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Enhancing The Efficacy And Thermotolerance Of Selected Beauveria Bassiana Isolates For Management Of Sunn Pest, Eurygaster Integriceps, Using Electrofusion Techniques

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ENHANCING THE EFFICACY AND THERMOTOLERANCE OF SELECTED *BEAUVERIA BASSIANA* ISOLATES FOR MANAGEMENT OF SUNN PEST, *EURYGASTER INTEGRICEPS*, USING ELECTROFUSION TECHNIQUES

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ABSTRACT

Sunn Pest, *Eurygaster integriceps* Puton (Heteroptera: Scutelleridae), is a major pest of wheat and barley in West and Central Asia and East Europe. The insect causes a significant reduction in yield and quality of grains. Entomopathogenic fungi, especially *Beauveria bassiana* are among the most dominant microbial agents that have been used against Sunn Pest. However, there are concerns related to their efficacy and thermotolerance particularly in countries with high temperature regimes.

Nine *B. bassiana* isolates were tested against Sunn Pest to estimate their virulence and speed of infection under laboratory conditions. The isolates were also exposed to 25, 36, 38 and 40 °C, for 24, 48, 72 and 120 hours. The isolate with highest virulence against Sunn Pest and the one with the highest thermotolerance were selected and their conidia were paired using a cell electrofusion technique to obtain a strain that was both highly virulent and thermotolerant. The infused conidia were first exposed to the foregoing temperature regimes and the most thermotolerant ones were selected to test against Sunn Pest. Three Sunn Pest populations at early, mid and late overwintering period were treated with electrofusion products and the two parental isolates. The efficacy of hybrid isolates was also tested against western flower thrips, *Frankliniella occidentalis* Pergande (Thysanoptera: Thripidae) as another test organism.

The results showed that among the electrofused conidia, some of them tolerated the highest temperature regime (120 h at 40 °C) and their virulence was similar and in some cases even higher than the most efficacious parental isolate. The susceptibility of the Sunn Pest to hybrids was dose dependent as considerable mortality occurred with the highest concentration (1×10^8 spores/ml). In addition, Sunn Pest populations were more susceptible to fungal infection at the end of the overwintering period. That is mainly because Sunn Pest adults do not feed during this period and their survival is entirely depended on the amount of energy and nutrition stored in their body. Depletion of nutrition at the end of the overwintering period and the harsh environmental conditions in overwintering sites make the insect more susceptible to the fungal pathogen. Assessing the genetic differentiation of the hybrids with the parental isolates is an important task that requires more investigation in the future.

In a separate experiment, the thermotolerance of two selected *B. bassiana* isolates for the electrofusion were tested under 40 and 45 °C wet and dry-heat conditions for 4, 8, 16, 24 and 48 h. Additionally, the thermotolerance of conidia and mycelia of the two isolates were compared at 38 and 40 °C for 24, 48, 72 and 120 h. In general, fungal isolates tolerated the dry-heat better than the wet-heat condition and the thermotolerance of fungal mycelia was significantly higher than the fungal conidia.

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To my lovely husband Arash,
and the joy of my life, Nikki

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CHAPTER 1: A REVIEW OF RESEARCH ON SUNN PEST, *EURYGASTER INTEGRICEPS* PUTON [(HEMIPTERA: SCUTELLERIDAE)] MANAGEMENT PUBLISHED 2004-2016

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ABSTRACT

Wheat is an important food crop that provides over 40% of the per capita dietary supply of calories and proteins in many developing countries. Wheat production has a crucial role in food security and the global economy. With the world's population estimated to reach 9.6 billion by 2050, the demand for wheat is expected to increase 60%. Sunn Pest, *Eurygaster integriceps* Puton (Hemiptera: Scutelleridae), is one of the major constraints to wheat production in Central and West Asia, Eastern Europe and North Africa. The economic impact of Sunn Pest is around 42 million USD for the region and that is only the cost of the chemicals used for its management. Application of chemical insecticides has been the main strategy for management of Sunn Pest. However,

emergence of resistance in Sunn Pest populations against most pesticides and increased awareness of their adverse impacts on the environment have prompted investigation of alternative approaches. This review provides information on the most current literatures on Sunn Pest management with emphasis on practices such as application of chemical insecticides, insect growth regulators (IGRs) and anti-juvenile hormones, use of Sunn Pest egg parasitoids, entomopathogenic nematodes, entomopathogenic fungi, use of digestive enzyme inhibitors and development of resistant wheat varieties.

Keywords: Anti-juvenile hormones, Egg parasitoid, Entomopathogenic fungi, Entomopathogenic nematodes, *Eurygaster integriceps,* Insect growth regulators (IGRs)

1.1. The importance of Sunn Pest as an insect pest

Sunn Pest is a common name given to a guild of true bugs in the shield bug (Scutelleridae) and stink bug (Pentatomidae) families. Genus *Eurygaster* occurs in the shield bug family (Hemiptera: Scutelleridae) with the species *Eurygaster integriceps* Puton being one of the most economically important insect pests of wheat and barley. Sunn Pest infests over 15 million ha in southeast Turkey, Iran, Iraq, Syria, Lebanon, Jordan, Israel, Kazakhstan, Uzbekistan, Kyrgyzstan, Tajikistan, Afghanistan, and Pakistan (Parker et al., 2002; Trissi et al., 2006; Parker et al., 2011). Estimated yield loss caused by high populations of Sunn Pest is 20-30% in barley and 50-90% in wheat (El Bouhssini, 2002; Tafaghodinia, 2006; Darkoh et al., 2010). This economic loss can reach 100% in the absence of control actions (Kivan and Kilic, 2005). In Iran only, the

qualitative and quantitative damage of Sunn Pest exceeds 9 million tons in wheat and barley (Abdollahi, 2004).

Both nymphs and adults of Sunn Pest cause damage to wheat by feeding on leaves, stems and grains that leads to a direct reduction in grain yield (Hosseininaveh et al., 2009; Fathi et al., 2010; Fathi and Bakhshizadeh, 2014). Besides direct damage, during feeding Sunn Pest injects salivary enzymes into the grains to enhance pre-digestion. This is a function that enables the insect to avoid seed defensive chemicals such as digestive enzyme inhibitors and antifeedants (Mehrabadi et al., 2012 and 2014). Proteolytic and amylolytic enzymes are among the most digestive groups that are injected into the grain through Sunn Pest saliva. These salivary enzymes destroy the gluten of the grain and reduce the baking quality of the flour prepared from damaged grains (Hosseininaveh et al., 2009; Allahyari et al., 2010; Mehrabadi et al., 2012). Grain damage as low as 2-3% is enough to make the entire lot of flour unacceptable for baking purposes (Radjabi, 2000). The bread prepared from the poor quality flour burns quickly, fails to rise and has an unfavorable flavor (Allahyari et al., 2010; Parker et al., 2011).

Sunn Pest has a monovoltine life cycle (one generation per year) that contains two major periods and the duration of each varies with geological location (El Bouhssini et al., 2004; Iranipour et al., 2010) (Fig. 1). The active (feeding) period in which Sunn Pest growth and development occurs in the fields on wheat and barley and an inactive (non-feeding) period that starts after crop harvesting and the beginning of the hot and dry months (Parker et al., 2011). The active period begins with migration of overwintered adults to the fields once the average daily temperature exceeds 10 $^{\circ}$C. This period lasts about 30 days and it can be longer in areas with lower temperatures and frequent rains

(Radjabi, 2000; Sheikhi Garjan et al., 2007; Alizadeh et al., 2010). After feeding on various parts of the crop, females lay 70-80 eggs on leaves, stalks or weed vegetation. Egg development takes 6-28 days and there are five instars that feed on wheat from the booting stage to the end of the dough development stage. The first four instars feed on leaves and buds, whereas fifth instars and adults feed mostly on the developing grains. Adults continue to feed on grains to accumulate enough fat reserves for the overwintering period. Sunn Pest adults migrate back to mountains and higher elevations after wheat harvest when there is no food available and the temperature becomes too hot in the fields. Adults aestivate underneath pasture plants such as *Artemisia* spp. and *Astragalus* spp. and trees such as oak and conifers in mountains that are cooler and wetter than the fields (Parker et al., 2011). Aestivation sites are usually at 1700–2200 m elevation and northern slopes are preferred (Karimzadeh et al., 2014). Sunn Pest hibernates under the snow between the litter layer and soil surface (Critchley, 1998; Parker et al., 2011; Toprak et al., 2014). Sunn Pest adults remain inactive in the overwintering sites for 9-10 months and then migrate back to the fields in spring and begin to feed (Radjabi, 2000, Parker et al., 2011). The survival of the overwintering adults depends on the amount of fat reserves, minimum ambient temperatures and density dependent mortality factors (Canhilal et al., 2005; Iranipour et al., 2011; Parker et al., 2011; Kocak et al., 2014).

There are reports of different overwintering behaviors of Sunn Pest depending on climatic conditions. In areas with cold and severe winters, Sunn Pest first migrates to higher elevations early in the summer to aestivate and then descends to lower elevations closer to the fields when the cold season starts (Arnoldi, 1995; Parker et al., 2011). However, in moderate and dry climates, Sunn Pest migrates to high elevations in the

summer and stays there until the following spring (Brown, 1962; Iranipour et al, 2010; Parker et al., 2011). Apart from elevation, geographical features of each region influence the movement of Sunn Pest. For instance, the presence of Lake Orumiyeh in the northwest of Iran hinders the movement of Sunn Pest at aestivation time by reducing the ambient temperature in that region (Parker et al., 2011).

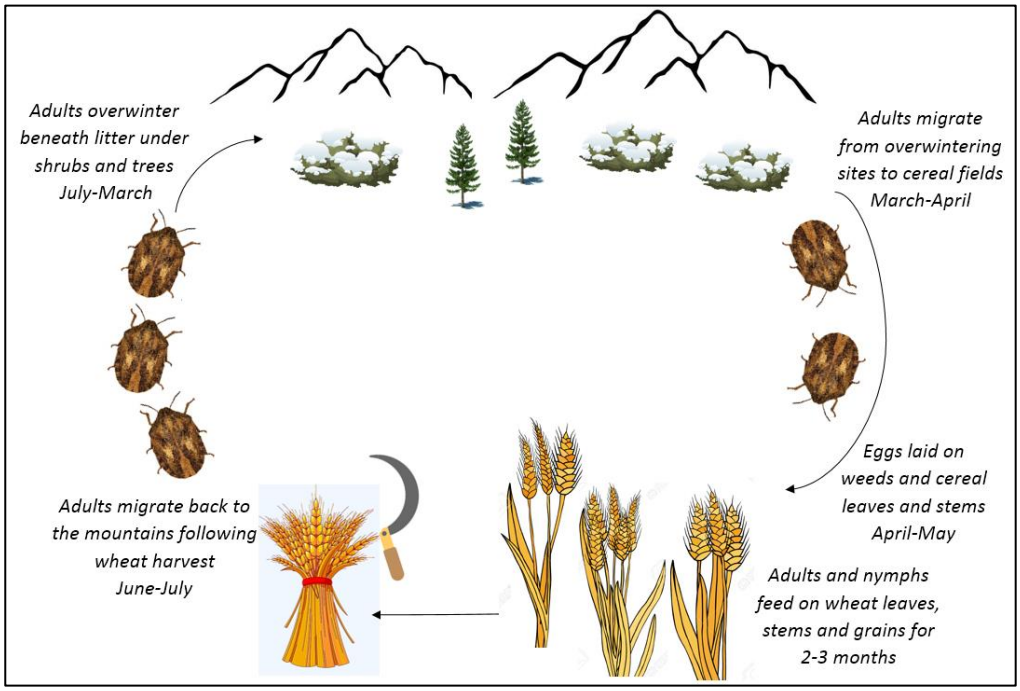

Fig. 1. Sunn Pest (*Eurygaster integriceps*) life cycle.

This review documents the update of progress in the management of Sunn Pest between the years 2004-2016 as a continuation study of the publication "Sunn Pest Management: A Decade of Progress 1994-2004" (Parker et al., 2007). Several techniques including chemical pesticides and biopesticides, natural enemies such as egg parasitoids and nematodes, microbial control agents, digestive enzyme inhibitors and resistant wheat varieties are reviewed.

1.2. Sunn Pest management

1.2.1. Chemical insecticides and biopesticides

The use of chemical pesticides to control Sunn Pest has a long history perhaps due to their effectiveness and ease of application. About 4 million acres are sprayed annually in the Near East and West Asia at a cost of ~$150 million (Miller and Morse, 1996; Gul et al., 2006; Gianessi, 2013). Deltamethrin, fenitrothion, trichlorfon and lambda cyhalothrin are some of the main registered insecticides that are commonly used against Sunn Pest in Iran (Mohammadipour et al., 2015). Toxicity and effectiveness of different insecticides varies among Sunn Pest species and life stages of the insect. For example, no resistance in *E. integriceps* has been reported against alpha-cypermethrin and fenitrothion (Bandani et al., 2005), however, there are reports of development of resistance in *E. maura* against alpha-cypermethrin in Anatolia, Turkey (Ugurlu-Karaagac et al., 2011).

Neonicotinoids are a group of insecticides whose mode of action is similar to nicotine. Since nicotinic acetylcholine receptors (nAChR) are more common in invertebrates compared with other animal groups, they are widely used for management of insect pests. Neonicotinoids have better ingestion toxicity compared with contact toxicity. The type of neonicotinoid compound, method of application and the life stage of Sunn Pest targeted have significant impacts on the effectiveness of these insecticides (Jafarpour et al., 2011). Imidacloprid a systematic insecticide belongs to the neonicotinoid group and widely used against insects with piercing and sucking

mouthparts such as Sunn Pest, aphids, whiteflies and planthoppers (Placke and Weber, 1993; Nauen et al., 1998).

Properly timed applications of chemical insecticides are crucial to improve efficiency (Gozuacik et al., 2016) and must be based on the pest's biology, location and climate conditions. Sunn Pest eggs and early nymphal stages are generally more susceptible than overwintered adults to insecticides. For instance, some neonicotinoids, such as acetamiprid are ineffective against Sunn Pest adults (Kocak and Babaroglu, 2006), whereas diafenthiuron and thiamethoxam have low effectiveness against late instars (Ali et al., 2005). Because the rate of insect development is faster in warmer years or regions, application must be precisely planned based on constant monitoring in each location. Degree-day models (total effective temperatures), thermal constant (Th. C.) and lower development threshold values are valuable tools to predict pest development and activity (Herms, 2004; Gozuacik et al., 2016).

Excessive application of chemical pesticides has environmental risks such as their potential effect on non-target species including natural enemies. Scelionid egg parasitoids (Hymenoptera: Scelionidae) are the most important biological control agents of Sunn Pest (Radjabi, 1995). Widespread application of pesticides such as fenitrothion, fenthion, trichlorfon, chlorpyrifos, and deltamethrin influences the establishment of scelionid egg parasitoids in agricultural fields (Zeren et al., 1994; Radjabi, 1995; Kivan, 1996). For instance, fenitrothion and deltamethrin decrease the emergence rate of the egg parasitoid *Trissolcus grandis* Thompson from host eggs by 18% and 34.4%, respectively (Saber et al., 2005).

The challenge of correct timing of insecticide applications, their inefficacy to suppress dense populations and their incompatibility with non-target organisms are reasons that research on insecticide applications have received less attention in recent years. Recently, attention has shifted to developing biopesticides such as insect growth regulators (IGRs), anti-juvenile hormones and botanicals e.g. pyrethrins and their analogs for Sunn Pest control (Zibaee and Bandani, 2009, 2010; Mojaver and Bandani, 2010; Saadati et al., 2011; Amiri et al., 2012; Zibaee et al., 2011). There is evidence that juvenile hormones play significant roles in the growth, development, reproduction, diapause and behavior of insects (Amiri et al., 2010, Burov et al., 1972). In many insect groups, anti-juvenile hormone agents inhibit the biosynthesis of juvenile hormones and can be used as insecticides. Amiri et al. (2013) reported that the impact of the anti-juvenile hormone agent, precocene I, on Sunn Pest was entirely stage and age-specific. It only increased mortality and physiological abnormality of third instars. Juvenile hormones are more effective at the beginning stages of metamorphosis and embryogenesis. Eggs treated with the juvenile hormone fenoxycarb, resulted in cellular and organelle disruption (Tunaz and Uygun, 2004; Dhadialla et al., 1998). Similar results were observed for the insect growth regulator, pyriproxyfen including lethal effects on eggs and nymphs and sub-lethal effects on adults (e.g. increase in adult weights, sex ratio disruption and the emergence of abnormalities) (Mojaver and Bandani, 2010; Zibaee et al., 2011).

Secondary organic compounds have shown promise for the control of Sunn Pest (Shekari et al., 2008; Zibaee and Bandani, 2010). *Artemisia annua* L. (Asteracea) extract has toxic, antifeedant and biochemical effects on Sunn Pest by disturbing the activity of

enzymes such as esterase, alkaline, acid phosphatase and acethylcholinesterase. Similarly, the seed extract of nonhost plants of Sunn Pest such as *Cicer arietinum* (Fabaceae), *Phaseolus vulgaris* var. naz (Fabaceae), *Triticosecale wittmack* (Poaceae), *Celosia argentea* (Amaranthaceae), caused a significant reduction in the insect`s protease activity *in vitro* (Saadati et al., 2011). However, this activity was strongly dose and time dependent (Zibaee and Bandani, 2010).

Research has also been focused on assessing the economic injury level (EIL) and economic threshold of Sunn Pest in different regions of Iran (Lavani, 2015; Noori and Shahrokhi, 2012) and Turkey (Canhilal et al., 2005). EIL is particularly important to reduce problems with pesticide resistance in Sunn Pest and also to minimize negative effects of pesticides on natural enemies in IPM systems. EIL varies depending upon the expected yield, variety and developmental stage of the wheat, farming practice used and the insect's life stage. Fig. 2 demonstrates the EIL of Sunn Pest in wheat fields of Kermanshah, Iran based on two farming practices and expected crop yield (Bahrami et al., 2002, Lavani, 2015).

