Potential Application of Entomopathogenic Nematodes for Management of Stable fly, *Stomoxys calcitrans* (L.) under Laboratory Conditions

Niyaporn Khwanket

A Thesis Submitted in Fulfillment of the Requirements for the

Degree of Master of Science in Entomology

Prince of Songkla University

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Candidate

I hereby certify that this work has not been accepted in substance for any degree, and is not being currently submitted in candidature for any degree.

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ABSTRACT

The stable fly (*Stomoxys calcitrans*) is an important insect pest of livestock because it causes annoyance and vector-borne diseases to livestock. Farmers deploy several methods to control stable flies. Entomopathogenic nematodes (EPNs) are used to control stable fly as they are soil-borne insect pathogens. In this study, eight substrates including 0–12 hours old fresh cow manure (FCM12), seven days old cow manure (CM7), seven days old cow bedding (CB7), manure (Man), seven days old fermented timothy hay (FT7), seven days old fermented cow manure (FCM7), seven days old cow manure and fermented timothy hay (CMFT7) and water-soaked cotton (WSC = control) were performed for the preference of larvae and gravid female of *S. calcitrans*. *Stomoxys calcitrans* larvae showed the highest preference for substrate CM7 with 26.70%. The highest preference of gravid female was FCM12 with 55.00%. The pathogenicity of six EPN isolates (*Steinernema scarabaei* EPNKU60, *Heterorhabditis indica* EPNKU64, *H. indica* EPNKU67, *H. indica* EPNKU82, *H. bacteriophora*, *S. carpocapsae* and *S. siamkayai*) at the rates of 0, 25, 50, 100, 200 and 400 Infective Juveniles /cm^2 were tested against second, third instar larvae and pupae of *S. calcitrans* in filter paper bioassays. *Heterorhabditis bacteriophora* killed 100.00% of the second instar larvae at the rate of 200 IJs/cm^2. *Heterorhabditis bacteriophora* and *H. indica* EPNKU82 showed 80.00% and 100.00% mortality of third instar larvae at the rate of 400 IJs/cm^2 while mortality of insect larvae was less than 30.00% for other EPNs. Low efficacy of EPN infection on pupae of *S. calcitrans* was observed and only 56.00% pupal mortality was found at the rate of 400 IJs/cm^2. Persistence of three selected EPNs (*H. indica* EPNKU82, *H. bacteriophora* and *S. siamkayai*) was estimated at 1, 3, 5, 7 and 9 days of exposure on eight substrates

where water-soaked cotton was replaced by 10% moist sand by baiting larvae at the last instar larvae of *Galleria mellonella*. *Heterorhabditis indica* EPNKU82 showed high persistence on SAN and FT7 causing 83.33–100.00% and 90.00–100.00% mortality at 1–9 days of exposure. Similarly, *H. bacteriophora* showed high persistence of EPN on SAN and CB7 and the insect mortality ranged from 93.33–100.00% and 90.00–100.00% at 1–9 days of exposure. Only on the 1 day of exposure *S. siamkayai* still had 70.00% mortality of the last instar larvae of *G. mellonella*.

ชื่อวิทยานิพนธ์ ศักยภาพการประยุกต์ใช้ไส้เดือนฝอยศัตรูแมลงเพื่อการจัดการแมลงวันคอกสัตว์ *Stomoxys calcitrans* (L.) ภายใต้สภาพห้องปฏิบัติการ

ผู้เขียน นางสาวนิยาภรณ์ ขวัญเกตุ

สาขาวิชา กีฏวิทยา

ปีการศึกษา 2564

บทคัดย่อ

แมลงวันคอกสัตว์ (*Stomoxys calcitrans*) เป็นแมลงวันที่มีความสำคัญทางปศุสัตว์ เนื่องจากสร้างความรำคาญและเป็นพาหะนำโรคต่อปศุสัตว์ เกษตรกรจึงต้องใช้วิธีการที่หลากหลายในการควบคุมแมลงวันคอกสัตว์ ไส้เดือนฝอยศัตรูแมลง เป็นวิธีการหนึ่งที่นำมาใช้ในการควบคุมแมลงวันคอกสัตว์ได้ เนื่องจากไส้เดือนฝอยศัตรูแมลงเป็นชีวภัณฑ์ที่ก่อโรคแก่แมลงที่ดำรงชีวิตอยู่ตามพื้นดิน การศึกษานี้ได้นำวัสดุบริเวณคอกสัตว์จำนวน 8 ชนิด ประกอบด้วย มูลวัวอายุไม่เกิน 12 ชั่วโมง (FCM12) มูลวัวอายุ 7 วัน (CM7) หญ้ารังนอนอายุ 7 วัน (CB7) ปุ๋ยคอก (Man) หญ้าธิโมธีหมักอายุ 7 วัน (FT7) มูลวัวหมักอายุ 7 วัน (FCM7) มูลวัวและหญ้าธิโมธีหมักอายุ 7 วัน (CMFT7) และสำสีชุบน้ำ (ชุดควบคุม, WSC) มาทดสอบความชอบในการเข้าหาวัสดุของแมลงวันคอกสัตว์ในระยะหนอนและตัวเต็มวัยที่ตั้งท้อง พบว่าแมลงวันคอกสัตว์ระยะหนอนมีความชื่นชอบต่อมูลวัวอายุ 7 วันมากที่สุด คิดเป็น 26.70 เปอร์เซ็นต์ ตัวเต็มวัยเพศเมียที่ตั้งท้องมีความชื่นชอบในการวางไข่ที่มูลวัวอายุไม่เกิน 12 ชั่วโมง มากที่สุด คิดเป็น 55.00 เปอร์เซ็นต์ การทดสอบประสิทธิภาพการก่อโรคของไส้เดือนฝอยศัตรูแมลงจำนวน 6 สายพันธุ์ (*Steinernema scarabaei* EPNKU60, *S. carpocapsae*, *S. siamkayai*, *Heterorhabditis indica* EPNKU64, *H. indica* EPNKU67, *H. indica* EPNKU82 และ *H. bacteriophora*) ที่อัตรา 0, 25, 50, 100, 200 และ 400 infective juveniles (IJs) /cm^2 ที่มีต่อระยะหนอนแมลงวันคอกสัตว์วัย 2, 3 และระยะดักแด้ ด้วยวิธี filter paper bioassays พบว่าไส้เดือนฝอยศัตรูแมลงสายพันธุ์ *H. bacteriophora* เข้าทำลายระยะหนอนของแมลงวันคอกสัตว์วัย 2 ได้ถึง 100.00 เปอร์เซ็นต์ ที่ความเข้มข้น 200 IJs/cm^2 นอกจากนั้นไส้เดือนฝอยศัตรูแมลงสายพันธุ์ *H. bacteriophora* และ *H. indica* EPNKU82 ที่อัตรา 400 IJs/cm^2 เข้าทำลายระยะหนอนของแมลงวันคอกสัตว์วัย 3 เป็น 80.00 และ 100.00 เปอร์เซ็นต์ ตามลำดับ ในขณะที่ไส้เดือนฝอยศัตรูแมลงชนิดอื่นทำให้ระยะหนอนของแมลงวันคอกสัตว์มีอัตราการตายน้อยกว่า 30.00 เปอร์เซ็นต์ สำหรับระยะดักแด้ของแมลงวันคอกสัตว์ พบว่ามีอัตราการตายเพียง 56.00 เปอร์เซ็นต์ ที่ความเข้มข้น 400 IJs/cm^2 เท่านั้น นอกจากนั้น การประเมินความคงค้างของไส้เดือนฝอยศัตรูแมลง 3 สายพันธุ์ (*H. indica*

