (45℃). The exposure for 4 h at 45℃ referred as lethal death point for *B. bassiana* OMNS150429-2, *B. brongniartii* TNO6, *C. fumosorosea* BPS2, *C. javanica* Czy-LP and *M. pingshaense* MS3. A higher dominance in thermotolerance was observed with *Metarhizium* strains in extended heat exposure, while *C. javanica* Czy-LP, *C. fumosorosea* BPS2, *B. brongniartii* TNO6 and *M. pingshaense* MS3 remained highly susceptible.

Obviously, there are several factors affecting persistence potential of entomopathogenic fungal strains under field conditions like the inherent capacity of the fungal strain, soil type, culture media and others. However, this study was handled regardless of the biotic factors. It focused only temperature to define the relationship between *in vitro* heat tolerance and field persistence. Field observation demonstrated that the three *Metarhizium* species, *M. pingshaense* F2685, *M. pingshaense* MS2 and *M. brunneum* F709 showed significant difference among the assayed strains with survival of CFUs at 7 DPI and 28 DPI in both hot and cold season trials. Whereas strains of *B. brongniartii* TNO6 and *C. javanica* Czy-LP were vulnerable under field condition. It detailed that direct relationship between the two parameters and concluded *in vitro* heat assay could be used in selection of field persistent fungal strains for hot season applications.

Laboratory based virulence assessment of 16 fungal strains against lepidopteran pest was carried out with the reference to insect pest, *S. litura*. It is one of the devastating pests worldwide. Last instar larvae and pupae were used for bioassays at the concentration of 1×10^8 conidia/mL. Data on mortality, percent of mycotized larvae and pupae, and adult emergence were recorded. Fungal strains exhibited varying levels of virulence regarding the treated life stages of the target pest with up to 100% mortality at 10 DPI. Strains of *M. rileyi* Nr4, *M. pingshaense* MS1, *M. brunneum* ARSEF 3294 and *M. pingshaense* ARSEF 8736 with the uppermost average mortality for last instar larvae, however, their virulence against the pupae was comparatively lower. The higher pupal susceptibility was observed with the strains of *B. brongniartii* TNO6, *C. javanica* Czy-LP, *M. brunneum* F709, and *M. pingshaense* MS2. Those strains scored virulence ≥90% to pupae and ≥85% to last instar larvae and seem composite strains and could be intensively investigated and developed as potential mycopesticides in integrated pest management programs against *S. litura*. In addition, the reference/commercial *B. bassiana* GHA accounted among the least efficient strains during the lab bioassays.

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