1.2.2. Natural enemies

Even though the current strategies for management of Sunn Pest rely mainly on chemical and cultural controls, tremendous progress over the past decade has been made on the use of natural enemies. Hymenopteran egg parasitoids, especially those in the genus *Trissolcus*, are important biological control agents and there are several reports on the successful control of Sunn Pest populations using them (Radjabi 2000, 2007; Kivan and Kilic 2002, 2004a, 2004b; Iranipour et al., 2010; Kutuk et al., 2010).

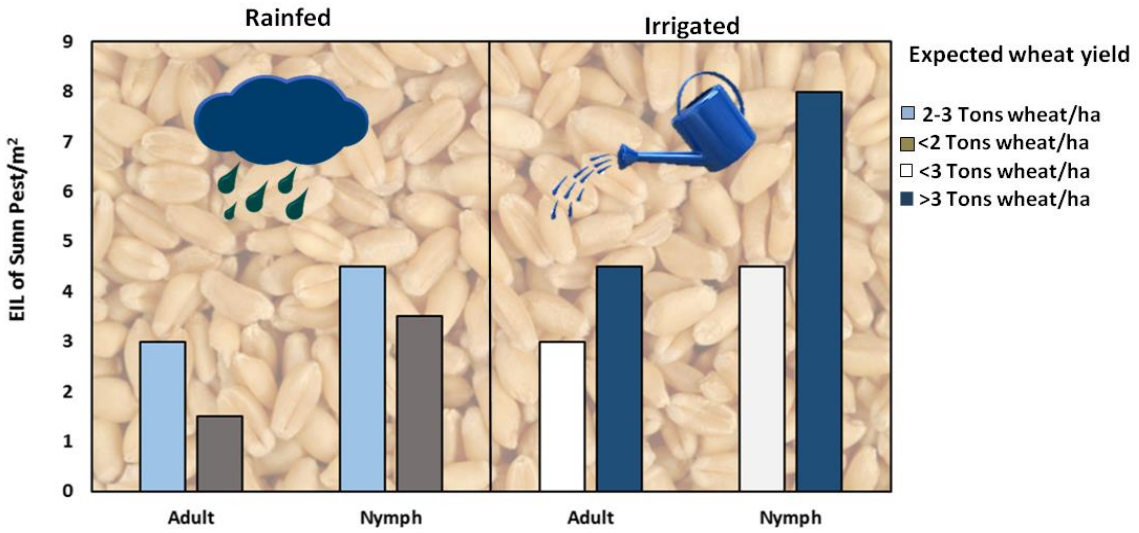

Fig. 2. Economic injury level (EIL) of Sunn Pest at different life stages and different farming practices based on the expected wheat yield.

that 90% of the Sunn Pest eggs collected from wheat fields in Iran were parasitized by this species. Parasitism rates by *T. semistriatus* Nees were 28.5-50% in Syria (Abdulhai, 2003) and 69.3% in Turkey (Kocak and Kilincer, 2001). There are also other species of egg parasitoids such as *T. rufiventris* Mayr, *T. vassilievi* Mayr and *T. festivae* that have been reported as biological control agents of Sunn Pest (Allahyari et al., 2004; Tarla and Kornosor, 2009; Iranipour et al., 2010).

Due to the importance of *Trissolcus* spp. in Sunn Pest biological control, considerable effort has been made to study their spatial distribution, factors that improve establishment rates and population growth in the fields, and developing techniques for mass rearing on artificial diet (Shirazi, 2006; Fathi et al., 2010; Amir-Maafi and Parker, 2011; Wand, 2011; Islamoglu and Tarla, 2013). *T. grandis* is the most common egg parasitoid in Iran and in some southern regions comprises 95% of wasps emerging from parasitized Sunn Pest eggs (Honarmand et al., 2007). The parasitism rate of *Trissolcus* spp. is greatly affected by environmental conditions particularly temperature and

humidity (Islamoglu and Tarla, 2013). Mild winters, warm summers and arid regimes are the most suitable climatic conditions for *T. semistriatus* in Iran (Samin et al., 2010; Yasemi et al., 2015), in Syria (Trissi et al., 2006), in Turkey (Kocak and Kilincer, 2001) and in Iraq (Wand, 2011). *T. semistriatus* has also been observed in Mediterranean subregions including many parts of central and western Asia (El-Bouhssini et al., 2004; Rajmohana, 2006; Anwar-cheema et al., 2010) and in some regions of Europe (Popovici, 2004; Kocak, 2007). The fecundity rate of *T. grandis* is greater at higher levels of temperature and rainfall (Islamoglu and Tarla, 2013; Iranipour et al., 2010; Kivan and Kilic, 2006). Considering different climatic conditions in each region, it is extremely important to study parasitism rates of *T. grandis* site-specifically for augmentation biological control programs. Wasps from warmer regions have shorter development time than those from the cooler regions, and their thermal threshold for initiating development is normally higher (Iranipour et al., 2010).

The parasitism rate of Sunn Pest by *Trissolcus* spp. is influenced by biotic factors including the ratio of host to parasitoid, age of the host and exposure time. A low host to parasitoid ratio, young eggs (usually younger than a day) and short exposure time are ideal situations to obtain a high parasitism rate (He and Wang, 2006; Knat et al., 2008; Amir-Maafi and Parker, 2011). The results of field studies in Turkey revealed that the parasitism rate of *Trissolcus* spp. strongly depended on the density of overwintered Sunn Pest adults. Sunn Pest nymphal populations were successfully suppressed when the density of overwintered adults was equal or lower than 1.5 adults/m^2 (Simsek and Sezer, 1985; Kutuk et al., 2010). The parasitism rate of *T. grandis* when one, two, or three egg parasitoids were exposed to 2 or 4 Sunn Pest adults/m^2 was not significantly different.

However, at 6 Sunn Pest/m^2 the parasitism rate was strongly dependent on the density of the egg parasitoids (Trissi et al., 2006). Based on these results, the parasitism rate of *T. grandis* is density-dependent for a minimum 6 Sunn Pest adults/m^2 exposed to a minimum 2 *T. grandis*/m^2.

Another component for successful biological control of Sunn Pest is developing efficient techniques to mass-produce the insect for inundative releases of *T. grandis* in the field. Because Sunn Pest is univoltine and is not present in the fields throughout the year, mass rearing of *T. grandis* cannot be entirely dependent on Sunn Pest (Allahyari et al., 2004). In addition, naturally occurring levels of egg parasitoids are low during the initial stages of Sunn Pest oviposition. This is a critical period for *T. grandis* to become established in the fields (Amir-Maafi and Parker, 2003; Tarla and Kornosor, 2009). *T. grandis* has been successfully reared on the shield bugs, *Graphosoma lineatum* L., *Graphosoma semipunctatum* and the true bug, *Podisus maculiventris* (Say) (Allahyari et al., 2004; Nozad Bonab and Iranipour, 2012). However, offspring of the parasitoid that emerges from the factitious host *P. maculiventris*, spends excessive time handling and parasitizing Sunn Pest eggs and therefore has lower efficacy. This might be due to its smaller size compared with *T. grandis* (Allahyari et al., 2004).

There are various artificial diets to mass-produce *T. grandis* in the laboratory and most of them contain insect hemolymph, insect-derived molecules, chicken egg yolk, yeast, a malt solution, amino acid, antibiotics, minerals and other nutritional compounds (Consoli and Parra, 2002; Shirazi, 2006). The rate of successful growth and development of the parasitoid varies depending on the artificial diet. Hemolymph is an oviposition stimulant that supports growth and development and is essential for pupal formation (Liu

et al., 1979, 1983). However, the combination of insect hemolymph, chicken egg yolk and salt solution resulted in growth of *T. grandis* only to second instar (Shirazi, 2006). In general, *in vitro* reared parasitoids showed reduced pupal weight and adult size, reduced fecundity, lower reproductive capacity and dispersion ability compared with those reared on natural hosts (Mellini and Campadelli, 1996; Morales-Ramos et al., 1998).

A limited number of studies have focused on Sunn Pest management using natural enemies other than egg parasitoids. The potential of a new species of mermithid nematode, *Hexamermis eurygasteri* n. sp. to parasitize *E. maura* and *E. integriceps* has been investigated in Turkey. The parasitism rate of *Hexamermis* was 20% and 16% for females and 31.6% and 7.1% for males in *E. maura* and *E. integriceps*, respectively (Tarla et al., 2010, 2011).

1.2.3. Microbial control agents

Microbial control agents are a group of biological control agents that have received attention in Sunn Pest IPM programs. Aside from low environmental impacts in comparison with chemical insecticides, the complex mode of action of microbial agents makes the development of resistance very difficult for pests (Khan et al., 2012). For example, entomopathogenic fungi have evolved several strategies to evade the host immune defenses. These pathogens use a combination of various enzymes and a wide range of secondary metabolites to breach the host's cuticle and colonize the hemocoel (Butt, 2002).

Microbial control of Sunn Pest was facilitated by the isolation and identification of entomopathogenic fungi, especially *Beauveria bassiana* (Rehner and Buckley, 2003;

Wan, 2003; Liu et al., 2003; Parker et al., 2003; El Bouhssini et al., 2004; Aquino de Muro et al., 2005; Kouvelis et al., 2008). The pathogenicity and virulence of different entomopathogenic fungi have received great attention over the last two decades (Table 1) (Edgington et al., 2007; Zibaee et al., 2009; Oliveira et al., 2010; Trissi et al., 2012; Gouli et al., 2013; Sedighi et al., 2013; Mustu et al., 2014). Moreover, the effect of culture media and growth conditions on sporulation and virulence of fungal pathogens are the subjects of many recent studies (Meyling and Eilenberg, 2007; Talaei-Hassanlou et al., 2009; Kim et al., 2010; Mohammadbeigi, 2012; Mohammadbeigi and Port, 2013).

Genetic variation was detected among *B. bassiana* isolates associated with overwintered Sunn Pest adults in Iran, Turkey, Kazakhstan, the Kyrghyz Republic, Russia, Syria and Uzbekistan (Parker et al., 2003). Despite a similar geographical pattern of *B. bassiana* occurrence between foregoing countries, there was considerable genetic variability among summer (isolates collected from wheat fields) and winter (isolates collected from overwintering sites) isolates in each country (Trissi et al., 2013). The mortality of Sunn Pest caused by the summer isolates was higher (100% after 10 days) than the winter isolates (60% after 10 days) under laboratory conditions (Trissi et al., 2012). The genetic association of *B. bassiana* to both summer and overwintering Sunn Pest populations was not clear. There is a possibility that this association is very casual and not necessarily due to the evolution of *B. bassiana* with the host. It might also be interpreted that *B. bassiana* has a wide host range and is pathogenic not only to overwintered adults of Sunn Pest but to many other hosts in the same habitat (Bidochka et al., 2002; Aquino de Muro et al., 2005; Kouvelis et al., 2008).

One important requirement for using an entomopathogenic fungus is the susceptibility of the pest to the fungus followed by the virulence of the fungus as a biocontrol agent (Sandhu et al., 2012). Fungal virulence depends on the genetic properties of each isolate and requires investigation through laboratory and field assays. Insects show different susceptibility to fungi at different life stages. The pathogenicity of two *Metarhizium anisopliae* isolates to Sunn Pest eggs, second instars and adults showed that the nymphal stage was more susceptible to fungi than the other life stages (Mohammadbeigi, 2012; Sedighi et al., 2013). Similarly, Mohammadbeigi and Port (2013) reported the fifth instar was the most susceptible life stage of Sunn Pest to *B. bassiana*. This is likely due to the hardness and thickness of the cuticle in adult insects that makes the spore penetration process more difficult (St Leger et al., 1989; Ghazavi et al., 2002).

It has been reported that the pathogenicity of entomopathogenic fungi is significantly different between summer and overwintering populations of Sunn Pest adults. Diapausing adults are more susceptible to fungal infection due to unfavorable environmental conditions, lower amount of reserved energy in their bodies (body fat) and lack of nutrition in comparison to summer populations (Moore and Edgington, 2006; Trissi et al., 2012; Sedighi et al., 2013). Interestingly, different parts of the insect body have a different degree of susceptibility to fungal infection. Susceptibility of different parts of the Sunn Pest body including antennae, tarsi, ventral abdomen and pronotum were compared (Talaei-Hassanloui et al., 2009). Mortality was significantly lower when a fungal suspension was applied to the insect pronotum compared with antennae, tarsi and ventral abdomen.

Table 1. Current literature related to the application of entomopathogenic fungi against Sunn Pest (*Eurygaster integriceps*).

Fungus	Isolate code	Sunn Pest life stage	Country	Reference
Metarhizium anisopliae	M14, IRAN 437c, IRAN 715c	5th instar/Overwintering adults*	Iran	Sedighi et al., 2013
Metarhizium anisopliae	M14	2nd instar	Iran	Roshandel et al., 2016
Beauveria bassiana	DEBI 001	5th and 4th instars/adults	Iran	Mohammadbeigi and Port, 2013
Metarhizium anisopliae	715C	5th and 4th instar	Iran	Mohammadbeigi and Port, 2013
Beauveria bassiana	NA	Laboratory reared adults	Iran	Zibaee and Bandani, 2009
Isaria farinosa	IfA1	Overwintering adults	Turkey	Mustu et al., 2011
Beauveria bassiana	BbA1	Overwintering adults	Turkey	Mustu et al., 2014
Beauveria bassiana	SP22, GHA, SP566, SPSR2	Overwintering adults	Syria	Trissi et al., 2012
Beauveria bassiana	S1, S2, SPDR1-3, SPSH1, SPSH2, SPSR1, SPSR2, SPSQ, SPSS	Overwintering adults	Syria	Abdulhai et al., 2010
Chaetomidium arxii	NA	Overwintering adults	Iran	Arzanlou et al., 2012
Beauveria bassiana	KCF102	Overwintering adults	Iran	Talaei-Hassanloui et al., 2009
Beauveria bassiana	NA	3rd instar	Iran	NouriAiin et al., 2014
Isaria farinosa	NA	3rd instar	Iran	NouriAiin et al., 2014
Metarhizium anisopliae	NA	3rd instar	Iran	NouriAiin et al., 2014
Beauveria bassiana	NA	Laboratory reared adults	Turkey	Kivan, 2007
Metarhizium anisopliae	NA	Laboratory reared adults	Turkey	Kivan, 2007
Beauveria bassiana	DEBI001, DEBI002, DEBI006, DEBI008, ARSEF 201	5th instar	Iran	Haji Allahverdi Pour et al., 2008
Beauveria bassiana	Spt-566, Spt-22, IRK-40, Fashand	Overwintering adults	Iran	Ghamari Zare et al., 2011
Beauveria bassiana	NA	Laboratory reared adults	Iran	Bandani and Esmailpour, 2006
Beauveria bassiana	IRK-10, 23, 31, 36, 37, 40, 43, 53, 58, 22	Overwintering adults	Iran	Kazemi Yazdi et al., 2011

* Sunn Pest adults collected directly from overwintering sites.

Beside biological characteristics of the target insect, virulence of a fungus is influenced by environmental conditions. For example, *M. anisopliae* isolates that were grown under conditions of nutritional stress showed higher virulence, increased

germination rate and greater conidial adhesion to the host cuticle (Andersen et al., 2006; Rangel et al., 2008; Ghamari Zare, 2011). The production of blastospores significantly increased on media with high amounts of rice bran (Roshandel et al., 2016) and oil formulations of *M. anisopliae* and *B. bassiana* suspensions enhanced fungal virulence and resulted in a lower LT_{50} in Sunn Pest adults compared with a distilled water and a wetting agent mixture (Tween 80) (Bandani and Esmailpour, 2006; Sedighi et al., 2013).

There is little information available on the effect of temperature on the virulence of *B. bassiana* isolates to Sunn Pest. The mortality rate of overwintered adults treated with *B. bassiana* was significantly different at various temperatures. The highest growth rate and conidial production for most *B. bassiana* isolates was at 20-25 ˚C (Abdulhai et al., 2010). Considering the trend of climate change over the past few years and the occurrence of unusual high temperatures in regions with Sunn Pest outbreaks, investigation of the thermotolerance of fungal isolates is crucial for Sunn Pest IPM programs.

1.2.4. Digestive enzyme inhibitors

One of the promising strategies for management of Sunn Pest is the use of digestive enzyme inhibitors. Sunn Pest pierces the plant tissue and injects digestive enzymes especially endoase enzymes (amylases and proteases) through its salivary canal into the plant tissue. This enables the insect to predigest the food and facilitates the sucking and ingesting process (Allahyari et al., 2010). Due to the polysaccharide-rich diet of Sunn Pest, they are highly dependent on α-amylase and glucosidases to convert starch to maltose or other oligosaccharides. Oligosaccharides are hydrolysed to glucose by α-

glucosidase and absorbed through the insect gut (Mehrabadi et al., 2010). The dependency of Sunn Pest on α-amylase for survival has led to the idea that α-amylase inhibitors could be an effective strategy for management (Mehrabadi et al., 2010). These inhibitors are naturally found in many plant species and act as a defense against phytophagous insects. However, the amount and specificity of these inhibitors in plants differs and requires investigation before they can be used in insect management practices (Franco et al., 2002).

α-Amylase is found in the salivary glands and in the midgut of Sunn Pest. Polysaccharides are digested partially with salivary secretions and the breakdown of starch is completed in the midgut (Boyd et al., 2002; Kazzazi et al., 2005). α-Amylase activity varies at different life stages of the Sunn Pest. The highest enzyme activity is in the third to fifth instars, which is their most active period of phytophagy (Radjabi, 2000; Kazzazi et al., 2005). Temperature, pH and the presence of particular ions and compounds in the insect midgut are some other factors that affect α-amylase activity in Sunn Pest (Mohammad, 2004; Kazzazi et al., 2005; Bandani et al., 2009).