EPNKU82, *H. bacteriophora* และ *S. siamkayai*) ในวัสดุบริเวณคอกสัตว์จำนวน 8 ชนิด ที่ระยะเวลา 1, 3, 5, 7 และ 9 วันหลังการใช้ โดยใช้ทรายที่มีความชื้น 10 เปอร์เซ็นต์ (SAN) เป็นชุดควบคุมทดแทนการใช้สำสีชุบน้ำ และใช้หนอนกินรังผึ้ง (*Galleria mellonella*) วัยสุดท้ายเป็นเหยื่อ พบว่าไส้เดือนฝอยศัตรูแมลงสายพันธุ์ *H. indica* EPNKU82 มีอัตราการตายของหนอนกินรังผึ้งสูงในทราย และหญ้าธิโมธีหมักอายุ 7 วัน อยู่ระหว่าง 83.33–100.00 และ 90.00–100.00 เปอร์เซ็นต์ ตามลำดับ ที่ระยะเวลา 1–9 วัน หลังการใช้ ในทำนองเดียวกันไส้เดือนฝอยศัตรูแมลงสายพันธุ์ *H. bacteriophora* มีอัตราการตายของหนอนกินรังผึ้งในทราย และหญ้ารังนอนอายุ 7 วัน อยู่ระหว่าง 93.33–100.00 และ 90.00–100.00 เปอร์เซ็นต์ ที่ระยะเวลา 1–9 วันหลังหลังการใช้ สำหรับไส้เดือนฝอยศัตรูแมลงสายพันธุ์ *S. siamkayai* พบว่า ทำให้หนอนกินรังผึ้งตายมากกว่า 70.00% เพียงระยะเวลา 1 วัน หลังการใช้

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INTRODUCTION

Nine percent of seven families of Diptera are known to be biting flies that feed on the blood of livestock (Wallace, 2009). Biting flies affect the liveliness of the animals that caused annoying, blood loss, animal pain and also reduces feed intake that affects on weight gain and milk production (Todd, 1964). Most the biting flies are cosmopolitan insects in the world except Antarctica (Wallace, 2009). The important biting flies are horn flies (Muscidae; genus *Haematobia*), deer and horse flies (family Tabanidae) and stable flies (family: Muscidae; genus: *Stomoxys*) (Cortinas and Jones, 2006). Stomoxyinae flies (Diptera: Muscidae) are blood-sucking insects with some species considered critical economic pests of livestock. Among 51 described species in 10 genera, five genera are of outstanding importance including *Stomoxys*, *Haematobosca*, *Haematobia*, *Haematostoma*, and *Stygeromyia* (Malaithong *et al.*, 2019; Pont and Dsouli, 2008; Pont and Mihok, 2000; Steelman, 1976; Zumpt, 1973). Among the 11 species of Stomoxyinae flies in Thailand, *Stomoxys calcitrans* (L.) or known as the stable fly is considered to be the most medically and veterinary important and also of economic concern (Malaithong *et al.*, 2019).

The stable fly *S. calcitrans* is a serious insect pest of veterinary importance that is widely distributed worldwide. Adults of stable fly feed on the blood of livestock through piercing-sucking mouthparts that cause pain and annoyance to livestock. The economic impact and losses caused by *S. calcitrans* and other related species can be uncountable. The damage of their bite can decrease 60% production of milk and 19% weight gain of animal (Campbell *et al.*, 2001). Severe biting activity by these flies reduces animal weights resulting in significant losses in meat and milk production (Greenberg and Povolny, 1971). Moreover, stable flies are considered insect vectors of livestock harboring the virus, bacteria, protozoa, parasites and helminthis (Baldacchino *et al.*, 2013; Foil, 1989).

Farmers employ several methods to control stable flies such as sanitation method, trapping with or without chemical insecticides and direct application of chemical insecticides. However, insecticides have a broad-spectrum activity and can cause environmental contamination, chemical residues, insecticide resistance and

animal irritation. Although, insecticides can reduce stable fly populations, they are a poor substitute for manure, trash and straw disposal. Insecticides are a common method in fly control programs but are becoming less effective. Hence, the increasing of insecticide resistance in stable fly population led most research attending to find the other option. Biological control method is an important non-chemical method in stable fly control. Several ways of controlling stable fly using biological agents such as parasitic insects, bacteria, fungi and entomopathogenic nematodes have been studied elsewhere (Leal *et al.*, 2017; Lysyk *et al.*, 2012; Machtinger *et al.*, 2016; Smith *et al.*, 1989). One of the effective biological agents for the control of insect pests, the stable fly on bedding substrates in stables is the entomopathogenic nematodes (Pierce, 2007).

Entomopathogenic nematode is being interested in stable fly control because they are soil-borne insect pathogens that can survive in soil. Entomopathogenic nematode can infect many important soil-borne insect pests worldwide, mainly in the immature stage of insect pests and they cause insect hosts to die within 24–48 hours by releasing symbiotic bacteria from their gut (Griffin *et al.*, 2005). The immature stage of the stable fly lives and feeds on animal manure, organic matter, poultry litter, waste and moist straw bedding (Broce *et al.*, 2005; Moon, 2002) therefore, the application of EPN is suitable for the control of this insect. EPN of the genera *Steinernema* and *Heterorhabditis* are also reported with infection with instar larvae of various flies, especially the stable fly under laboratory conditions (Leal *et al.*, 2017; Mahmoud *et al.*, 2007; Taylor *et al.*, 1998).