Plants produce different defensive compounds against herbivores that might be non-proteinaceous such as alkaloids and terpenes or proteinaceous ones including chitinases, lectins, arcelins and glucosidases (Felton, 1996; Carlini and Grossi-de-sa, 2002; Rahimi-Alangi and Bandani, 2013). Wheat and barley contain small amounts of amylase inhibitors, which might explain their high susceptibility to Sunn Pest. The proteinous extracts from some plant seeds such as chickpea, bean, cowpea, amaranthus and triticale (an interspecific cross between wheat (*Triticum aestivum*) and rye (*Secale cereale*)), have high α-glucosidase levels and have and inhibitory effect in Sunn Pest

(Saadati et al., 2011; Mehrabadi et al., 2012; Rahimi-Alangi and Bandani, 2013). These proteinous extracts could also disrupt Sunn Pest growth, development, adult weight and survival (Saadati et al., 2011). The inhibitory activity of the seed proteinaceous compounds is highly dose and pH dependent. The higher the dose of seed extract, the greater is the inhibitory effect on the enzyme activity (Rahimi-Alangi and Bandani, 2013; Rahimi and Bandani, 2014). The highest inhibition activity occurred at pH 5, which is similar to the pH of Sunn Pest gut (pH 5-6) (Bandani et al., 2009; Rahimi-Alangi and Bandani, 2013). Moreover, the stability of the inhibitors during the pre-oral and oral digestion process is important and is considered an essential criterion for an efficient inhibitor. This inhibitor stability has been reported in some plant seeds such as triticale (Mehrabadi et al., 2012).

There are also other characteristics that an efficient digestive enzyme inhibitor should have for Sunn Pest management. First, it should be able to inhibit insect digestive enzymes at low concentrations and at the pH found in the insect gut. Secondly, the inhibitor should be resistant to gut proteases (Valencia et al., 2000). Since there is more than one α-amylase isozyme in Sunn Pest, the specificity of the inhibitor is also important (Mehrabadi et al., 2010). These inhibitors in triticale (T-αAI) could be applied effectively against Sunn Pest because they have most of the above criteria.

α-Glucosidase is another digestive enzyme in Sunn Pest that could be targeted by enzyme inhibitors. However, its characteristics and mode of action needs to be studied thoroughly. α-Glucosidase actively occurs both in the midgut and salivary glands of Sunn Pest but occurs in higher concentration in the midgut (Bandani et al., 2009). The optimal activity of α-glucosidase occurs at pH 5 and temperatures between 40 and 45 $^{\circ}$C

(Mehrabadi et al., 2009). This information could be employed in finding appropriate enzyme inhibitors to use for Sunn Pest control.

Proteinase inhibitors are enzymes that bind to digestive proteases of phytophagous insects and cause adverse effects on insect growth and development (Lawrence and Koundal, 2002; Oppert et al., 2003). They are synthesized by plants and considered a defense mechanism against herbivores and pathogens (Silva et al., 2006). Since these inhibitors are the products of single genes, there is a high potential for transferring them to the target plant to induce resistance against herbivore insects (Falco and Silva-Filho, 2003; Alfonso-Rubi et al., 2003). The effectiveness of inhibitors such as serine protease varies against Sunn Pest at different growth and development stages (Saadati and Bandani, 2011). Retardation of development in insects is dose dependent and is not observed during the entire life cycle of Sunn Pest. It is only observed in the third instars and is probably due to the adaptation of the fourth and fifth instars to the presence of inhibitors in their diet (Oppert et al., 2003; Saadati and Bandani, 2011).

1.2.5. Sources of resistance in wheat varieties

Resistance of host plants against Sunn Pest has received substantial consideration in IPM programs. The International Center for Agricultural Research in the Dry Areas (ICARDA) has been developing participatory programs through which resistance varieties of wheat are investigated, selected and held in gene banks for further studies (El Bouhssini et al., 2007; Ali et al., 2009). A continuous selection of wheat varieties in Russia since 1911 resulted in development of varieties with a high level of resistance to Sunn Pest. The level of gluten in the resistant wheat remains high even with 15-20%

Sunn Pest damage (Krupnov, 2012; Kamenchenko et al., 2010). However, a recent study in Israel showed that the quality loss of gluten happens once the percentage of damaged kernels of wheat increases from 0.8% to 11%, and the gluten index, as a parameter of gluten quality, decreases from 60% to 20% (Kostyukovsky et al., 2014). A field study of selected wheat genotypes from the ICARDA gene bank in Syria led to identification of one durum wheat and eight bread wheat varieties with acceptable resistance to Sunn Pest damage (El Bouhssini et al., 2009). Studying the resistance level of wheat varieties in Turkey showed variable loss in yield and quality (Kinaci and Kinaci, 2007). Wheat genotypes with hard red grains showed higher resistance to Sunn Pest damage than soft white grains. In general, winter wheat varieties are more resistant than spring varieties and there might be a relationship between cold tolerance and resistance to Sunn Pest (Najafian et al., 2008; Rezabeigi, 1996; Sanaey and Najafi-Mirak, 2012). Accordingly, genotypes that start heading earlier in the season incurred more damage compared with those that initiate heading later. This is why the winter varieties which start heading later showed higher resistance (Sanaey and Najafi Mirak, 2012).

Wheat varieties with more spike density, more height, longer awn (a needle-like element of the wheat spike) and late maturing time are more prone to Sunn Pest damage (Geits and Pavlov, 1977; Susidko and Felko, 1997; Najafi-Mirak and Mohammadi, 2004; Rezabeigi et al., 2004). The effect of different planting methods of early, mid-season and late wheat varieties did not result in changes in Sunn Pest nymphal density and grain yield in Turkey (Gursoy et al., 2012). However, it was determined that early durum wheat varieties were considerably more resistant to Sunn Pest damage and density. These

results provide valuable information for selecting potential gene sources for wheat breeding programs.

1.3. Conclusion

Sunn Pest has been a major insect pest of cereals for hundreds of years. Research has focused on management techniques to keep the pest population under the economic threshold level. However, increasing demand for cereal-based foods especially in developing countries carries concerns about the future of food security in Sunn Pest-prone regions. Rising awareness of the negative effects of synthetic chemical pesticides over the past several decades and the emergence of environmental movements to protect human health and nature conservation has drawn attention to environmentally safe practices. Currently, research on Sunn Pest is mainly focused on the combination of chemical, cultural and biological techniques within an integrated management program.

Sunn Pest populations vary from year to year due to changes in climatic conditions and outbreaks occur periodically. Therefore, scouting populations on a yearly basis could minimize the use of insecticides. Furthermore, application of selective pesticides with minimum impacts on non-target species, their application when pest populations reach economically damaging levels and development of biopesticides are promising approaches that require more consideration by IPM program leaders.

Research on Sunn Pest management using Scelionidae egg parasitoids, especially *Trissolcus* spp., has shown promising results. The parasitism rate of *T. grandis* reaches 100% in cold and moderate climatic conditions (Iranipour et al., 2010). However, arid areas with mild winters and hot summers are less suitable for this parasitoid. Therefore,

knowledge on the ecology of *Trissolcus* spp. and development of distribution models can facilitate the introduction of these biological agents to new locations. This also requires frequent sampling of *Trissolcus* spp. and selection of resistant individuals that could eventually be used in mass rearing and inundative release programs in locations with less favorable climates. Developing techniques to establish natural enemy populations in the fields needs to be considered in conservation biological control programs. Preserving areas around the fields and growing flowering plants and fruit tress could be helpful in attracting natural enemies and providing alternate food sources and shelter for them.

The unique overwintering behavior of Sunn Pest is a factor that enables researchers to take advantage of it in favor of minimizing the pest populations before attacking the fields. Locations that Sunn Pest adults overwinter are hillsides and nearby mountains that are usually 10-20 km from wheat fields. Extension service personnel and farmers in each region have investigated these locations many years and have initiated participatory programs for collecting Sunn Pest. Based on the knowledge gained on the overwintering behavior of Sunn Pest, fungal pathogens could also be applied at this stage of the insect`s life cycle (Skinner et al., 2007). Application of entomopathogenic fungi against Sunn Pest in overwintering sites has several advantages. First, overwintered adults are highly susceptible to fungal infection due to the lower amount of reserved energy in their bodies (Amiri and Bandani, 2013). Second, application of fungal pathogens where the density of pest is relatively high increases the possibility of horizontal transmission of fungi and mortality due to density-dependent factors. Finally, treating Sunn Pest adults with pathogenic fungi in overwintering sites reduces the adverse effects of these pathogens on non-target organisms especially egg parasitoids.

Collection and identification of entomopathogenic fungi have received great attention over the past few years. The promising outcomes of the application of fungi such as *B. bassiana*, *M. anisopliae* and *I. farinose* to control Sunn Pest populations accelerated the potential of this group of pathogens for further research and investigation. Selection of fungal isolates that contain favorable properties such as high sporulation and pathogenicity against Sunn Pest at extreme temperatures are the beginning steps in their development. The latter is particularly important because resistance of fungal isolates to low temperatures is a prerequisite trait for effective control of Sunn Pest in overwintering sites. On the other hand, due to the current trend of global warming and the possibility of having unpredictably high temperatures in the future, identification of thermotolerant isolates is another important factor. Application of novel molecular techniques such as protoplast fusion and cell electroporation for strain improvement of fungi is another consideration. This requires investigation and collection of superior isolates and formation of fungal gene banks similar to gene banks of valuable wheat varieties. These genetic resources could be used for fungal transformation programs in the future.

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CHAPTER 2: CELL ELECTROFUSION TO IMPROVE EFFICACY AND THERMOTOLERANCE OF THE ENTOMOPATHOGENIC FUNGUS, *BEAUVERIA BASSIANA*

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ABSTRACT

Beauveria bassiana is among the most dominant microbial agents that have been used against insect pests. However, there are concerns related to the virulence and thermotolerance of most isolates that can be improved through molecular techniques. Nine *B. bassiana* isolates were tested against western flower thrips, *Frankliniella occidentalis* (Thysanoptera: Thripidae) (WFT) to estimate their pathogenicity and speed of infection under laboratory conditions. The isolates were also exposed to 25, 36, 38, and 40 °C, for 24, 48, 72 and 120 h. Two isolates with the highest virulence against WFT and greatest thermotolerance were selected and paired using a cell electrofusion technique to obtain a single strain that was both highly virulent and thermotolerant. To determine the hybrid conidia among the fusion cells, each selected isolate was loaded with a different nuclei-staining fluorescent dye before the fusion process. Hybridized fungal strains were acquired by a series of sequential dilutions and were tested for

thermotolerance and virulence against WFT. Those that demonstrated both characteristics (high thermotolerance and virulence) similar to or greater than the parental isolates were considered hybrids. These results demonstrated that genetic transformation using electrofusion can be used to obtain hybrid isolates with various desired properties for integrated pest management and industrial purposes in the future. Further DNA-based analysis is required to determine the genetic variation between hybrid and the parental isolates.

Keywords: Cell electrofusion, *Beauveria bassiana*, nuclei staining dye, thermotolerance, western flower thrips, *Frankliniella occidentalis*

2.1. Introduction

Fungi are normally classified based on their life cycle, sexual and asexual phases and morphological characteristics (Castrillo et al., 2005). The entomopathogenic fungus, *Beauveria bassiana*, is the asexual stage (anamorph) of *Cordyceps bassiana*, a sexual (telemorph) species that has been collected only in eastern Asia (Li et al., 2000; Sandhu et al., 2012). *B. bassiana* produces mitosporic conidia that are passively dispersed by wind and rain (Inglis et al., 2001; Shah and Pell, 2003). The absence of sexual recombination in *B. bassiana* results in clonal populations with limited genetic variation. Therefore, natural variability among individual isolates due to mutation is the only source of new genotypes (Arahana et al., 2013; Papierok, 2003). Recombination of fungi without sexual reproduction has been facilitated through a process called parasexual recombination (Couteaudier et al., 1996). This is not a natural process and has been conducted for genetic improvement of various fungal species in the laboratory (Estrada et

al., 2007; Bello and Paccola-Meirelles, 1998; Castrillo et al., 2004). Parasexuality involves transferring nuclei between two intra- or interspecific individuals and results in the formation of a heterokaryon (cell with two genetically distinct nuclei) that is extremely unstable. Eventually, chromosome exchange takes place due to mitotic crossing over and haplodization during mitotic division and haploid cells with different genotypes will be attained (Clutterbuck, 1996; Milgroom et al., 2014). The first step is close proximity between two individual cells and then degradation of the cell walls with the help of various chemical or physical fusion inducing agents (Verma and Kumar, 2000) such as polyethylene glycol (PEG) or electric stimulation (Hallsworth and Magan, 1999; Kawai et al., 2010; Magae et al., 1986). Electric pulses can be used for transferring nuclei between cells by making structural rearrangement in the lipid bilayer and producing permeable pores in the cell membrane (Rems et al., 2013). Electrofusion has several advantages over chemical fusogens such as PEG. The former provides a higher fusion yield, the hybrid cells display more vigorous growth and minimal alteration in the biological structure of targeted cells occurs (Karsten et al., 1988; Rems et al., 2013; Pigac and Schrempf, 1995).

Genetic recombination has been reported in *B. bassiana* through protoplast fusion of aerial mycelium using PEG to increase their virulence against insect pests (Bello and Paccola-Meirelles, 1998, Dalzoto et al., 2003, Castrillo et al., 2004; Couteaudier et al., 1996). A hybrid strain of *B. bassiana* and *B. sulfurescens* showed three days reduction in LT_{50} of European corn borer, *Ostrinia nubilalis* (Lepidoptera: Crambidae), but the yield of fusion was relatively low (Couteaudier et al., 1996). Aiuchi et al. (2008) confirmed the potential of protoplast fusion in *Lecanicillium lecanii* to improve their pathogenicity to

insect pests. Protoplast fusion between isolates of *B. bassiana* and *Metarhizium anisopliae* resulted in formation of hybrid strains with higher sporulation and faster mycelium growth than their parental isolates (Sirisha et al., 2010).

A limited number of studies have focused on the fusion of fungal conidia using nonchemical fusogens. Tanaka et al. (2011) applied electric shock to introduce linearized plasmid into the secondary conidia of rice false smut fungus, *Villosiclava virens*. Conidia of *Neurospora crassa* were successfully transformed by introducing an exogenous DNA using a cell electroporator (Navarro-Sampedro et al., 2013). An interspecific electrofusion of protoplasts from two *Aspergillus* species resulted in formation hybrid strains with higher proteinase productivity for industrial purposes (Ushijima and Nakadai, 2014). Due to the importance of the entomopathogenic fungus, *B. bassiana*, as a successful microbial biocontrol agent, genetic modification could increase the potential of this fungus for control of insect pests. There are concerns about the virulence of many *B. bassiana* strains under extreme environmental conditions and their virulence against some important insect pests (Foster et al., 2010). The goal of this study is to optimize the potential of *B. bassiana* through cell electrofusion with emphasis on improving its thermotolerance and virulence against western flower thrips, *Frankliniella occidentalis* (Thysanoptera: Thripidae) (WFT). WFT was used as a model organism because it is a widely distributed insect pest worldwide that causes serious damage on greenhouse-grown ornamentals and vegetables.

2.2. Material and methods

2.2.1. Fungal isolates

Four isolates of *B. bassiana*, IRK-22, IRK-59, IRK-8 and IRK-53 were obtained from the fungal collection located at the Iranian Research Institute of Plant Protection (IRIPP), Sunn Pest Research Department, Tehran, Iran. In addition to isolates from Iran, two isolates of *B. bassiana*, JEF007 and JEF006 from Chonbuk National University, Jeonju, South Korea and two isolates, SP-22 and SP-566 from the Entomology Research Laboratory Worldwide Collection of Entomopathogenic Fungi, Burlington, VT were used in this study. These isolates have been shown to have considerable promise to control insect pests in our previous studies and are mainly acquired from regions with temperature extremes. A commercial strain of *B. bassiana*, GHA which is the active ingredient in BotaniGard®, was included as the standard (Table 1). All fungal isolates were tested against WFT and exposed to different thermal regimes to identify the most virulent and thermotolerant ones.

2.2.2. Bioassay of WFT

Conidia of fungal isolates were harvested from the surface of 15-days old potato dextrose agar (PDA) medium and suspended in a test tube containing 5 ml sterile distilled water and 0.02% Tween 80® solution. Suspensions were stirred and filtered through a single layer of linen to remove culture debris and mycelia. Conidial concentrations were adjusted to 1×10^6, 1×10^7 and 1×10^8 spores/ml.

Table 1. *Beauveria bassiana* isolates used in the study.

Isolate Code	Origin	Isolated species
IRK-53	Kermanshah, Iran	Sunn Pest, *Eurygaster integriceps*
IRK-22	Kermanshah, Iran	Sunn Pest, *Eurygaster integriceps*
IRK-59	Kermanshah, Iran	Sunn Pest, *Eurygaster integriceps*
IRK-8	Kermanshah, Iran	Sunn Pest, *Eurygaster integriceps*
JEF007	Jeonju, South Korea	Lepidopteran pest
JEF006	Jeonju, South Korea	Lepidopteran pest
SP-22	Turkey	Sunn Pest, *Eurygaster integriceps*
SP-566	Turkey	Sunn Pest, *Eurygaster integriceps*
GHA	Active ingredient in BotaniGard®, Laverlam International Corp	*Diabrotica* spp.

Second-instar WFT were aspirated from an even-age insect colony reared on bean plants, *Phaseolus vulgaris cv*. Royal Burgundy. The colony was held at 22 ±1 °C, 40–50% rH and 16:8 (L:D) regime in wooden chambers (45 ×30 × 30 cm) in an insectary at the Entomology Research Laboratory, University of Vermont. A 2-cm diam. disc of bean leaf was sprayed with 1 ml of conidial suspension using a Potter spray tower (Burkard, Rickmansworth, Herts., UK) and placed on moistened filter paper in a plastic 2.5 cm diam. Petri dish. Ten second-instar WFT were added to each Petri dish, sealed with parafilm and incubated at 25 ± 1 °C. Mortality was counted 3, 5, 7 and 10 days after treatment. Each isolate and each concentration was replicated three times and the entire experiment repeated three times within a 4 months period.

2.2.3. Assessment of fungal thermotolerance

To investigate the effect of temperature on fungal growth, 50µl of the fungal suspension with a concentration of 1×10^6 spores/ml was placed onto a 10-mm diam. disc of filter paper placed on the center of PDA in a 60 mm Petri dish. Fungal isolates were incubated at 25 (the control), 36, 38 and 40 °C for 24, 48, 72 and 120 h. After each thermal stress period, Petri dishes were incubated at 25 °C for another 20 days. During this time, the surface radial growth (mm) of each isolate was measured and marked daily from the center of the Petri dish to the outer edges of the fungus. Each isolate was replicated three times and the entire experiment repeated three times within a 6 months period.