In, Thailand, control of stable fly with EPN has not been reported, therefore the use of Thai EPN to control stable fly is a major challenge. Numerous isolates of Thai EPN have been reported in Thailand and some of them are known to be excellently potential biological agents. The efficacy of Thai EPN against stable fly should be studied under laboratory conditions to provide guidance for field-scale application. In addition, the application of EPNs may replace the use of synthetic insecticides and lead to reduce the use of synthetic insecticides and increase animal welfare for sustainable livestock system in the country. Entomopathogenic nematodes also provide sustainable agriculture as they can survive in soil, are specific to their hosts, harmless to mammals and naturally maintain ecological balance.

OBJECTIVES

1. To investigate the preference of laboratory reared larvae, oviposition of wild-caught *S. calcitrans* adult on bedding substrates under laboratory conditions.

2. To evaluate the efficacy of EPN against larvae and pupae of *S. calcitrans* under laboratory conditions.

3.To investigate the persistence of EPN applied on bedding substrates under laboratory conditions.

LITERATURE REVIEW

1. Stable fly

1.1 Classification of the stable fly

Kingdom: Animalia

Phylum: Arthropoda

Class: Insecta

Order: Diptera

Suborder Brachycera

Family: Muscidae

Subfamily: Muscinae

Genus: *Stomoxys*

Species: *calcitrans*

Binomial name: *Stomoxys calcitrans* (L.)

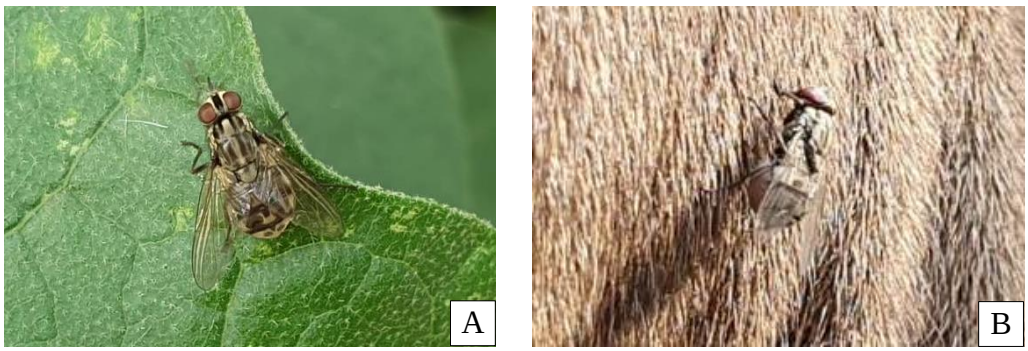

Figure 1 *Stomoxys calcitrans*, A: an adult of *Stomoxys calcitrans*; B: *Stomoxys calcitrans* is feeding on the cow.

Stomoxys calcitrans (L.) is generally called “stable fly” which originated in Africa (Zumpt, 1973) (Figure 1A). The stable fly is reported as a serious economic insect pest of livestock when it present in large numbers in the population (Rugg, 1982). Both male and female stable flies are hematophagous insect pests (Figure 1B) that cause painful bites by their piercing sucking mouthparts onto their host skin (Foil and Hogsette, 1994). They prefer to feed on host blood on legs, knees, or hocks, preferring the underside of various livestock (Dougherty *et al.*, 1994; Hogsette *et al.*, 1987; Lavoipierre, 1965; Moorhouse, 1972; Zumpt, 1973). Stable flies are more numerous in

the summer season than the other thus of the year (Dougherty *et al.*, 1994; Hogsette and Farkas, 2000). They also suck blood meal with many domestic animals (Hogsette and Farkas, 2000; Skovgård and Nachman, 2017) and they can live on a sugar-based diet (Salem *et al.*, 2012; Taylor and Berkebile, 2008).

1.2 Biology and behavior

Adults of *S. calcitrans* are diurnal feeders from morning to the afternoon (10:00 am–4:00 pm) under favorable environmental conditions (Charlwood and Lopes, 1980; Hoffmann, 1968). They are inactive at night, but they can be found in some resting places to avoid the wind such as on support beams, ceilings, near barns, shaded structures and trees (Broce, 1988). They feed on blood within 3–4 minutes until their stomachs are full (Foil and Hogsette, 1994). They rest on the undersides of plant leaves, trees, fences and other areas near the host. They must feed on blood more than 1 time before laying eggs (Bishopp, 1913; Kuzina, 1942; Lotmar, 1949; Moobola and Cupp, 2008; Venkatesh and Morrison, 1980) Adult females require 3–5 times of blood from the host to complete their reproduction (Chia *et al.*, 1982; Kuzina, 1942; Moobola and Cupp, 2008) and adult males also require at least one blood meal to complete their seminal fluid operation (Anderson, 1978; Jones *et al.*, 1992).

Habitat: Normally, breeding sites of stable flies are located near animal manure, poultry litter, fermenting vegetables, kitchen waste, wet straw bedding, garbage, and poorly composted manure (Broce *et al.*, 2005; Moon, 2002; Solórzano *et al.*, 2015). They are found in tropical and subtropical regions and rely on overwintering in temperate regions (Moon, 2002) but distribution is cosmopolitan (Skidmore, 1985; Soós and Papp, 1984; Zumpt, 1973).

Adult females of the stable fly feed on the blood for egg production and lay their eggs within 5–8 days after mating (Hoffmann, 1968). The eggs disperse in varying amounts and patterns (Simmons and Dove, 1941; Skovgård and Nachman, 2017). Females lay clutches of approximately 25–50 eggs to 100–300 eggs on plant material, animal manure, silage with organic matter, rotting hay and bedding mixed with urine and feces (Axtell, 1986; Friesen and Johnson, 2012). Fecundity rate depends on various factors such as temperature, rearing conditions and reproductive rate (Gilles *et al.*, 2005;

Lysyk, 1998). Eggs are white, 1 mm long and hatch to the first-instar larva within 12–48 hours at 26˚C (Foil and Hogsette, 1994; Showler and Osbrink, 2015; Skovgård and Nachman, 2017).