2.2.4. DNA staining and electrofusion procedure

Based on the results of the bioassays and thermotolerance tests, isolates with the greatest thermotolerance and virulence to WFT were selected. Each selected isolate was cultured on PDA and prepared for electrofusion. Because the electrofusion technique seldom results in 100% fusion yield and some cells remain unchanged, it is necessary to detect and quantify fusion products. First, the conidia of the two parental isolates were marked with different nuclei-staining fluorescent dyes Acridin Orange (AO) (Cayman chemical, MI) and Hoechst 33342 (AAT Bioquest, Inc., CA). These are cell-permeant nucleic acid stains that emit green (excitation/emission500/525 nm) and blue fluorescence (excitation/emission 350/460 nm) when bound to DNA, respectively.

For the staining purpose, conidial suspensions were harvested and suspended in 10 ml of sterile distilled water with 0.02% Tween 80®. The concentration of each

suspension was adjusted to 1×10^7 spores/ml. Suspensions were transferred to a 1.5-ml Eppendorf tube and centrifuged for 5 min at 3000 rpm. The supernatant was discarded and the conidial pellet was suspended in 1 ml of 4 °C sterile distilled water. This washing process was repeated three times. After the final wash, conidial pellets of the greatest thermotolerant and virulent isolates were suspended in a dilute solution of Hoechst 33342 and AO, respectively (0.1 ml stock solution in 0.5 ml distilled water), for 30 min at room temperature. The conidial pellet of each fungal isolate was acquired by centrifuging the suspension for 5 min at 3000 rpm and the supernatant was removed and replaced with 1 ml 4 $^{\circ}$C buffer (1 M sorbitol). A drop of conidial suspension (50 µl) from each tube was placed on a clean glass slide, squashed under a cover glass and observed under a Olympus BX60 upright compound microscope with a 100× objective lens to ensure that the staining process was successful. Two filter cubes, UMNU and UMNIB were used on the fluorescent microscope. The former filter allows ultraviolet excitation and a broad range of emissions whereas the latter allows for blue excitation and green and red emission.

Electrofusion was done using the Bio-Rad gene pulser Xcell^{TM} electroporation apparatus (Bio-Rad Laboratories, Inc.). For this purpose, 40 µl of each stained conidial suspension was added to a 4 mm electroporation cuvette in ice. The suspension was agitated gently to mix the two conidial suspensions. The Bio-Rad gene pulser was then adjusted to 2000 V and 200 Ω with a constant capacitance at 25 µF with an exponential decay pulse type. The 4-mm cuvette containing conidial suspensions was placed in the holder of the electroporation apparatus and shocked with an exponential decay pulse. In exponential decay pulse the length of the time that cells are exposed to the electric field is

controlled by capacitance of the instrument and the resistance within the circuit and not directly by the setting. Immediately after the electric shock, 1 ml 4 °C sorbitol was added to the electroporated suspension and incubated at 30 °C for one hour to let the spores recover. After incubation, 20 µl of the conidial suspension was placed on a glass slide, squashed under a cover glass and observed under a fluorescent microscope with a 100x objective lens to evaluate the yield of fusion. The assessment of fusion outcomes was achieved by making at least three slides, adding 20 µl of the final suspension and counting the number of conidia that contained both fluorescent dyes in at least three areas of each slide. The counts revealed the average number of hybrid conidia in 20 µl of the electroporated suspension. The viability of parental and electrofused conidia was assessed after staining and electrofusion to ensure that the fluorescent dye and the electric pulse did not affect their viability. This was done by spreading 100 µl of suspension on PDA and counting germinated conidia with a microscope at 400× after 24 h.

2.2.5. Isolation of hybrid conidia and bioactivity tests

Hybrid conidia were isolated from the electroporated suspension by a series of sequential dilutions to reduce and adjust the number of conidia to 10 conidia/ml. Because the initial concentration of electroporated suspension was 1×10^7 spores/ml, the equivalent number was estimated for the 80µl suspension that was added from parental suspensions into the electroporation cuvette. By having the number of conidia in the electroporated suspension (80 µl) and the yield of fusion, the probability of having at least one hybrid spore among 10 conidia/ml was estimated. The ten conidia that presumably contained at least one hybrid conidia were cultured on PDA and incubated at 25 °C for 5 days. Each colony forming unit (CFU) was then isolated and cultured in a separate Petri dish and

tested for virulence against WFT second instars and thermotolerance according to the procedure described in sections 2.2.2 and 2.2.3. Among ten fungal colonies, those that survived 40 $^{\circ}$C for 120 h and caused mortality to WFT similar to the parental isolates were assumed to be hybrid.

2.2.6. Statistical analysis

All thermotolerance and WFT mortality data before and after electrofusion were compared by one-way analysis of variance (ANOVA) followed by Tukey's studentized test and linear regression analysis when significant differences were found at $P \leq 0.05$ using SPSS ver. 24.0 (SPSS, 2009).

2.3. Results

2.3.1. Effect of fungal isolates on WFT mortality

To select isolates for hybridization, the virulence of *B. bassiana* isolates was tested against WFT second instars. In all fungal isolates the rate of mortality of WFT increased over time after treatment. Additionally, mortality of WFT was dose-dependent and with increase in fungal concentration more mortality was achieved (Fig. 1-3). At 1×10^6 spores/ml the virulence among the fungal isolates was not significantly different at day 10 (Fig. 1) ($F_{8,72}$=1.81, P=0.09). However, at 1×10^7 spores/ml ($F_{8,72}$=7.48, P=0.00) and 1×10^8 spores/ml ($F_{8,72}$ = 10.54, P=0.00) there was a significant difference among the virulence of fungal isolates (Fig. 2 and Fig.3). JEF007, IRK-22, IRK-8 and GHA were the most virulent at 1×10^7 spores/ml whereas JEF007, IRK-22 and IRK-8 were the most virulent at 1×10^8 spores/ml. In general, among all fungal isolates JEF007, IRK-22 and

IRK-8 were the most virulent isolates against WFT at the end of the experiment with 86.7±4.7, 82.2±2.7 and 92.2±3.2 % mortality after 10 days, respectively (Fig. 3).

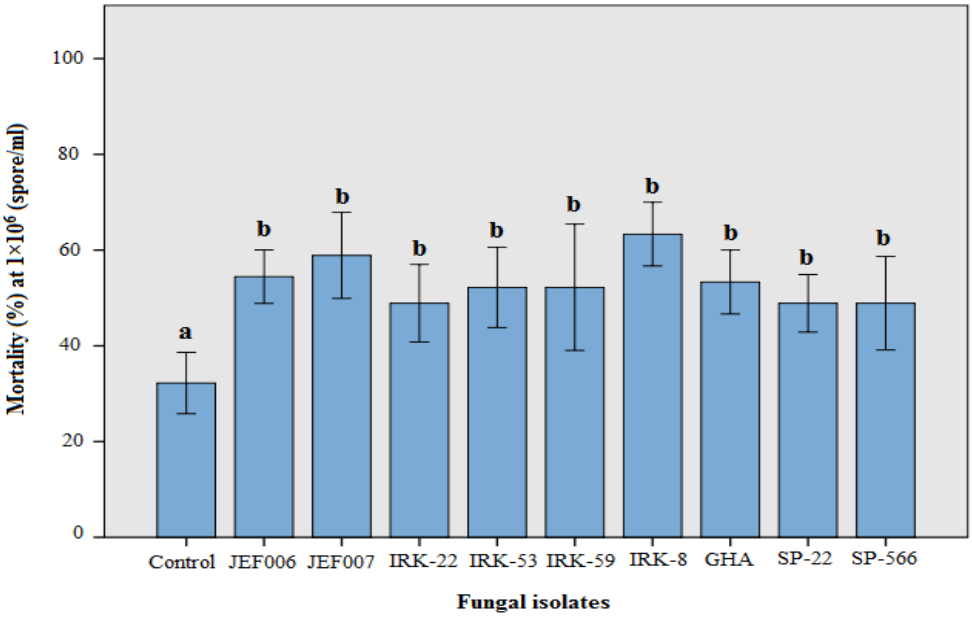

Fig. 1. Mean (±SE) percentage mortality of WFT second instars over time at 1×10^6 (spores/ml). Same letters above bars indicate no significant difference at day 10 ($p < 0.05$) according to Tukey test.

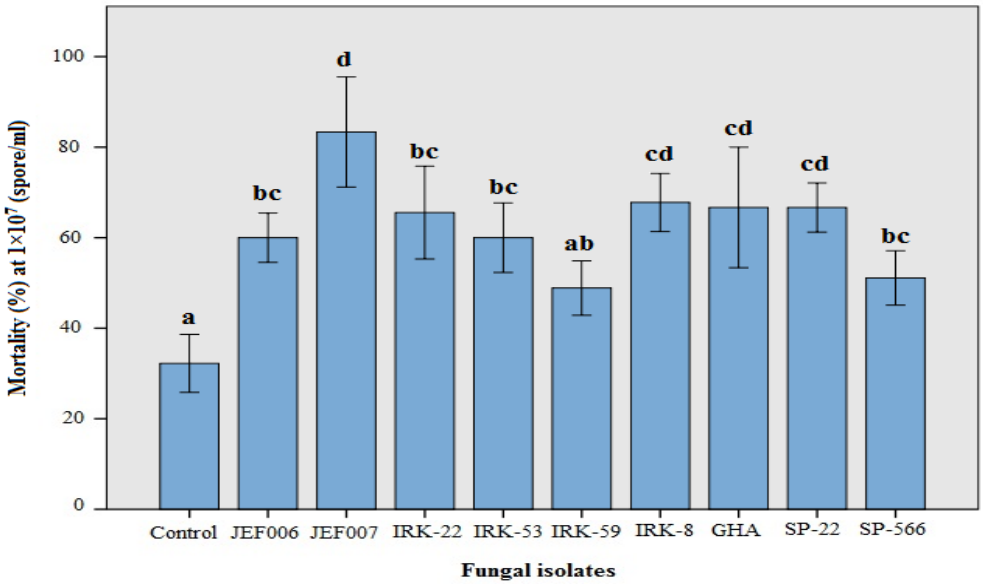

Fig. 2. Mean (±SE) percentage mortality of WFT second instars over time at 1×10^7 (spores/ml). Same letters above bars indicate no significant difference at day 10 ($p < 0.05$) according to Tukey test.

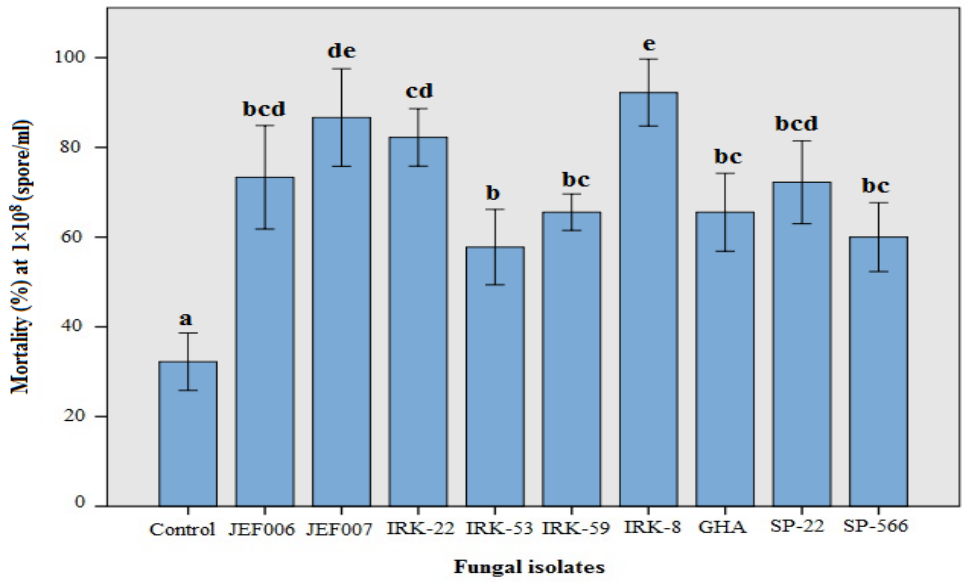

Fig. 3. Mean (±SE) percentage mortality of WFT second instars over time at 1×10^8 (spores/ml). Same letters above bars indicate no significant difference at day 10 ($p < 0.05$) according to Tukey test.

2.3.2. Thermotolerance of fungal strains

All fungal strains grew after 24, 48, 72 and 120 h exposure to 25, 36 and 38 ˚C and the radial growth (mm/day) 20 days after incubation at 25 ˚C is presented in Table 1. At 40 ˚C, all strains grew after 24, 48 and 72 h exposure except IRK-53 that did not survive this temperature more than 48 h. Survival of fungal strains was significantly different after 120 h exposure at 40 ˚C ($F_{8,72}$ = 2539.70, P=0.00) (Table 2). IRK-59 and JEF007 were the only isolates that survived at this temperature with relatively high radial growth, 5.3±0.2 and 5.1±0.3 mm/day, respectively, after 20 days post experiment (Table 2).

Table 2. Mean (±SE) radial growth (mm/day) of fungal strains at different temperatures and time of exposure at the end of the experiment.

25 °C

Exposure time	Exposure time			
	24h	48h	72h	120 h
IRK-53	4.5±0.4^{b}	4.1±0.3^{a}	3.9±0.4^{ab}	3.9±0.6^{ab}
IRK-22	3.9±0.2^{a}	4.0±0.3^{a}	3.8±0.1^{a}	3.7±0.2^{a}
IRK-59	5.0±0.4^{bc}	5.3±0.2^{c}	5.1±0.3^{c}	5.1±0.2^{d}
IRK-8	5.3±0.2^{c}	4.8±0.5^{bc}	5.0±0.2^{c}	4.5±0.3^{bcd}
JEF007	5.1±0.2^{c}	4.9±0.3^{c}	4.9±0.2^{c}	4.7±0.4^{cd}
JEF006	5.2±0.2^{c}	5.2±0.3^{c}	5.2±0.2^{c}	4.8±0.3^{cd}
SP-22	5.1±0.2^{c}	5.1±0.2^{c}	5.0±0.3^{c}	4.8±0.2^{cd}
SP-566	4.9±0.4^{bc}	4.2±0.3^{ab}	4.3±0.3^{b}	4.5±0.3^{bc}
GHA	5.2±0.2^{c}	5.0±0.2^{c}	4.9±0.4^{c}	4.9±0.2^{cd}

36 °C

Exposure time			
24h	48h	72h	120 h
4.9±0.3^{b}	4.9±0.5^{b}	4.5±0.2^{b}	4.4±0.3^{bc}
4.1±0.3^{a}	4.0±0.3^{a}	3.9±0.3^{a}	3.8±0.3^{ab}
5.4±0.1^{b}	5.2±0.2^{b}	5.3±0.1^{c}	5.3±0.1^{c}
5.1±0.2^{b}	5.0±0.2^{b}	5.0±0.2^{bc}	3.1±1.4^{a}
5.3±0.2^{b}	4.7±0.3^{b}	5.1±0.2^{bc}	5.1±0.1^{c}
5.3±0.2^{b}	5.2±0.1^{b}	4.9±0.4^{bc}	4.8±0.2^{bc}
5.1±0.3^{b}	5.2±0.1^{b} h	4.9±0.3^{bc}	4.9±0.1^{c}
5.0±0.2^{b}	5.1±0.2^{b}	4.9±0.2^{bc}	4.8±0.3^{bc}
5.4±0.1^{b}	5.2±0.2^{b}	4.9±0.3^{bc}	4.8±0.2^{c}

38 °C

	Exposure time			
	24h	48h	72h	120 h
IRK-53	4.8±0.4^{cd}	4.3±0.4^{a}	3.9±0.4^{b}	0.6±1.0^{a}
IRK-22	5.1±0.2^{cd}	5.2±0.1^{bc}	3.9±0.8^{b}	1.8±1.3^{ab}
IRK-59	5.3±0.2^{cd}	5.5±0.1^{c}	5.4±0.1^{d}	5.3±0.3^{d}
IRK-8	5.3±0.2^{cd}	5.3±0.2^{c}	5.2±0.2^{cd}	3.2±1.4^{cd}
JEF007	5.3±0.3^{d}	5.4±0.1^{c}	5.3±0.2^{cd}	4.4±0.3^{d}
JEF006	4.7±0.4^{bc}	4.5±0.4^{ab}	3.7±0.6^{b}	0.8±0.2^{a}
SP-22	3.9±0.1^{a}	4.0±0.7^{a}	1.8±0.9^{a}	1.5±1.3^{ab}
SP-566	4.2±0.3^{ab}	4.0±0.2^{a}	3.9±0.3^{b}	1.8±1.7^{ab}
GHA	5.3±0.2^{cd}	4.3±0.7^{a}	4.4±0.3^{bc}	0.2±0.6^{a}

40 °C

	Exposure time		
24h	48h	72h	120 h
5.4±0.1^{b}	3.0±1.6^{a}	0^{a}	0^{a}
5.0±0.4^{b}	4.6±0.6^{ab}	5.1±0.3^{c}	0^{a}
5.4±0.2^{b}	5.3±0.2^{b}	5.3±0.2^{c}	5.3±0.2^{b}
5.4±0.2^{b}	4.8±0.4^{ab}	4.4±0.2^{c}	0^{a}
5.4±0.1^{b}	5.3±0.2^{b}	5.3±0.1^{c}	5.1±0.3^{b}
4.3±0.3^{a}	3.6±0.3^{ab}	2.7±0.9^{b}	0^{a}
5.4±0.1^{b}	4.4±1.3^{ab}	0.4±0.6^{a}	0^{a}
5.4±0.2^{b}	3.1±1.8^{a}	0.7±0.8^{a}	0^{a}
5.3±0.2^{b}	5.3±0.1^{b}	0.3±0.6^{a}	0^{a}

Means within a column labeled with the same letter are not statistically different, ($p < 0.05$) according to Tukey test.