The larval stage is a vermiform shape with creamy color and is divided into three larval stages (Foil and Hogsette, 1994; Showler and Osbrink, 2015). The larvae live in decaying organic matter, hay residue, algae, spilled feed, dung, manure mounds, silage mounds, near trench, silos and feed troughs in cattle (Lysyk, 1993). Suitable temperatures for growing of this insect growing range from 10–30˚C in temperate countries (Gilles *et al.*, 2005). The prepupal stage is 10 mm in size and spends 12–26 days (Larsen and Thomsen, 1940; Showler and Osbrink, 2015). The pupa is capsular with a reddish-brown color (Skovgård and Nachman, 2017) and it takes 5–26 days to pupate (Foil and Hogsette, 1994).

The appearance of both adult males and adult females of the stable fly resembles the house fly (*Musca domestica* L.) but they are slightly larger than the house fly (Foil and Hogsette, 1994; Todd, 1964). Adults are 4–7 mm long (Foil and Hogsette, 1994). They usually have four black stripes on the thorax and a black checkerboard on the abdomen with a distinct pattern of black spots (Foil and Hogsette, 1994; Service, 1980; Zumpt, 1973) and they have a slight upward bend on the M1+2 vein of the wing vein (Castro, 1967; Foil and Hogsette, 1994).

In general, female stable flies can produce an egg batch of about 60–130 eggs and they can lay a large number of eggs on the same day (Foil and Hogsette, 1994). Adult life longevity is 30–35 days under laboratory conditions and 14 days under field conditions (Killough and Mckinstry, 1965). Under ideal conditions, the life cycle can be completed within an average of 20–25 days (Pugh *et al.*, 2014) but under high temperatures and humidity, stable flies can complete within 15–27 days (Lysyk, 1998; Taylor and Berkebile, 2011).

1.3 Economic importance

Stable flies have a major impact on reducing production in livestock because they cause pain, dermal allergies, hoof damage, hazard the animal host and reduce

growth development of cattle (Krinsky, 1976; Muzari *et al.*, 2010; Taylor *et al.*, 2012). They are a blood feeder of cattle, sheep, goats, cows, camels, horses, primates, canids and felids (Hogsette and Farkas, 2000). For example, a ratio of one stable fly per cattle could reduce 0.7% of milk production in the United States (Bruce and Decker, 1958). The stable fly outbreak of 50 and 100 flies per cattle reduced the weight gain of cattle by 13.2% and 20.0%, respectively compared to healthy cattle (without stable flies) (Campbell *et al.*, 1977).

Two flies per leg have been reported as an economic loss in heifer feeding (Campbell *et al.*, 1987; Marcon *et al.*, 1997). In 1981, (Campbell and McNeal) reported that 5 stable flies per cow foreleg was an economic threshold while in 1997 an economic threshold of 7 per cow per leg was reached (Catangui *et al.*, 1997). Moreover, Campbell *et al.* (2001) reported that biting damage can cause a 40–60% reduction in milk production and 19% reduction in weight gain. In eastern Nebraska, infestation levels of 50%, 25% and lower of the feedlots were found when stable fly numbers were 100, 50 and <15 flies per animal, respectively. Taylor *et al.* (2012) reported that the impact of the stable fly outbreak is estimated to be up to $2,211 million per year.

In addition, stable flies are considered insect vectors of livestock containing a virus, bacteria, protozoa, parasites and helminths (Baldacchino *et al.*, 2013; Foil, 1989). Stable flies can cause digestive diseases consisting of Equine infectious anemia (EIA or swamp fever), Vesicular stomatitis (VS), African horse sickness (AHS), Pigeon fever, Anthrax and Habronemiasis (Krinsky, 1976; Littlewood, 1999; Pusterla *et al.*, 2003). They can transmit pathogenic nematodes to livestock when they feed on wounds or the genitalia, eyes, nasal cavity, lips and foreskin (Schuster *et al.*, 2010).

The foraging time of stable flies has been reduced by promoting defensive behaviors such as foot treading, head throwing, jerky skinning and tail flapping (Dougherty *et al.*, 1993; Mullens *et al.*, 2006). These behaviors lead to impaired metabolism, reduction in animal feeding, fly-induced stress (fly fear), immunological responses to antigens (Schole *et al.*, 2011), plasma cortisol increasing and also loss of productivity (Vitela-Mendoza *et al.*, 2016). Nevertheless, stable fly outbreaks are associated with an increase in the sugarcane industry and sugarcane crops (Cançado *et*

al., 2013). The immature stage of the stable fly is located inside the sugarcane stalk after harvest, which is used as a breeding site for larval development because it is rich in nutrients and contains many quantities (Cançado *et al.*, 2013; Rolim *et al.*, 2013).

1.4 Management

Management of Stable flies is complicated and depends on the behavior of stable flies (Pitzer *et al.*, 2011). Nowadays, the successful methodology to control a stable fly population in livestock, horse and poultry farms is an integrated pest management program (IPM). This program consists of mechanical methods, sanitation, insecticide treatments, natural products, physical controls and biological control agents (Isman, 2006; Kaufman and Rutz, 2002; Lazarus *et al.*, 1989; Meyer *et al.*, 1991; Schmidtmann, 1991). Several different IPM programs are effective methods for controlling this insect pest (Axtell, 1986). The goal of management is to reduce the density of the stable fly population. The monitoring system is important to assess the situation of stable fly before deciding on the use of natural enemies, beneficial organisms and insecticides (Skovgård and Nachman, 2017).

Sanitation is one of the management programs that are key to reduce the population of stable flies by controlling abiotic factors such as setting unsuitable temperature and humidity, changing unattractive conditions, drying out litter, removing of bedding site at least once a week, cleaning up spilled feed, rotating areas where animals are fed (Axtell, 1986). Bedding material made from wood chips is better than using straw or hay because it does not decompose or compact quickly when soiled.

Trapping is a mechanical control that uses color, host odor, light, heat and smell as attractants. This method has been used to control stable flies such as the Williams trap (sticky traps) which is an effective method of monitoring a stable fly population (Broce, 1988; Williams, 1973). Hoy (1970) found that the Malaise traps baited with CO_2 were able to catch more stable flies than non-Malaise traps baited with CO_2. Cilek (1999) found that alsynite cylindrical traps with CO_2 and dry ice provided a very powerful attractant for stable flies.

In addition, alsynite fiberglass traps (AFTs) are visible and attractive to stable flies (Agee and Patterson, 1983; Hogsette and Ruff, 1990). Broce's cylindrical trap (Hogsette and Ruff, 1990) and blue-black fabric targets are attracted to stable flies in the United States (Foil and Younger, 2006; Hogsette and Foil, 2018; Mihok *et al.*, 1995). Moreover, Gilles *et al.* (2005) collected adult stable flies up to 1,250 files per day in Vavoua traps. Ultraviolet light traps are also evaluated as stable fly placed around entrances 1–2 m on the ground.

Chemical control of the stable fly in the last decade has focused on controlling the adult population through the use of insecticides that induced resistance in the stable fly to DDT (dichloro-diphenyl-trichloroethane), lindane and chlordane (Drummond, 1977; Sömme, 1958). Later, the resistance of stable flies to dieldrin (Mount, 1965) was reported in the United States, while resistance to toxaphene and lindane was also reported in South Africa in 1972 (Harris *et al.*, 1972). Cilek and Greene (1994) reported that stable flies in southeastern Kansas were tolerance to chlorvos (237.6–fold), stirofos (4.6–fold), and permethrin (1.8–fold) while Olafson *et al.* (2019) found first the *kdr* allele at position L1014F in stable flies from France and Thailand which was molecular indicators of pyrethroids resistance.

Many insecticides and repellents have been used in livestock to control fly populations (Herholz *et al.*, 2016). Tainchum *et al.* (2018) reported wild *S. calcitrans* were caught at each study site in southwestern France showed knock-down effects of Phoxim. Permethrin in emulsifiable concentrate and wettable powder formulations was residue-free effective on unpainted wood in shaded locations where stable flies feed (Hogsette *et al.*, 1987). Foil and Hogsette (1994) revealed that spraying permethrin on stable fly habitat or breeding sites such as building walls, bunks, and shelters affected fly control.

The product of permethrin or pyrethrin could control the central nervous system of stable fly systems (Debboun *et al.*, 2007). Both insecticides as arousal stimulators that repel and cause biting behavior to disappear (Hogsette *et al.*, 1987). In some cases ear tags and ear bands have been impregnated with insecticides to reduce stable fly populations (Hogsette and Ruff, 1986). The use of sprays or whips applied to

the abdomen and legs of horses have been shown to reduce stable fly bites (Pugh *et al.*, 2014).

Essential oils of camphor, peppermint, chamomile and onion were used to repel stable fly for 6 days (Khater *et al.*, 2009). Zhu *et al.* (2010) found that catnip oil repelled stable flies for 3 hours when the farmer used it as a wax-based formulation. Stable fly population was reduced by leggings, leg bands, and the citronella spray in horse farms.

However, insecticide resistance in stable fly populations has been reported in the field for decades (Marcon *et al.*, 1997; Pitzer *et al.*, 2011; Pitzer *et al.*, 2010; Weinzierl and Jones, 1998). Therefore, biological control methods are more useful for controlling stable flies. Many natural enemies such as beetles and predatory mites feed on the eggs and small larvae of the fly. Over 10 species of pteromalid wasps kill on stable fly pupae by penetrating a pupa with their ovipositor to lay their egg (Moon, 2002).

A parasitic insect, *Spalangia cameroni* Perkins is the most common parasitic insect against stable fly pupae in Kansas, Nebraska and Florida (Meyer and Petersen, 1983; Petersen, 1989) while *Muscidifurax raptorellus* Kogan and Legner is a pupal ectoparasitoid of the stable fly in North American (Floate *et al.*, 2000; Skovgård, 2004). Nevertheless, some parasites (Hymenoptera, *Muscidifurax*, *Pteromalidae*, *Spalangia cameroni* Perkins) have been proved to be biological control agents in fly control programs (Jones and Weinzierl, 1997; Meyer *et al.*, 1990).

Entomopathogens including bacteria, fungi and nematodes are presented as a method to control stable fly populations as non-chemical alternatives under laboratory and field conditions (Leal *et al.*, 2017; López-Sánchez *et al.*, 2012; Moraes *et al.*, 2008; Moraes *et al.*, 2010; Taylor *et al.*, 1998; Watson *et al.*, 1995). For example, the formulation of *Metarhizium anisopliae* (Metchnikoff) Ma134 could control *S. calcitrans* and reduce defensive behavior in cattle (Cruz-Vázquez *et al.*, 2015). Stable fly populations are susceptible to entomopathogenic fungi when applied to cattle under confinement or grazing conditions (Moraes *et al.*, 2008; Moraes *et al.*, 2010; Watson *et al.*, 1995). Especially, entomopathogenic nematodes from 5 species of the genus

Steinernema infected third instar larvae of stable flies at 100 IJs while 8 species of the genus *Heterorhabditis* killed larvae of stable flies at a low level (Taylor *et al.*, 1998). Mahmoud *et al.* (2007) found that *Steinernema feltiae* (Filipjev) infected the second and third instar larvae of stable fly. Leal *et al.* (2017) reported that *H. bacteriophora* Poinar HP88 infected stable fly maggots in all treatments (25, 50, 100, 150, and 200 IJs/larva) in the laboratory experiment.

2. Entomopathogenic Nematode (EPN)

2.1 Classification of EPN

Kingdom: Animalia
Phylum: Nematoda (roundworms)
Class: Chromadorea
Order: Rhabditida
Family: Steinernematidae
Genus: *Steinernema*
Family: Heterorhabditidae
Genus: *Heterorhabditis*

Entomopathogenic nematodes are insect pathogens which have been used as biological control agents of insects. They are non-segmented roundworms in the phylum Nematoda and are also known as eelworms or thread-worms (Shapiro *et al.*, 2017). Most of the 30 families of nematodes are found as parasites and some of them are an association with insects (Gaugler *et al.*, 1997; Nickle, 1972; Poinar, 1990; Poinar and Nelson, 1973). However, only two genera of *Steinernema* and *Heterorhabditis* and their associated symbiotic bacteria (genus *Xenorhabdus* and *Photorhabdus*, respectively) are successfully used as for biological control agents of agricultural insect pests (Blaxter *et al.*, 1998; Boemare *et al.*, 1993; Dorris *et al.*, 2002; Lewis and Clarke, 2012; Poinar, 1990). Nowadays, EPN in the genera *Steinernema* and *Heterorhabditid* are described as over 100 species and some of these EPN are being developed for biological control (Shapiro *et al.*, 2017).