2.3.3. Electrofusion outcomes

Based on the results of the bioassay and thermotolerance tests, IRK-59 and IRK-8 were selected as the most thermotolerant and the most efficacious isolates, respectively. Isolate JEF007 was not selected because it demonstrated both high thermotolerant and virulence and selection of hybrid isolates after electrofusion would have been easier if each parental isolate had only one of the desired characteristics. Fig. 2 shows the results of DNA staining of IRK-59 and IRK-8 with Hoechst 33342 and AO, respectively, and the hybrid conidia after electrofusion. Based on the viability test of fungal suspensions after staining and electrofusion, germination of all them was >90% confirming that staining and electrofusion did not decreased the viability of fungal conidia.

Fusion frequency (or yield of fusion) was obtained from the electrofused suspension with 1×10^7 spores/ml by randomly counting the number of conidia that contained both fluorescent stains on at least three areas of six microscope slides using a 100x objective lens. The mean percentage of hybrid conidia in the final suspension that contained both colors was 40% and this number was used to calculate the likelihood of having at least one hybrid among 10 conidia, based on the binomial theorem (Table 3). The suspension containing 10 fungal conidia was cultured on PDA and incubated at 25 $^{\circ}$C. After 5 days fungal colonies were transferred to individual Petri dishes for more growth. Fungal colonies were then used for WFT bioassay and thermotolerance tests according to previously described procedure (Fig. 4).

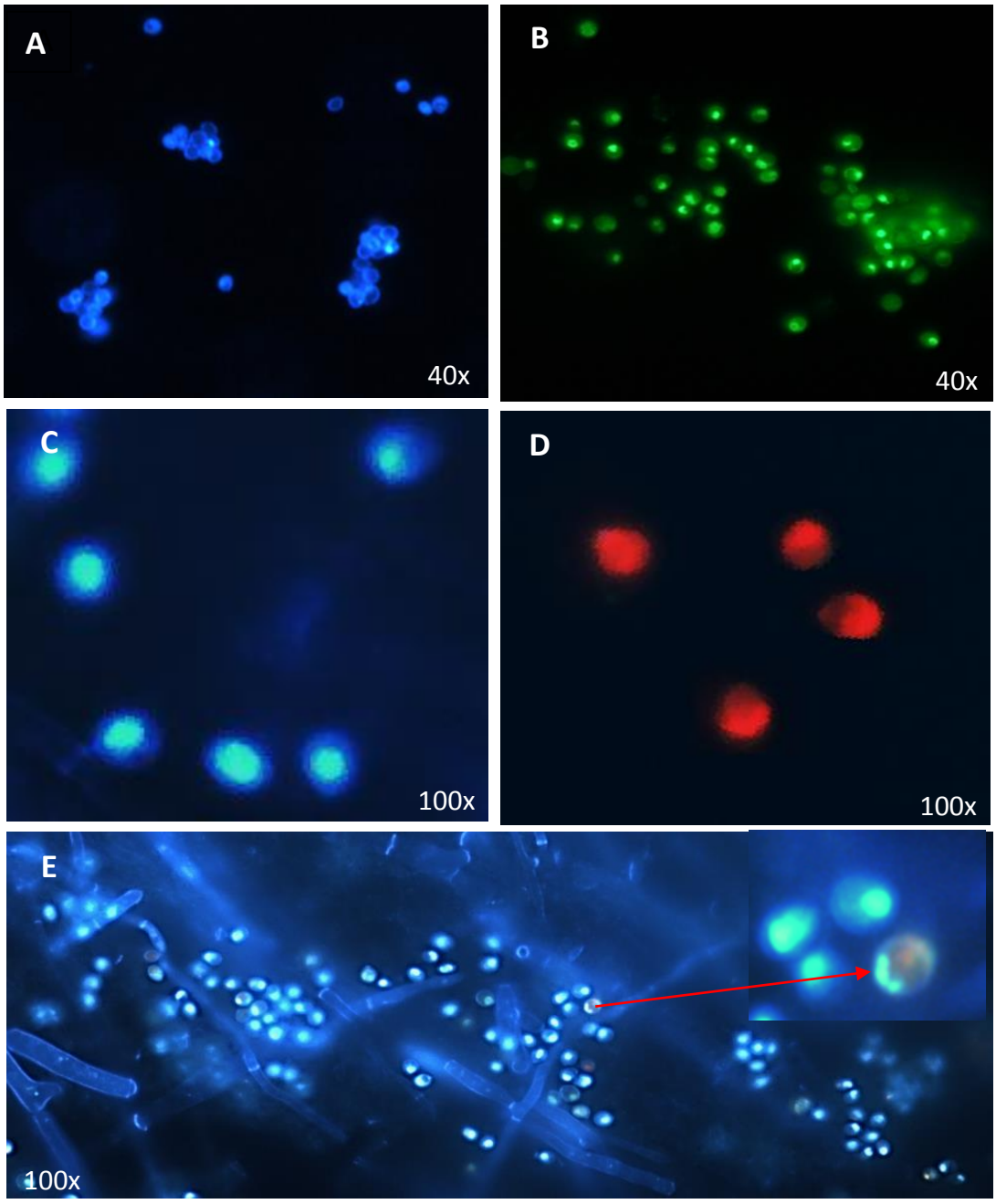

Fig. 4. Stained DNA of *B. bassiana* conidia. A and C) Stained conidia of IRK-59 with Hoechst 33342 observed with UMNU filter (excitation/emission 360/420 nm), B) Stained conidia of IRK-8 with AO observed with NIB filter (excitation/emission 480/510 nm), D) Stained conidia of IRK-8 with AO observed with the NU filter. E) Electrofused suspension containing parental conidia (stained with Hoechst 33342 and AO dyes) and hybrid conidia observed with the UMNU filter. The red arrow points to the hybrid conidia that contain both fluorescent dyes.

Table 3. The probability of having a hybrid conidia based on the binomial theorem.

# of cells remaining:	Probability of having 0 hybrid cells in remaining cells	Probability of having at least 1 hybrid cell in remaining cells
1	0.6	0.4
2	0.36	0.64
3	0.216	0.784
4	0.1296	0.8704
5	0.07776	0.92224
6	0.04666	0.95334
7	0.02799	0.97201
8	0.0168	0.9832
9	0.01008	0.98992
10	0.00605	0.99395

2.3.4. Bioactivity test of hybrid isolates and comparison with parental ones

After isolation of 10 CFUs and incubation at 25 $^{\circ}$C for one week, all were exposed to 25, 36, 38 and 40 $^{\circ}$C for 24, 48, 72 and 120 h. All 10 cultures survived at 25, 36 and 38 ^{0}C for 24, 48 and 72 h. Only three survived at the highest temperature (40 $^{\circ}$C) and the longest exposure time (120 h) (Fig. 5). These three isolates were called strain A, strain B and strain C and were tested against WFT second instars with the two parental isolates (IRK-59 and IRK-8) and GHA as the standard. The results showed that at concentrations of 1×10^{6} spores/ml ($F_{3,\ 32}$= 9.61, P=0.00) and 1×10^{8} spores/ml ($F_{3,\ 32}$= 39.88, P=0.00), strain A caused significantly higher mortality in WFT compared to the other new strains (Fig. 6).

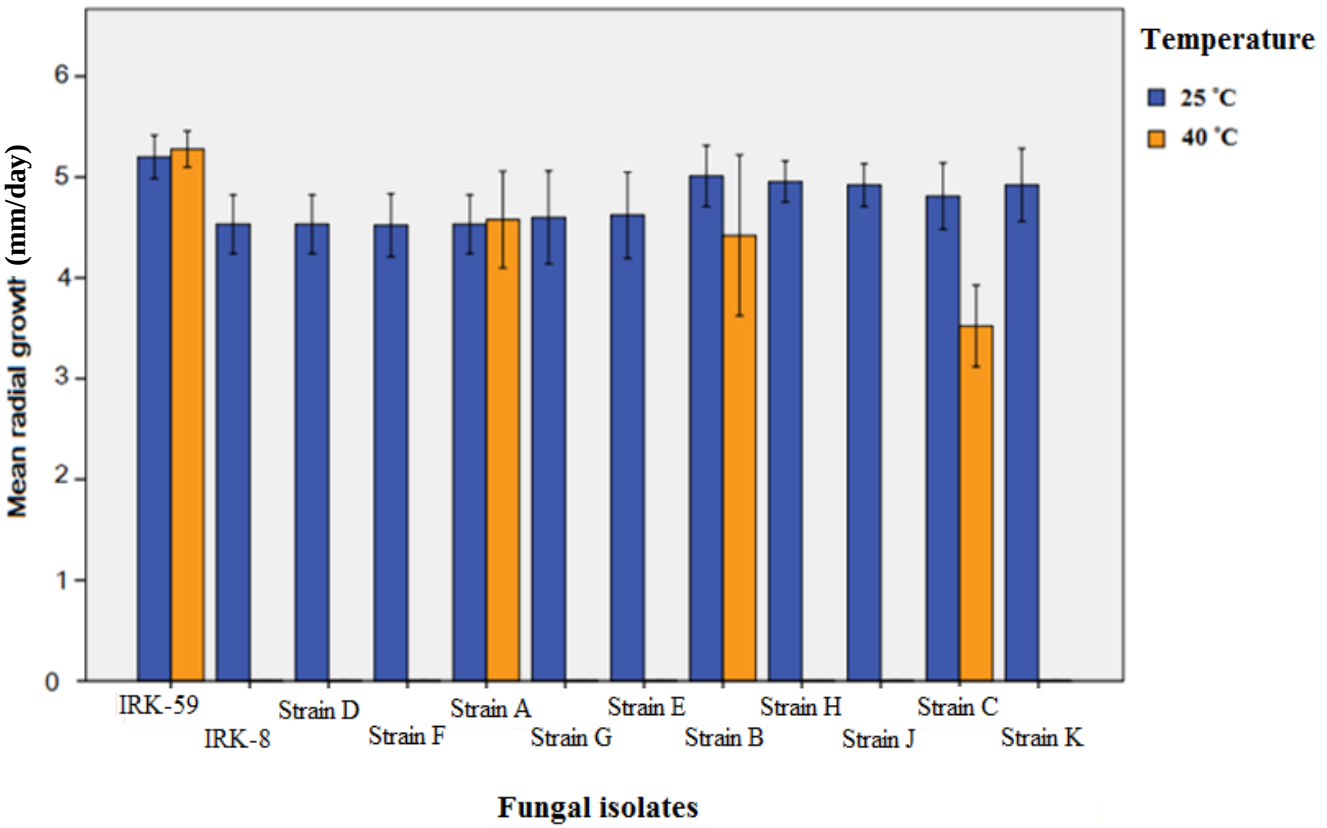

Fig. 5. Mean (±SE) radial growth (mm/day) of fusion products and the parental isolates at the highest thermal treatment and the longest exposure time.

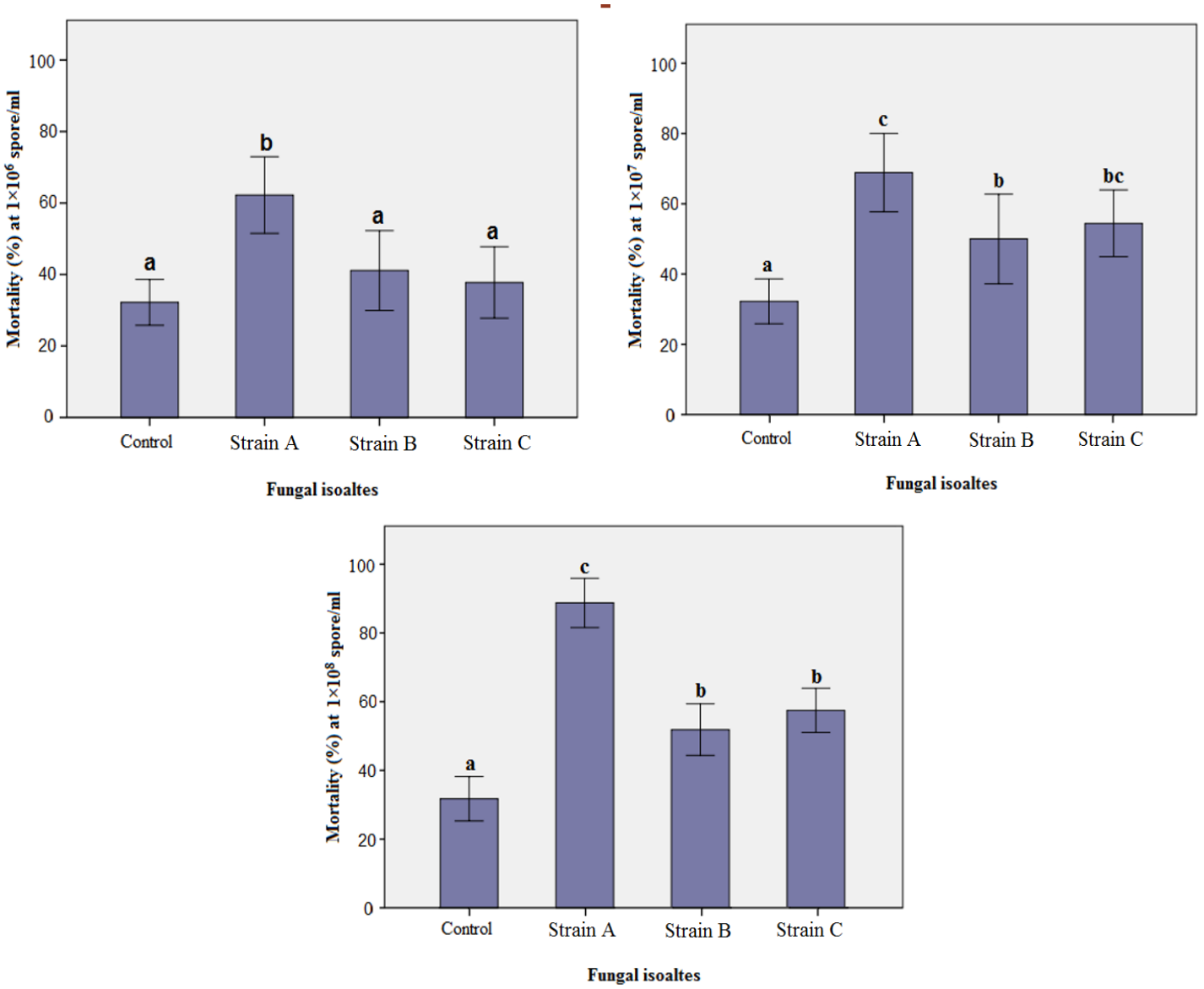

Fig. 6. Mean (±SE) percentage mortality of WFT second instars over time at three fungal concentrations of electrofusion products. Same letters above bars indicate no significant difference a day 10 ($p < 0.05$) according to Tukey test.

The efficacy of three thermotolerant strains was also compared with the two parental isolates and GHA (Fig. 7). At concentration of 1×10^6 spores/ml, there was no strain A and the most efficacious parental isolate (IRK-8) caused a significantly higher mortality to WFT compare to other fungal isolates ($F_{6,\ 56}$ =10.91, P= 0.00). The mortality of WFT treated with strain B and strain C was significantly lower than Strain A once the fusion products where compared with IRK-8. The virulence of strain A was not statistically different from the other two strains at 1×10^7 spores/ml ($F_{6,\ 56}$= 10.28, P=0.00). However, at 1×10^8 spores/ml, strain A and IRK-8 were significantly different from other new strains and from GHA with mean (±SE) percentage mortality of 83.3% and 90.0%, respectively at day 10 ($F_{6,\ 56}$= 36.75, P=0.00) (Fig. 7). Strain A was not only the most efficacious strain among the three electrofusion products at day 10, it also caused the highest percentage mortality to WFT at days 3, 5 and 7 when compared with strains B and C, IRK-59 and GHA (Table 4).

Table 4. Mean (±SE) mortality of WFT over time at different fungal concentrations after electrofusion.

	Day 3			Day 5			Day 7			Day 10		
	Concentration (spore/ml)			Concentration (spore/ml)			Concentration (spore/ml)			Concentration (spore/ml)		
	1×10^6	1×10^7	1×10^8	1×10^6	1×10^7	1×10^8	1×10^6	1×10^7	1×10^8	1×10^6	1×10^7	1×10^8
IRK-59	1.9 ± 0.6^{b}	2.7 ± 1.0^{b}	2.6 ± 0.8^{bc}	2.8 ± 0.8^{ab}	4.0 ± 1.1^{c}	4.1 ± 1.1^{bcd}	3.7 ± 1.0^{abc}	4.6 ± 0.9^{b}	5.2 ± 1.1^{cd}	4.7 ± 0.9^{abcd}	5.2 ± 0.8^{b}	6.4 ± 0.4^{b}
IRK-8	1.3 ± 0.9^{ab}	2.1 ± 1.1^{b}	3.7 ± 0.4^{c}	3.0 ± 1.1^{ab}	3.3 ± 0.9^{bc}	5.2 ± 1.3^{d}	4.7 ± 0.7^{c}	5.1 ± 1.0^{b}	7.2 ± 1.3^{d}	6.3 ± 0.7^{d}	7.6 ± 1.0^{c}	9.0 ± 0.7^{c}
GHA	1.3 ± 0.8^{ab}	1.6 ± 0.7^{ab}	2.0 ± 0.5^{ab}	2.0 ± 0.8^{a}	2.1 ± 0.7^{ab}	3.1 ± 0.9^{bc}	3.0 ± 1.0^{abc}	4.3 ± 0.9^{b}	5.0 ± 0.9^{bcd}	5.1 ± 0.6^{bcd}	6.1 ± 1.1^{bc}	6.4 ± 0.8^{b}
Strain A	1.8 ± 0.5^{b}	2.4 ± 0.7^{b}	2.8 ± 0.8^{bc}	3.8 ± 0.9^{b}	3.6 ± 0.9^{bc}	4.3 ± 1.0^{cd}	4.2 ± 0.9^{bc}	4.8 ± 0.9^{b}	6.3 ± 0.9^{cd}	5.7 ± 0.5^{cd}	6.9 ± 1.1^{bc}	8.3 ± 0.8^{c}
Strain B	0.9 ± 0.4^{ab}	1.3 ± 0.7^{ab}	1.7 ± 0.7^{ab}	2.1 ± 0.7^{a}	2.1 ± 1.1^{ab}	2.7 ± 0.6^{abc}	3.1 ± 0.9^{abc}	3.6 ± 1.0^{ab}	3.8 ± 0.7^{bc}	4.1 ± 1.1^{abc}	5.0 ± 1.3^{ab}	5.2 ± 0.7^{b}
Strain C	1.6 ± 0.4^{ab}	1.9 ± 0.6^{ab}	1.6 ± 0.4^{ab}	2.0 ± 0.5^{a}	2.9 ± 0.4^{abc}	2.6 ± 0.6^{ab}	2.8 ± 0.5^{ab}	4.3 ± 0.7^{b}	4.3 ± 0.7^{ab}	3.8 ± 1.0^{ab}	5.4 ± 1.0^{bc}	5.8 ± 0.7^{b}
Control	0.5 ± 0.6^{a}	0.6 ± 0.5^{a}	0.6 ± 0.5^{a}	1.3 ± 0.8^{a}	1.3 ± 0.8^{a}	1.3 ± 0.8^{a}	2.0 ± 0.8^{a}	2.0 ± 0.9^{a}	2.0 ± 0.9^{a}	3.2 ± 0.7^{a}	3.2 ± 0.6^{a}	3.2 ± 0.6^{a}

Means within a column labeled with the same letter are not statistically different, ($p < 0.05$) according to Tukey test.