The entomopathogenic nematodes belonging to the family Steinernematidae and Heterorhabditidae are effective biological agents as these EPN can kill a wide range of insect pests. The first EPN, *S. kraussei* Steiner was discovered in 1986 (Poinar, 1990). Later, *S. glaseri* Glaser was the first to be successfully used against the Japanese beetle (*Popillia japonica* Newman) in New Jersey in May 1929. *Heterorhabditis bacteriophora* was the first EPN species in the family Heterorhabditidae in 1976 (Poinar, 1975). However, only the infective juvenile stage or the third juvenile stage juvenile can be tolerated outside their host (Mullens *et al.*, 2006; Poinar, 1990) and moves to find a suitable host (Gaugler *et al.*, 1997; Griffin *et al.*, 2005; Lortkipanidze *et al.*, 2016). They can survive in the natural environment and can use for to the insect control method (Poinar, 1990). When EPNs approach the insect host they release their symbiotic bacteria into the insect's hemocoel, the insects are killed by blood-bacterial infection within 24–48 hours by blood-bacterial infection (Adams and Nguyen, 2002; Gaugler *et al.*, 1997; Li *et al.*, 2017; Poinar, 1990).

2.2 Morphology

The third juvenile stage or infective juvenile (Mullens *et al.*, 2006) or dauer juvenile of EPN is usually sheathed by the cuticle but thicker than the second juvenile stage juvenile with the lateral field shape (Adams and Nguyen, 2002). IJs have 4 cephalic papillae on the head pharynx and intestine overlap and the excretory pore locates posterior to the basal bulb. The tail is short, conoid and taper to a small spike-like tip (Poinar, 1990). Their symbiotic bacteria are located in a specific bacterial vesicle in the family Steinernematidae (*Xenorhabdus* spp.) or in the alimentary tract in the family Heterorhabditidae (*Photorhabdus* spp.). IJs develop into an adult when they enter the insect cavity (Poinar, 1990).

IJs present longitudinal ridges along the entire body length of the cuticle and a tessellated pattern in the most anterior regions (Adams and Nguyen, 2002). The lateral field consists of is two ridges. A distinct dorsal cuticular tooth is present. They also have a hook or spine located on the dorsal. The pharynx and intestine are overlapped and the excretory pore locates posterior to the nerve ring. The IJs of the genus *Heterorhabditis* break off the integument of the insect cuticle with their protruding teeth (Bedding and

Molyneux, 1982). The difference of cuticular ridges in each EPN and the number of ridges can be classified as characteristic of their species (Kozodoi and Spiridonov, 1988).

Adult EPN of the family Steinernematidae is found only in insect cadavers. They have 6 points convex around the lips and non-stylet. There are 4 labial papillae on the lips. The stomata are short and wide. The esophagus is narrowed and swells to apex shape. A corpus is cylindrical and a metacorpus is not differentiated. An isthmus is a short and basal bulb pear shaped with the reduced valve. The excretory pore is usually located at the level of the basal knob. This genus becomes adult females and males in the first generation after infection. In the female, the vulva is located near the middle of body, with or without an epiptygma, with around end tail (Adams and Nguyen, 2002). They are ovoviviparous animal that their offspring consumed nourishment in the mother's body. For Steinernematidae male morphology is very important they have an enlarged head or not with 4 cephalic papillae, blade shaped spicules with arrowhead shaped gubernaculum, no-bursa, with or without mucron and the tail contains 20 genital papillae (Adams and Nguyen, 2002).

Adult EPN of the family Heterorhabditidae is hermaphroditic females with an ovotestis that is normally present only inside the insect host in the first generation and is at least 1 generation. They have an amputated round head and non-stylet. Females of Heterorhabditidae present 6 separate lips (Adams and Nguyen, 2002) that fuse at their base and have a labial papilla in each lip. Moreover, they are also devoid of pro, meso and metarhabdions and the posterior portion of the stoma is overlapped (Poinar, 1990). The pharynx is broad and barrel-shaped with a narrow isthmus. The nerve ring is located at mid-isthmus in the female and near the basal bulb in the male (Poinar, 1990). Hermaphroditic females contain sperm in the proximal portion of the ovotestis and functional vulva. During mating, amphimictic females are functional. Conoid tail and post-anal swelling are present. Male spicules are paired, symmetry, straight or arcuate, with pointed tips and rectal glands present. The male is in the amphimictic stage. Only one testis and the gubernaculum are slender, about half as long as the spicules. The bursa is open, and the peloderan is accompanied by nine pairs of papillae (Adams and Nguyen, 2002; Poinar, 1990).

2.3 Life cycle

The life cycle of the genus *Heterorhabditis* and genus *Steinernama* is mostly similar. The IJ stages of both EPN do not consume food and can survive in the natural habitats where they are outside the insect cadaver in search of a new host (Gaugler *et al.*, 1997; Silva *et al.*, 2021) which depends on biotic and abiotic soil factors consisting of soil texture, temperature and moisture (Lawrence *et al.*, 2006) as well as physical, chemical and biotic conditions (Hoy *et al.*, 2008). They are usually found in soil, wetlands or organic matter all around the world. The IJ stage of the two EPN contains specific symbiotic bacteria in their intestines that infests the insect host. The IJ stage penetrates the insect host via the natural opening such as spiracles, mouth, anus (Griffin *et al.*, 2005) or some *Heterorhabditis* species directly through their insect host by anterior tooth (Bedding and Molyneux, 1982).

A heat and protease-resistant, low-molecular-weight component from the insect hemocoel (Ciche *et al.*, 2006) activate the development of IJ and the release of symbiotic bacteria (Ciche and Ensign, 2003). The number of symbiotic bacteria increases in the insect blood and the insect host dies within 24–48 hours (Griffin *et al.*, 2005). The infected cadaver killed by EPN of the family Heterorhabditidae and the family Steinernematidae turns to red and dark brown, respectively (Kaya and Gaugler, 1993). After invading their host, EPN cooperates with symbiotic bacteria to overcome the host insect is immunity (Dowds and Peters, 2002). EPN grow in the insect body by obtaining food from bacteria, ingested cadaver tissue (Griffin *et al.*, 2005) and the bacteria also release the antibiotic to protect the insect scavenger. (Zhou *et al.*, 2002).

The infecting IJs feed on the insect's tissues, then develop into the fourth juvenile stage and adult, respectively. Adults of the family Heterorhabditidae become hermaphrodite (self-fertile), but the next generation becomes males and females. Adults of the family Steinernematidae become male and female in all generations (Grewal *et al.*, 2005). Therefore, in the family Heterorhabditidae only one EPN can reproduce in the host whereas the family Steinernematidae requires at least 2 EPNs to reproduce. Female EPN hatch in the uterus and grow with the mother's tissue (Johnigk and Ehlers, 1999) and obtain nourishment from the insect host in the juvenile stage consisting of

egg, juvenile 1 (J1), juvenile 2 (J2), juvenile 3 (J3), juvenile 4 (J4) to adult. EPN feed and develop inside the insect cadaver for 2–4 generations depending upon the availability of food (Adams and Nguyen, 2002). After the food is depleted, the pre-infective juvenile stage (J2) containing some symbiotic bacteria in their anterior intestine develop themselves into the IJ stage (J3 with moisture sheathed) (Adams and Nguyen, 2002). When they get limited resources juveniles to become infectives juvenile and emerge from the insect cadaver to find a new host (Dix *et al.*, 1992; Griffin *et al.*, 2005).