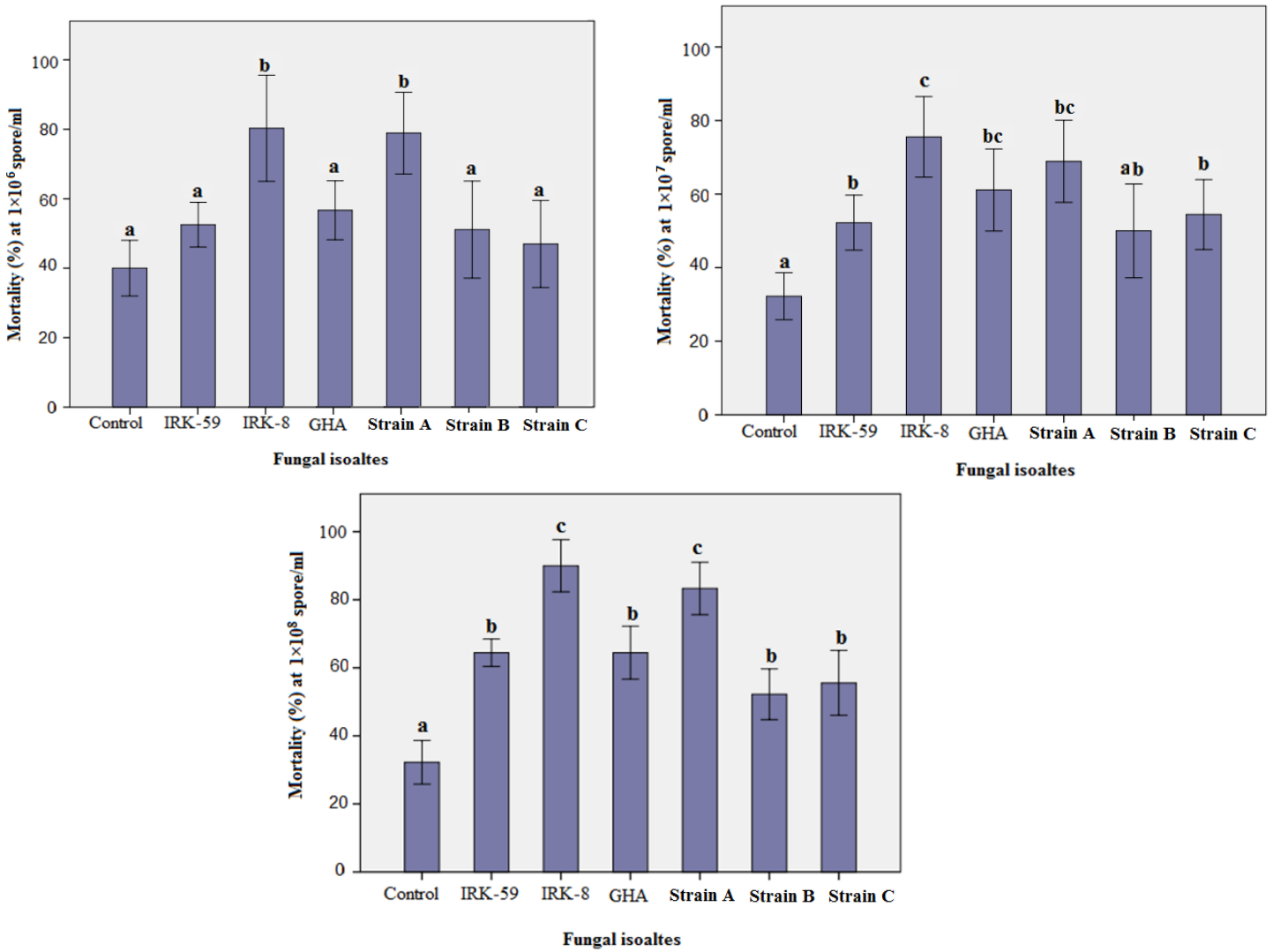

Fig. 7. Mean (±SE) percentage mortality of WFT second instars over time at three fungal concentrations of electrofusion products along with the parental isolates and GHA as the standard. Same letters above bars indicate no significant difference at day 10 ($p < 0.05$) according to Tukey test.

2.4. Discussion

Entomopathogenic fungi have considerable potential to suppress a broad range of arthropod pests (Khashaveh et al., 2010; Bonnie et al., 2009; Valero-Jimenez et al., 2014; Alvarez-Baz et al., 2015). However, sporulation, virulence and resistance to extreme environmental conditions are extremely diverse among various fungal strains. This variation is mainly due to the genetic properties and geoclimatic origins of fungal strains (Papierok, 2003). In the present study, *B. bassiana* strains were isolated from various regions including Iran, South Korea and Turkey with diverse geoclimatic conditions. Besides the difference in virulence, which is mostly associated with the genetic makeup of each strain, the most thermotolerant ones had been isolated from habitats with higher temperatures such as Iran. This variability in conidial thermotolerance isolated from different origins has been already confirmed in various species of entomopathogenic fungi (Rangel et al., 2005; Liu et al., 2003). In the current study, strains IRK-59 and JEF007 were exceptionally thermotolerant compared to other strains of *B. bassiana* that have been studied. Most *B. bassiana* strains tolerated temperatures above 38 ^{0}C for less than 24 hours (Kim et al., 2011; Fernandes et al., 2008; Ying and Feng, 2006), however, IRK-59 and JEF007 conidia resumed growth after 120 h incubation at 40 ˚C.

Percentage of WFT mortality induced by the most efficacious strains, JEF007 and IRK-8, was basically comparable to other *B. bassiana* strains that have been previously studied at similar spore concentrations (Thungrabeab et al., 2006; Gouli et al., 2008; Ugine et al., 2005; Gao et al., 2012). Higher thermotolerance and efficacy in IRK-59 and IRK-8 among all nine studied strains made these strains excellent candidates for the electrofusion process and acquiring a hybrid that contains both characteristics. This is the

first report of fusion between conidia of two *B. bassiana* strains using electric shock. One of the important components of a successful protoplast fusion using either chemical or physical fusogens is the frequency or yield of fusion after the fusion process. Srinivas and Panda (1997) reported 0.2-2% fusion frequency in *Trichodermma reesei* using PEG. Additionally, only 4 fusants were recovered from 150 protoplasts of two strains of *Sclerotium rolfsii* after protoplast fusion by use of PEG (Hayat and Christias, 2010). In our study, the number of fusants was relatively high 40% in a 50 µl droplet of final suspension. In general achieving higher yield of fusion is easier with electrofusion compared to the chemical processes (Verma and Kumar, 2000). This is mainly due to the versatility of the electrofusion process. The duration of electric pulse and voltage (two electrical parameters that govern pore formation) are adjustable based on the type and the concentration of cells in the electroporator (Potter and Heller, 2003).

The Bio-Rad Gene Pulser has different settings with recommended adjustment for various cell types. The optimized setting for fungal cells in this instrument is an exponential decay pulse type, capacitance 25 µF, parallel resistance 200 Ω and voltage range 1000-3000 V (Bio-Rad, www.bio-rad.com). All recommended voltages were screened and it was revealed that the highest yield of conidia containing both fluorescent dyes was obtained at 2000 V. We believe that voltages below 2000 V were too weak to produce pores in the conidia cell wall. Interestingly, at voltages above 2000 V the fusion products were completely obliterated in the buffer and the entire structure of the conidia was collapsed due to electric breakdown of the cell membranes and excessive leakage that lead to cell death. These results are similar to the optimal conditions for electrofusion

of *Colletotrichum gloeosporioides* that resulted in a 58% conidial survival (Robinson and Sharon, 1999).

Besides the number of fusants, the properties and the virulence of hybrid cells should be comparable or even more desired than the parental strains as the result of a successful hybridization. Among ten fungal conidia acquired after electrofusion, those that showed the same amount of resistance to 40 $^{\circ}$C were selected and assumed to be either hybrids or IRK-59. Only one strain (strain A) among three possible hybrids performed a comparable efficacy against WFT with the most efficacious parent IRK-8. In a similar study the fusion product obtained from a toxin-producing strain of *B. sulfurescens* and a highly pathogenic strain of *B. bassiana* against European corn borer showed three days reduction in LT50 (Couteaudier et al., 1996). Although in our study the hybrid strain was not superior in terms of efficacy and thermotolerance relative to the parental strains but it proved to possess both features after the fusion process.

There is no clear mechanism for the transformation of fungal strains using electrofusion. In general, application of external electric field leads to movement of ions in the surrounded medium of the cell membranes, accumulation of charges at the membrane surfaces and membrane polarization. The charges at the membrane surfaces create an electric field inside the membrane, which is much stronger than the field in the surrounding medium. The electric field inside the membrane interacts with the polarized membrane material and changes the structural rearrangements of the cell wall. Once the field strength is high enough these events can lead to the formation of pores in the membrane surfaces (Dimitrov and Christov, 1992).

The formation of pores leads to exchange of molecules including nuclei and formation of a heterokaryon that undergoes karyogamy and results in a diploid cell (Pontecorvo, 1956; Roper et al., 2013). Meiotic recombination and repeated chromosome loss leads to the formation of haploid cells and cells with different genomes from those of either parent nuclei (Pontecorvo, 1956; Strom and Bushley, 2016).

In this study, electrofusion was applied to produce a hybrid strain of *B. bassiana* that contains the desired genes from two different parental strains. The entire process of choosing hybrid strains was based on the biological activity of the fusion products and the statistical similarity between them and the parental strains. However, it is particularly important to investigate the genetic differentiation of the resulting hybrids with the parental strains at the molecular level using techniques such as AFLP, RAPD or ITS sequencing (Aquino De Muro et al., 2005). Moreover, performance and stability of the hybrids strains under field conditions requires further investigation. The current research is a pioneer study of genetic transformation in *B. bassiana* by use of electrofusion and it could be developed to create superior hybrid strains with various desired properties for integrated pest management and industrial uses in the future.

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CHAPTER 3*:* ENHANCE THE POTENTIAL OF *BEAUVERIA BASSIANA* ISOLATES FOR MANAGEMENT OF SUNN PEST, *EURYGASTER INTEGRICEPS*

3.1. Introduction

The Sunn Pest, *Eurygaster integriceps* Puton (Hemiptera: Scutelleridae) is a major pest of wheat and barley in Western and Central Asia, and Southeast Europe (El Bouhssini et al., 2002). Nymphs and adults of Sunn Pest feed on leaves, stems and grains and cause significant damage on both yield and quality of the grains. During feeding, Sunn Pest injects saliva into the grains through its piercing-sucking mouthparts. The injected saliva contains digestive enzymes that destroy the gluten in the grain and reduce the baking quality of the bread made from the flour. Due to the high dependency of people's diet in the Near and Middle East on cereal crops, Sunn Pest management strategies are needed for both food security and stability of wheat-based agricultural systems.

Chemical control is the primary strategy to control Sunn Pest particularly in developing countries (Miller and Morse, 1996). However, due to problems such as insecticide resistance, environmental pollution and human health hazards associated with chemical residues, more attention needs to be paid to environmentally friendly approaches to management of Sunn Pest. Research has been done on distribution,

ecology and physiology of Sunn Pest, but more needs to be done on developing a sustainable integrated pest management model (IPM). Special emphasis has been devoted over the past few years to cultural methods, development and cultivation of resistant wheat varieties, and detection and application of Sunn Pest natural enemies. Attempts on mass rearing and inundative releases of egg parasitoid wasps (Kutuk et al., 2010; Amir-Maafi and Parker, 2011; Trissi et al., 2006) and isolation and application of entomopathogenic fungi (Mustu et al., 2014; Edgington et al., 2007; Trissi et al., 2012; Mohammadbeigi and Port, 2013; Haji Allahverdi Pour et al., 2008), have shown promising results that require further investigation.

Entomopathogenic fungi have significant potential to suppress Sunn Pest populations (Abdulhai et al., 2010; Mustu et al., 2014; Sedighi et al., 2013; Zibaee and Bandani, 2009, Jenkins et al., 2007). However, despite a considerable amount of research on isolating, mass production and formulating fungal entomopathogens, there are still concerns related to the efficacy, speed of kill and their resistance to several abiotic factors. To minimize pest impact, a biological control agent must reduce or eliminate the pest quickly to prevent economic damage and must be effective at a range of environment conditions. This is particularly important because unlike other insect pathogens such as bacteria and viruses that require direct ingestion through insect mouthparts, the infection process by fungi occurs directly through the external cuticle. Once the conidia of a fungal pathogen attaches to the insect body, it germinates and breaches the cuticle to enter the insect hemocoel and causes the infection (Khan et al., 2012). Therefore, ambient conditions including temperature, humidity, UV radiation and rainfall have significant impact on successful germination of fungal conidium (Braga et al., 2001; Roberts and St.

Leger, 2004; Inglis et al., 1999). Even after host death, the growth of aerial mycelia of the fungi from the insect cadaver and formation of sporogenous cells take place only under favorable conditions (Goettel et al., 2000).

Entomopathogenic fungi are mesophilic and have a broad range of temperature tolerance (0-40 °C), however, the optimum temperature for germination, growth, infection and sporulation is generally restricted to 20-30 °C (Kassa, 2003; Rangel et al., 2005). Most entomopathogenic fungi are damaged or killed by temperatures higher than 36 °C, however, fungal tolerance to high temperatures is highly species and strain dependent.

In general, *Metarhizium* species have the widest thermal range of growth (5-40 °C), followed by *Beauveria* and *Paecilomyces* (5-30 °C) (Hallsworth and Magan, 1999; Rangel et al., 2005). Some strains of *M. acridum* resume growth after 12 days exposure to 38 °C and 40 °C (Rangel et al., 2008). Similarly, Rangel et al., (2005) reported that the conidia of some *M. anisopliae* strains survived and showed an acceptable germination rate (>80%) after 24 h exposure to 50 °C. Additionally, the thermal dead point of a *M. anisopliae* strain is reported to be between 50 and 55 °C after 30 mm (Zimmermann, 1982). Obviously, the duration of high temperatures and whether the temperature is constant or fluctuates is strongly determinative in survival of the conidia. In some isolates of *B. bassiana*, conidial germination began to decrease after 8 h at 35-38 °C (Uma Devi et al., 2006), however, another isolate showed relatively high germination rate (>70%) after 1 h at 45 °C (Fernandes et al., 2008). Geoclimatic studies revealed that there is a correlation between thermal tolerances of fungal strains isolated from different regions (Bidoshka et al., 2001). Generally, strains from higher latitudes have lower thermal

tolerance compare to those from lower latitudes (Kryukov et al., 2012; Rangel et al., 2005). Liu et al. (2003) reported that the conidia germination of a *M. anisopliae* isolate from high latitude (Chittenden County, VT, USA) significantly reduced by increase in temperature from 40 to 50 °C. Moreover, this isolate showed lower germination rate in comparison with a *B. bassiana* isolate from a lower latitude (Arkansas, USA).

Considering the maximum temperature in wheat fields during the outbreak of Sunn Pest (at the end of April and beginning of May), for instance, 37.5 ^{0}C in Romania (Popov et al., 1996), 33.8 °C and 36.6 °C in West and Central Iran (Anonymous, 2017/Wheatherspark.com), 36-38 °C in Iraq (FAO, 2011), fungal pathogens with higher thermal resistance seem to be better candidates for use in integrated Sunn Pest management programs. While temperature is an important factor in survival of fungal conidia, its association with high humidity can significantly affect conidial thermotolerance (Rangel et al., 2005). Protein denaturation and membrane disorganization are major problems that occur in cells subjected to wet heat (Setlow and Setlow, 1998). DNA damage and generation of mutants are the main consequences of dry heat (Nicholson et al., 2000). Ying and Feng (2006) reported that water-stressed conidia were significantly less thermotolerant in comparison with conidia exposed to wet heat. In addition, it is believed that fungal conidia are more resistant to high temperature than the vegetative cells.

Based on the current research on pest management with fungal pathogens, there is considerable diversity among fungal strains in terms of sporulation, virulence and resistance to extreme environmental conditions (Sedighi et al., 2013; Mustu et al., 2014; Mohammadbeigi and Port, 2013; Kryukov et al., 2012; Kim et al., 2010; Edgington et al.,

2007). Consequently, several collections of entomopathogenic fungi have been created all around the world that need to be effectively used. Similar to plant genetic materials, fungal strains should be genetically enhanced using current fungal genetic resources to address major concerns about their present and future application (Meyling and Eilenberg, 2007).