Although EPN attack insects and cause insects death, IJs can act as scavengers. For example, *S. kraussei* (Lewis *et al.*, 1996) and *H. megidis* Poinar, Jackson and Klein can reproduce in freeze-killed *Galleria mellonella* L. larvae (Blanco-Pérez *et al.*, 2017). The relationship between EPNs and bacteria is associated with the severity of complex destruction (Bisch *et al.*, 2015). The efficacy of EPN could be enhanced by the evaluation of complex bacterial nematodes that produce the proteins necessary to bypass the host or nematode immune systems and expel the bacterial symbionts with a short delays (Kenney and Eleftherianos, 2016). *Steinernema* species are axenic nematodes that cause host death by bacterial toxin containing high levels of proteases (serine carboxypeptidases, trypsins, eukaryotic aspartyl proteases, zinc carboxypeptidases) and release protease inhibitors into the insect hemocoel (Lu *et al.*, 2017; Peña *et al.*, 2015).

2.4 Behavior

Infective juveniles emerge from an insect cadaver to find the new host and various factors such as specific EPN behavior, soil depth, soil moisture and host habitat, influence IJs demand (Lewis *et al.*, 1996). EPN foraging behavior is classified into three groups as ambusher, cruiser and intermediate nematode (Gaugler *et al.*, 1997). Some *Steinernema* spp. (e.g., *S. carpocapsae* (Weiser), *S. scapterisci* (Nguyen and Smart), *S. siamkayai* (Stock, Somsook and Reid (n. sp.)) belong to the ambusher as they like to wait for the prey to move into their area and then they attack the prey without chemical cues. It has also been noted that these ambusher can stand on their tails in a straight position (nictating behavior) (Campbell and Gaugler, 1993) and they can jump with their

bodies to attach to the insect hosts (jumping behavior) (Campbell and Gaugler, 1993). Cruiser nematodes such as *H. bacteriophora* (Poinar), *S. glaseri* (Glaser), *S. cubanum* (Mracek), *S. longicaudum* (Shen and Wang), *S. oregonenses* (Liu and Berry) move in different environments to find their hosts with long-range chemical cues (carbon dioxide, vibration and other chemical cues) to find the location of the host or they do not nictate but they do not nictate more than two seconds and slowed movement compared to movement on smooth agar for ambusher are an intermediate group such as *S. ceratophorum* (Jian, Reid and Hunt), *S. feltiae* (Filipjev), *S. monticolum* (Stock, Choo and Kaya), *S. riobrave* (Lewis *et al.*, 1992).

2.5 EPN Applications

Normally, EPN sprayed on the soil surface are widely used commercial biological insecticides, especially against root weevils and soil insects (Gaugler *et al.*, 1997). Successful use of EPN to control many insect pests has been reported in commercial applications such as citrus (Diaprepes root weevil), greenhouses (black vine weevil, fungus gnats, thrips, and certain borers), turf (white grubs, billbugs, and mole crickets), and mushrooms (sciarid flies) (Georgis *et al.*, 2006). Campos-Herrera *et al.* (2012) reported that more than 60 species of the genus *Steinernema* and 20 currently species of the genus *Heterorhabditis* have been recognized. Entomopathogenic nematodes are smaller than 1 mm in length and they can be applied with a sprayer (Gaugler *et al.*, 1997). They can attack a wide range important insect pests (Shapiro *et al.*, 2002). Only a few species have been commercially produced in a biological control such as *S. carpocapsae*, *S. feltiae* and *H. bacteriophora* especially *S. carpocapsae* (Grewal *et al.*, 2005; Lacey *et al.*, 2015).

EPNs can infect many soil-borne insects in various stages. The efficacy, survivability and infectivity of EPN depend on the nematode species (Molyneux *et al.*, 1983). A limiting factor for EPN application is temperature, which is normally 15–25°C depending on the species (Shapiro *et al.*, 2017). Larval and pupa stage of insects are often more likely to be infected by EPN than adults (Lu *et al.*, 2017; Ramos-Rodríguez *et al.*, 2006; Williams *et al.*, 2015). Environmental factors such as temperature, humidity, plant type, and soil properties. They can influence the survival and severity

of nematodes (Grewal, 2002). Normally, nematodes can live better in sandy-loam soil than in clay (Kaya, 1990).

In addition, EPNs can be used for horticultural equipment such as handheld sprayers, mist blowers, electrostatic sprayers and irrigation systems (Grewal, 2002). The choice of equipment depends on the cropping system. In general, it is advisable to use large nozzles (holes) and high volumes (up to 400 gallons per acre). The screen filter must be removed and rotary disc from the spray device line to prevent IJs from clogging and must be shaken continuously during use. High pressure (> 300 psi) must be avoided and it must be maintained at a cool temperature. Studies have shown that IJs can interact with many pesticides and herbicides. Fresh manure and fertilizers such as urea have an effect on the survival and performance of EPNs. In recent years, EPN formulas have been developed, especially for soil-based applications such as nematode surfactants and water-soluble polymers (Shapiro *et al.*, 2010).

However, when EPN are applied in the field, abiotic and biotic factors, non-target hosts and potential competitors can affect other nematodes (Kaya, 2002; Shapiro *et al.*, 2017; Ulug *et al.*, 2014) but the environmental risks of using EPN generally have little impact on non-target animals (Akhurst and Smith, 2002). EPN used for bio-control also tend to interact with other organisms, particularly those in the host food web. Within a few days of infection, they release decontamination inhibitor (Scavenger deterrent factor, SDF), a chemical produced by parasitic nematode bacteria that can act as a potential corrosion inhibitor and help prevent host fouling (Gulcu *et al.*, 2012).