Genetic transformation of fungi was first reported in the budding yeast *Saccharomyces cerevisiae* (Hinnen et al., 1978). This transformation was done by removing the cell wall and introducing an exogenous DNA into the yeast cells. Several techniques have been investigated for permeabilization of cell wall in *S. cerevisiae* including spheroplast method, electrofusion, biolistic method, polyethylene glycol (PEG) and a glass bead method (Hashimoto et al., 1985; Kawai et al., 2010). Genetic transformation has also been reported in other yeasts such as *Saccharomyces pombe*, *Pichia pastoris*, *Candida albicans* (Moreno et al., 1991; Kurtz et al., 1986) and some species of the filamentous fungus *Aspergillus* (Dawe et al., 2000). Protoplast fusion is another molecular approach to gene transfer in microorganisms lacking the genetic exchange by conventional mating systems (Verma and Kumar, 2000). This includes many phytopathogenic and insect killing fungi. Salamiah et al. (2001) applied protoplast fusion on two pathogenic and non-pathogenic strains of *Alternaria alternate* using electrofusion and PEG and studied the pathogenicity of the hybrid strain. Protoplast fusion was conducted on plant pathogenic fungus *Sclerotium rolfsii* to develop a new hybrid strain (Hayat and Christias, 2010). Couteaudier et al. (1996) produced a somatic hybrid from two species of entomopathogenic fungi *B. bassiana* and *B. sulfurescens*

using PEG. The hybrid strain was hypervirulent against European corn borer (*Ostrinia nubilalis*) compared to parental isolates.

Most studies of fungal protoplast fusion have been done using PEG (Kawai et al., 2010; Couteaudier et al., 1996). However, the advantage of transformation by electrofusion is that this technique is a nonchemical method that does not alter the biological structure or function of the target cells. In addition, unlike chemical techniques, electrofusion is a more efficient and less tedious process (Pigac and Schrempf, 1995). The only drawback of electrofusion technique is the need for a cell electroporator, however, since the process is too short there is a possibility to share the instrument with different labs (Navarro-Sampedro et al., 2000). Electrofusion is a two stage process: I) bringing cells to close physical contact and II) application of the electric pulse to cause the formation of pores in cell membranes and inducing the cells to exchange their genetic material (Weaver, 1995) (Fig. 1). It is important to know that the result of fusion is not always 100% formation of hybrid cells and fusion products are a combination of hybrid and intact (parental) cells. The fusion yield is controlled by amplitude, duration, and number of applied pulses. Increase in any of the pulse parameters mentioned leads to a higher level of membrane fusion and consequently higher number of fused cells (Teissie and Ramos, 1998). Parameters of the electric pulses should be carefully chosen.

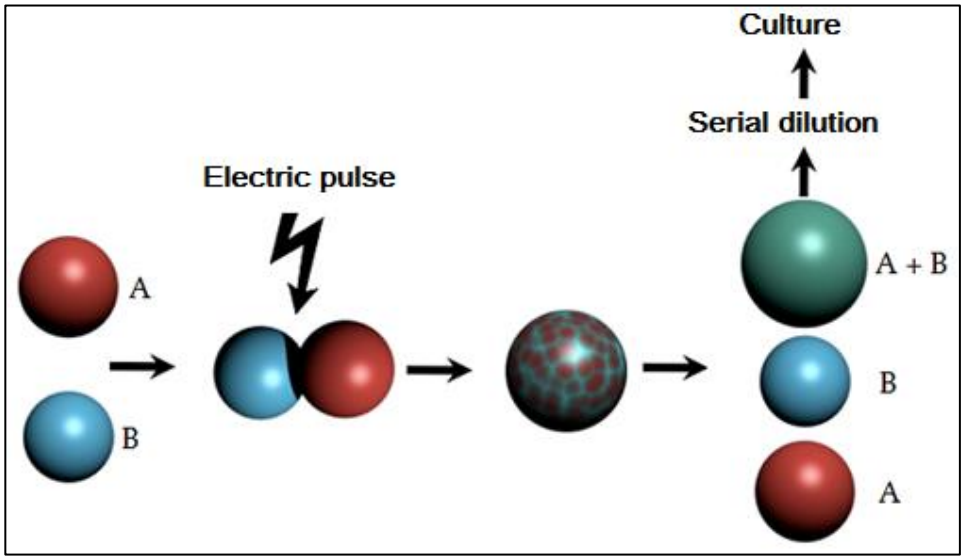

Fig. 1. Schematic of the cell fusion process using the electric shock.

The purpose of this study is to develop the potential of the entomopathogenic fungus, *Beauveria bassiana* as a component of IPM for management of Sunn Pest. The molecular technique electrofusion was applied to the thermotolerant and highly virulent isolates to combine these traits and create a hybrid strain that is highly thermotolerant and pathogenic against Sunn Pest. The electrofusion technique used to make hybrid isolates to apply against Sunn Pest is the same as illustrated in Chapter 2 and was not explained here to avoid redundancy.

3.2. Material and methods

3.2.1. Fungal isolates

Two isolates of *Beauveria bassiana*, IRK-59 and IRK-8 that were obtained from the fungal collection located at the Iranian Research Institute of Plant Protection (IRIPP), Sunn Pest Research Department, Tehran, Iran, were used for additional thermotolerance tests and efficacy against Sunn Pest. Three characteristics of isolates were evaluated:

conidial vs. mycelia thermotolerance, wet vs. dry conidial thermotolerance and the rate of infection or virulence against Sunn Pest. Fungal isolates were cultured on potato dextrose agar media (PDA) and incubated at 25 ± 1 °C for 15 days. Viability of fungal isolates was assessed prior to the experiment by spreading 100 µl of fungal suspensions on PDA and incubation at 25 ± 1 °C for 24 h.

3.2.2. Thermotolerance tests

3.2.2.1. Conidia vs. mycelia heat tolerance

To investigate the effect of temperature on mycelia growth, each isolate was cultured on PDA and incubated at 25 ± 1 °C for 4 days. A five-mm diam. disc from unsporulated mycelium of 4 days old fungal isolate was placed upside down on the center of PDA in a 60-mm Petri dish. Fungal isolates were incubated at 25 (the control), 38 and 40 °C for 24, 48, 72 and 120 h. After each thermal stress period, Petri dishes were incubated at 25 °C for another 20 days. For conidia thermotolerance, 50µl of the fungal suspension with a concentration of 1×10^6 spores/ml was cultured on the center of a PDA plate and incubated as above. The surface radial growth (cm) of each isolate was measured and marked every 5 days from the center of the Petri dish to the outer edges of the fungus. Each isolate was replicated three times and the entire experiment repeated three times.

3.2.2.2. Wet vs. dry heat tolerance

Fungal suspensions with the concentration of 1×10^6 spores/ml were transferred into Eppendorf tubes (500 µl in each tube) and placed in a water bath at 40 and 45 °C for

4, 8, 16, 24 and 48 h. After incubation at each thermal treatment, 300 µl of fungal suspensions were spread on the surface of PDA and incubated at 25 °C. The effect of dry heat on fungal conidia was assessed by spreading 300 µl of the 1×10^6 spores/ml suspension and incubation at the above mentioned temperatures with the same time durations. The percent germination was calculated 24 and 48 h post incubation.

3.2.3. Sunn Pest bioassay

Sunn Pest adults were collected one day before bioassaying (Parker, personal communication) from overwintering sites in Varamin, Iran at three stages of the overwintering period (June, November and March). Collected insects were placed in a glass container covered with a wet paper towel and transferred to the laboratory and held at 25±1 °C, 60-70% rH and a light: dark (L: D) of 14:10 hours at least 48 hours before the beginning of the bioassaying. Conidia of selected fungal isolates were harvested from the surface of 15 days old cultures and suspended in a test tube containing 5 ml sterile distilled water and 0.02% Tween 80® solution. The suspension was stirred and filtered through a single layer of linen to remove culture debris and mycelia. Conidial concentrations were adjusted to 1×10^6, 1×10^7 and 1×10^8 spores/ml.

The bioassay was conducted on 10 Sunn Pest adults (5 male, 5 female). Insects were placed on a strip of scotch tape dorsal side down and 5 µl of conidial suspension dropped onto the mesosternum. After 10 minutes of drying, insects were transferred to rectangular plastic containers (20×14×6 cm) containing wheat grains and a vial of water clogged with cotton and held in an incubator at 25±1 °C, 65±5% rH. Mortality counts were taken 5, 10, 15 days post application. Sunn Pest adults were considered dead if they

failed to move following slight probing. Dead insects from each treatment were surface sterilized by placing them in Ethanol 50% for 5 sec, in sodium hypochlorite for 30 sec and finally three times in sterile distilled water for 10 sec and kept separately in Petri dishes containing sterile paper towel moistened with 0.10% streptomycin sulfate and 0.02% penicillin G (Lacey and Brooks, 1997). Dishes were incubated at 22±2 °C and 65±5% rH for 2 weeks to observe fungal outgrowth. The fungal characteristics were evaluated and matched with those that were applied earlier (Koch's postulates). Each isolate, and each concentration were replicated three times and the entire experiment was repeated three times.

3.2.4. Statistical analysis

Mortality and thermotolerance were compared by one-way analysis of variance (ANOVA) followed by Tukey's studentized test and linear regression analysis when significant differences were found at $P \leq 0.05$ using SPSS ver. 24.0 (SPSS, 2009).

3.3. Results and discussion

3.3.1. Fungal thermotolerance

3.3.1.1. Conidial vs. mycelia thermotolerance

Based on the results of the thermotolerance test, the conidial tolerance of IRK-8 was clearly less than the IRK-59 at high temperatures. This difference is more obvious at 40 °C as after 120 h incubation at this temperature IRK-8 did not grow even after 20 days recovery at 25 °C (Fig. 2). Interestingly, this difference in thermotolerance was not

observed when mycelia of the two isolates were exposed to the temperature regimes. Although, the average growth of IRK-8 was lower than IRK-59 in most thermal treatments, the mycelium of this isolate tolerated even the longest incubation time and the highest temperature regimes (Fig. 3).

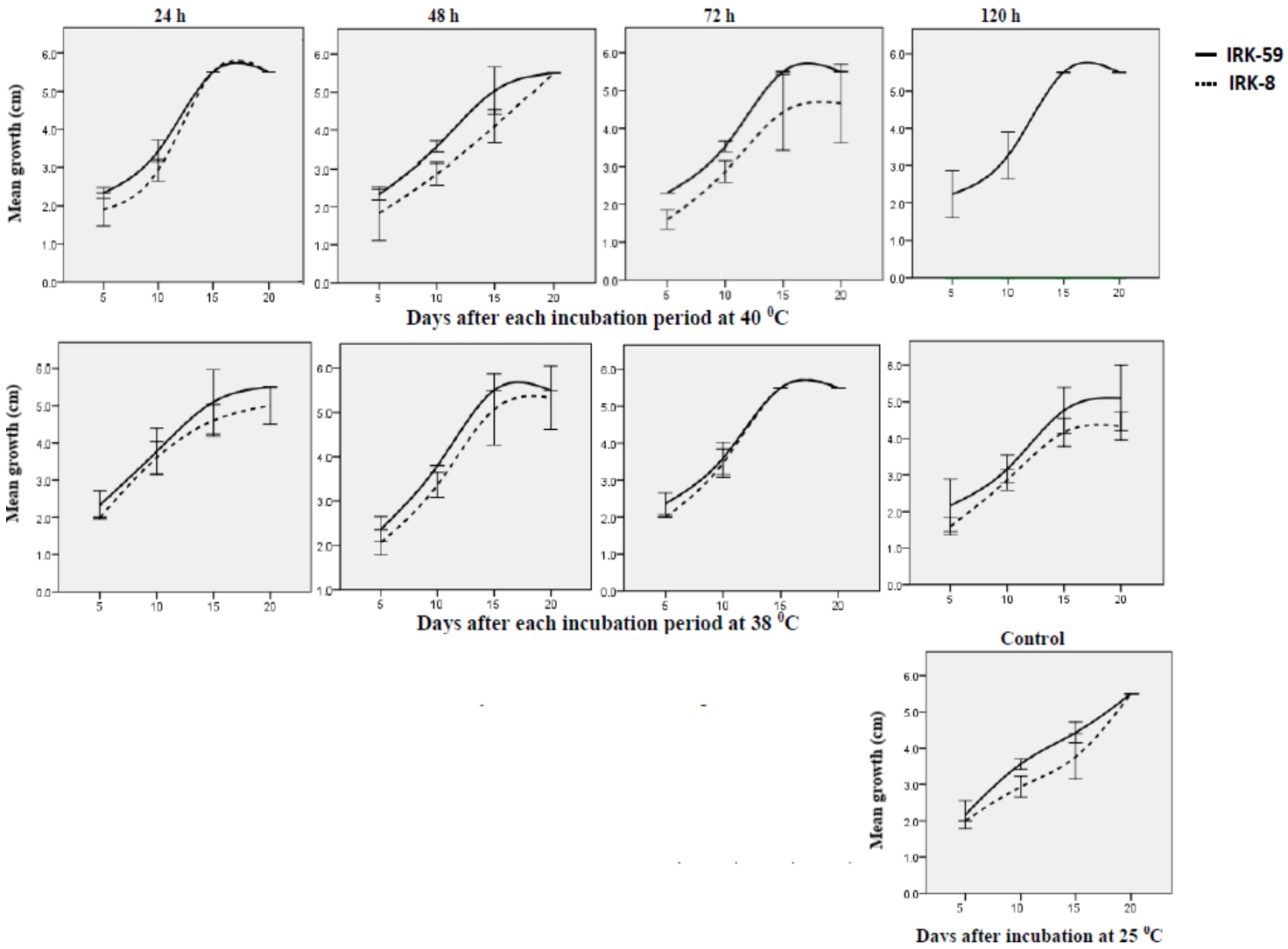

Fig. 2. Mean radial growth (cm) of fungal conidia at 40 ˚C and 38 ˚C.

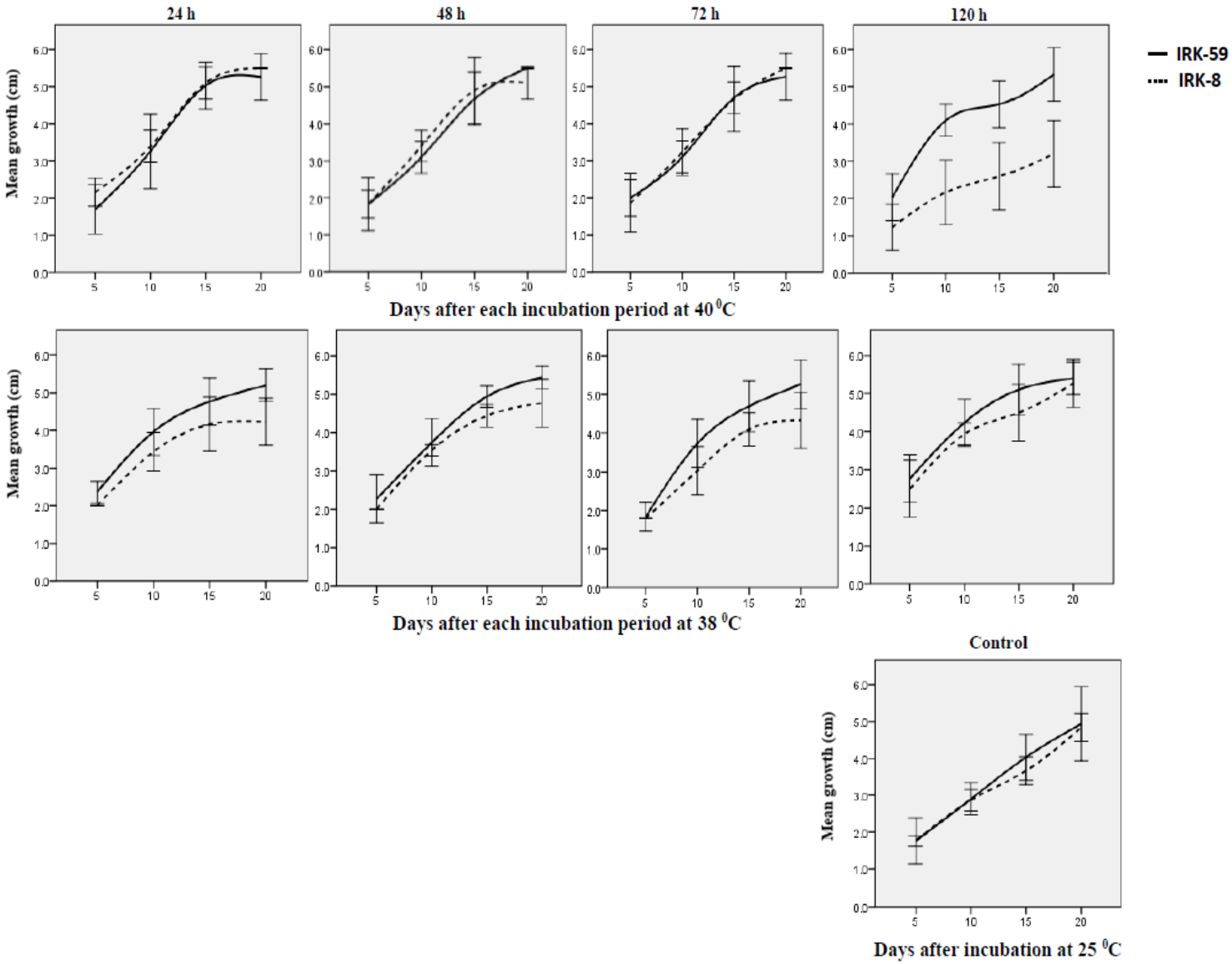

Fig. 3. Mean radial growth (cm) of fungal mycelia at 40 °C and 38 °C.

Based on the results of mycelia and conidia thermotolerance test, it was revealed that mycelia growth of both IRK-8 and IRK-59 is not affected by various thermal treatments. This shows that conidia of the studied isolates are probably more susceptible to the high temperatures than their mycelia. This was in contrast with our knowledge about the morphological features of the cell walls in conidia vs. mycelia and their levels of resistance to extreme temperatures. It is believed that conidia have relatively thicker cell walls compared to those of the vegetative hyphae and most fungi use conidia as a stress-resistant dispersal stage. However, fungal mycelium can form specialized survival structures known as a sclerotium. This is a firm, often rounded mass of hyphae with thickened cell walls that increases the fungal resistance under unfavorable conditions (Reinartz et al., 1989). Moreover, the conidia cell wall is a highly dynamic structure and is subject to changes and modifications with culture age (Ghamrawi et al., 2014). Conidia harvested from 11 days old cultures were less heat resistant than those of 25 days old cultures (Conner and Beuchat, 1987). This difference is due to the accumulation of stress tolerance compounds such as mannitol and trehalose. In the current study, conidia that were exposed to high temperatures were harvested from a 7 days old culture. Therefore, their higher susceptibility to the heat shock in comparison with the mycelia might be associated with their immature stage.

In addition, some parameters such as the sugar content of the growth medium, pH and the amount of organic acids in the medium increase the heat resistance of the conidia during the germination phase (King, 1990).

3.3.1.2. Conidial dry-heat vs. wet-heat

The results of the thermotolerance test between the conidia of IRK-8 and IRK-59 showed that both isolates are more resistant to the dry-heat than to the wet-heat (Fig. 4 and Fig. 5). The percent germination of both isolates at 40 °C was above 80% under dry-heat conditions. In addition, both isolates tolerated 4, 8 and 16 h exposure to wet-heat. Although the percent germination in IRK-8 was significantly lower ($F_{1,16}$= 9.12, P=0.00) that IRK-59 at 16 h treatment and no germination occurred in this isolate after 24 h incubation (Fig. 4). There was no germination for either IRK-8 or IRK-59 under wet conditions at 40 ^{0}C. In general, IRK-59 was more tolerable to 40 °C wet-heat than IRK-8. The difference between the two isolates was not obvious at 45 °C since none of them survived wet-heat more than 8 h with considerably low percent germination at this time duration (Fig. 5). The percent germination of both isolates decreased with an increase in incubation time at 45 °C under dry conditions. No germination was observed in IRK-59 and IRK-8 after 48 h incubation at 45 °C.

These results are consistent with those of Rangel et al. (2005) in which exposure of conidia to 45 °C wet-heat delayed germination of some isolates of *M. anisopliae*. Moreover, Zimmermann (1982) reported an increased thermotolerance in *M. anisopliae* by decreasing the relative humidity of the growth media. Temperature tolerance is strongly tied to the amount of water and wet heat is more damaging to the spores than dry heat. This is probably associated with the level of the damage and amount of time that is needed to repair damage before germination occurs (Nicholson et al., 2000).

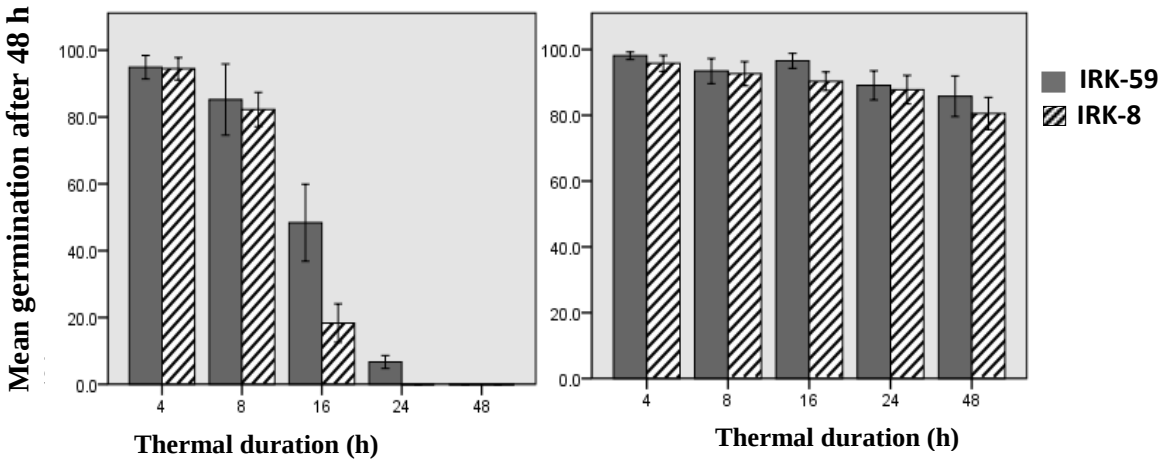

Fig. 4. Percent germination of IRK-59 and IRK-8 after 4, 8, 16, 24 and 48 hours incubation at 40 ˚C wet-heat (left) and dry-heat (right)

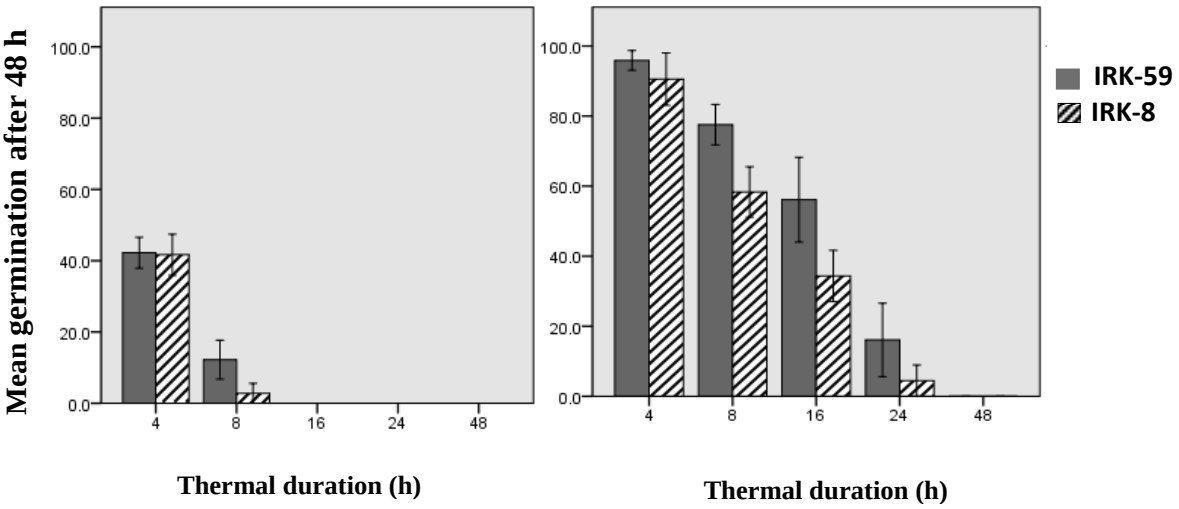

Fig. 5. Percent germination of IRK-59 and IRK-8 after 4, 8, 16, 24 and 48 hours incubation at 45 ˚C wet-heat (left) and dry-heat (right)

3.3.2. Sunn Pest mortality

Following the electrofusion process that was explained in Chapter 2, the first isolate that was considered as the hybrid (strain A) was tested against overwintering adults of Sunn Pest along with the parental isolates and the GHA. The result was similar to what was observed in the WFT bioassay. No significant difference was observed between fungal concentrations ($F_{9, 215}$= 0.65, P=0.75) and the mortality slightly increased at higher concentrations (Fig. 6). The results of the analysis of variance showed that there was significant difference ($F_{1,128}$= 7.04, P=0.00) between the parental isolates, IRK-59 and IRK-8, in Sunn Pest mortality regardless of the overwintering stage. However, no significant difference was observed among the efficacious parental isolate, IRK-8, strain A and GHA ($F_{2,178}$= 1.15, P=0.32). Considering the results of the thermotolerance tests from Chapter 2, this similarity of strain A to the efficacious parent isolate IRK-8 supports our hypothesis that strain A is most probably a hybrid.

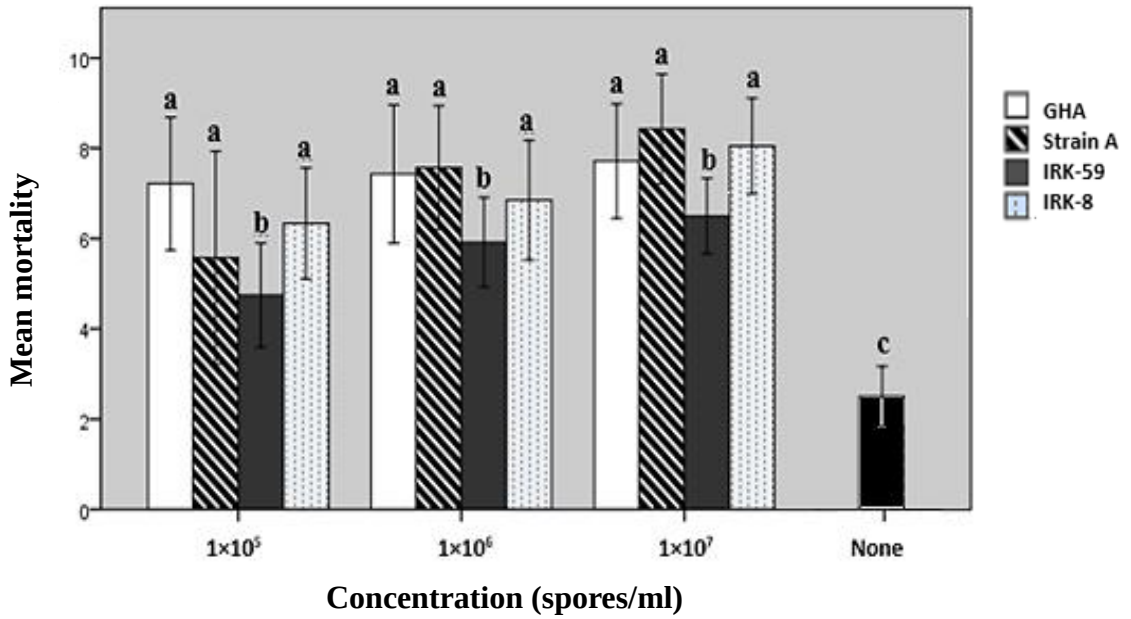

Fig. 6. Sunn Pest mortality a week after treatment with three fungal concentrations

The results of Sunn Pest bioassay at three stages of the overwintering period were compared using analysis of variance. Mean mortality of adults were compared by the Tukey-test and considered to be statistically different at the significance level of 95% ($F_{2, 230}$= 31.34, P=0.01) (Fig. 7). Sunn Pest mortality caused by fungal isolates was increased toward the end of the overwintering period as the percent mortality reached 100% in March for IRK-8 and strain A. These results are supported by other authors (Trissi et al., 2012; Saadati bezdi et al., 2012; Mojaver and Bandani, 2010). Sunn Pest adults do not feed during the overwintering period, therefore their survival entirely depend on the amount of energy and nutrition stored in their body. Therefore, depletion of nutrition at the end of the overwintering period (March) makes the insect more susceptible to the fungal pathogen. This critical life stage of Sunn Pest should be considered in the efficient management of the insect. It means that lower fungal material is required to suppress Sunn Pest populations in their vulnerable overwintering stage (Fig. 8).

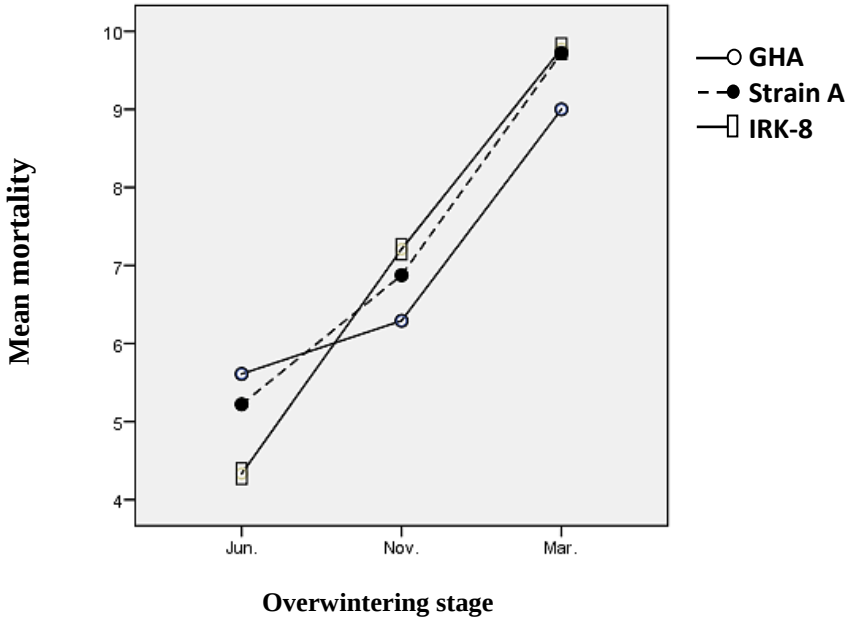

Fig. 7. Sunn Pest mortality a week after treatment with fungal isolates at various stages of overwintering period.

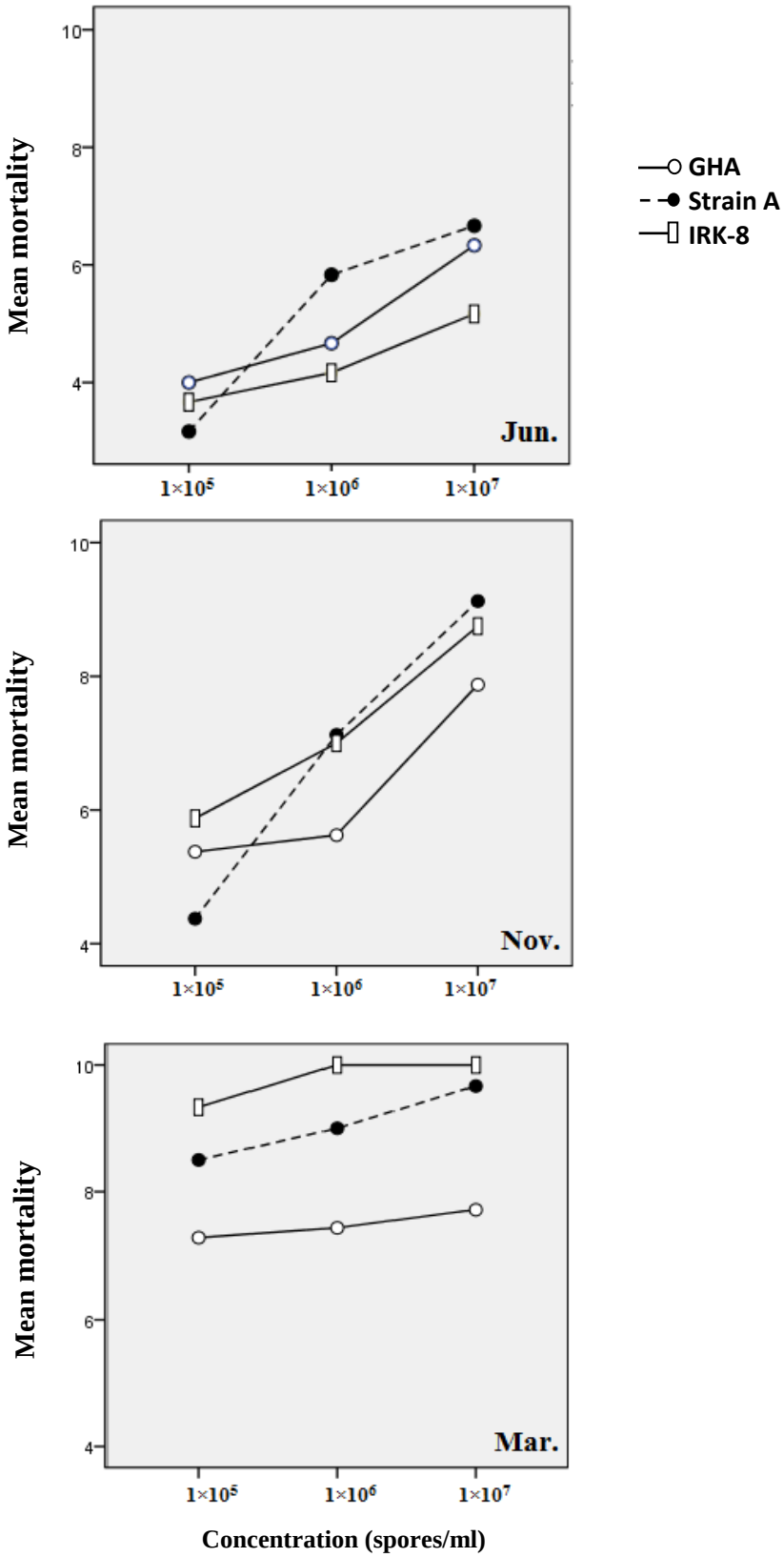

Fig. 8. Sunn Pest mortality a week after treatment with three fungal concentrations at various stages of overwintering period.

3.4. Conclusion and perspective

Application of entomopathogenic fungi especially *B. bassiana* has been increased in Sunn Pest management programs over the last decade. However, considerable effort is still focused on collection and storage of various fungal isolates. Considering the significant genetic variation among different isolates of *B. bassiana* isolates, studying the fungal characteristics to select superior ones is crucial. In the current study, two *B. bassiana* isolates that were significantly effective against Sunn Pest and WFT and also thermotolerant at high temperatures underwent the electrofusion process. Among fusion products only one contained both characteristics of the parental isolates and was considered a hybrid (strain A).

The above mentioned conclusion was based on the bioactivity of strain A and statistical analysis of mortality and thermotolerance data. These results need to be confirmed by molecular techniques and DNA sequence analysis of the hybrids in the future. Favorable characteristics can be selected through standardize bioassays and screening the growth and survival of fungal isolates at extreme environmental conditions. This requires excessive replication of laboratory tests and altering several parameters to obtain the most robust results. For instance, several factors are involved in the level of thermotolerance in fungal isolates. Beside the intensity, the duration of temperature, the amount of moisture in the medium and the sensitivity of mycelium vs. conidia were investigated in this study. However, there are many other factors that affect the thermotolerance of fungal isolates (i.e. pH, type and the amount of nutrition in the growth medium, temperature fluctuation, etc.).

Based on the results of this study, the susceptibility of Sunn Pest to various entomopathogenic fungi changes during the overwintering period. This should be considered to conduct more effective management of this insect. Migration of Sunn Pest adults to overwintering sites and diapausing a considerable amount of time (around 9 months) in those locations is a great opportunity to target more populations before adults actually attack the fields.

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