In Thailand EPN have been used with *S. carpocapsae* (Weiser) to control many insect pests of longkong tree (*Lansium domesticum* Correa.) including the bark eating caterpillar, *Cossus* sp. and *Microchlora* sp. and 80% mortality of the bark eating caterpillar was observed 24 hours of exposure at a rate of 200 IJs/ml (Somsook *et al.*, 1986). Then, EPN became popular, they were used to control *Ostrinia furnacalis* (Guenée), *Phyllotreta* sp. and also *Microcerotermes* sp. by *S. carpocapsae*, *S. siamkayai*, *H. bacteriophora* and *H. indica* (Maneesakorn *et al.*, 2010; Somsook, 1991; Somsook *et al.*, 1986). In 2003, Sasnarukkit (2003) reported that *S. siamkayai* killed *Plutella xylostella* (L.). Chongchitmate *et al.* (2005) reported that *S. carpocapsae* and *S.*

riobrave killed fourth-instar larvae of *Spodoptera litura* (F.), *Spodoptera exigua* (Hübner), *Helicoverpa armigera* (Hübner) Noosidum *et al.* (2010) found new Thai EPN that killed fourth-instar larvae of *G. mellonella*. Moreover Maketon *et al.* (2010) combined *S. carpocapsae, S. glaseri*, *H. bacteriophora* and some Thai EPN isolates (T1 and T2) with baits to control German cockroach *Blattella germanica* L. and found that *S. carpocapsae* infected with German cockroach more than 86% at the rate of 1×10^6 IJs concentrate. Later, two EPN, *S. carpocapsae* and *Steinernema* sp. isolate K8 (Thai isolate) infected third-instar larva of *S. litura* and *P. xylostella* (Noosidum *et al.*, 2016).

2.6 Culturing and handling

EPN can be produce in culture either in vivo or in vitro (solid and liquid) (Shapiro *et al.*, 2012). In vivo production is a simple method for EPN rearing by using live larvae of *G. mellonella* in the modified White traps (White, 1927). This method is small and suitable for study or laboratory purposes (Shapiro *et al.*, 2002). Normally, in vivo methods are used for maintenance of EPN under laboratory conditions in small scale production and require the rearing of host. In vitro culture is suitable for commercial purposes. In large scale production in vitro is better, EPN are cultured in media without need an insect host to grow but bacterial incorporation must be considered in this method (McMullen and Stock, 2014). IJs can collect with a sponge, polyurethane, water dispersion, vermiculite, gel, alginate and prey and they can keep in 2–5 months depending on the type of EPN and environmental factor. The quality of EPN culture can be determined by detecting nematode toxicity and shelf-life, age and occurrence of non-digestible nematodes (Grewal *et al.*, 2005).

The effectiveness of EPN is usually reduced by to improper handling, transportation and storage (Shapiro *et al.*, 2002). Biotic and abiotic factors can be detrimental during application. Entomopathogenic nematode is used well in sandy soils. Suitable pH ranges from 4–8, but they are very weak at very low temperatures and hot temperatures, UV or even too dry. *Steinernema riobrave* (Cabanillus), *S. glaseri* (Glaser) and *H. indica* (Poinar, Karunakar and David) are resistant strains while *H. megidis* (Poinar, Jackson and Klein) and *H. marelatus* (Liu and Berry) can be adapted to cool temperatures (Grewal *et al.*, 1994). The efficiency of EPN is increased by

matching the target species to the target pests. Proper rates and area must be at least 8 hours after spraying and use in the morning or evening to reduce the ultraviolet radiation. It is important to check whether the EPN is alive or not by using a 20X hand lens or stereo microscopes.

Interestingly, most researchers reported that stable fly females prefer to lay their eggs on animal feces, straw and substrates moistened with urine, bedding substrates and organic matter. However, there is no study on the efficacy of entomopathogenic nematodes against stable flies. Therefore, the application of EPN may be an option to control stable fly larvae on ovulation substrate.

MATERIALS AND METHODS

1. Stable fly

1.1 Collection site

Adults of stable flies (*S. calcitrans*) were collected from a local cattle farm in Suan Kaew Temple Bang Yai District, Nonthaburi Province, Thailand (x: 13.862081, y: 100.442367) (Figure 2A). Suan Kaew Temple is 19.6 km away from Kasetsart University. The study site consisted of mixed herd of cattle and goats.

1.2 Vavoua trap

Vavoua trap is a blue fabric trap that can capture Dipteran with the visual cue of the blue and black colors of polyester (Lendzele *et al.*, 2020). It has been used to assess the population of *Stomoxys* spp. (Gilles *et al.*, 2007; Holloway and Phelps, 1991). Vavoua traps were obtained from Professor Dr. Theeraphap Chareonviriyaphap (the Department of Entomology, Kasetsart University, Thailand). The design of the Vavoua trap was consisted of different pieces of textile cutting with black polyester (76×28 cm) and trapezium shape blue polyester (76×48×27×4 cm). The mosquito net section was designed in the shape of a cone with a bottom diameter of 82 cm and a top diameter of 4 cm, and a height of 76 cm. The mosquito net covered the collection cage has a size of 6.5×8.5×14 cm and the other one covered the cone has a diameter of 15 cm, a top diameter of 2×2 cm and a length of 10 cm the center of the bottom. The cone can be inserted by a circular galvanized wire with a diameter of 80 cm and the whole trap was held by a vertical iron rod of 1.5 m in length. The trap was set at 40 cm above the ground.

1.3 Stable fly collections

Adults stable flies (*S. calcitrans*) were surveyed (Figures 2B–2D) and collected from a local cattle farm between 2 and 5 p.m. using 2 methods, Vavoua traps (Figure 2E) and mouth aspirator (Figure 2F) (Laveissiere and Grebaut, 1990). Stable flies collected from each trap were identified as *S. calcitrans* according to Zump (Zumpt, 1973). Stable flies were transferred to a cage (6.5×8.5×14 cm) covered with a soaked cotton pad containing 10% sucrose syrup and maintained in a plastic container (30×35×20 cm) covered with a moist towel when transferring to the laboratory,

Department of Entomology, Faculty of Agriculture, Kasetsart University (Figures 3A–3B). All insects were maintained under the laboratory conditions at 27±2°C, 50±10% relative humidity (RH) and 12:12 hours L:D photoperiod.

Gravid females fed on animal blood using a modified bovine blood feeder (Figure 3C) (Salem *et al.*, 2012). A plastic glass feeder (16 oz) covered with two layers of pig intestine membrane was attached to the top of the cage and maintained with warm water (37±2°C) through an electronic boiler (YCowboys-TH, OEM, China) and a temperature controller (XK-W2001, SRUIS-33, Guangdong, China). During female feeding, warm water was passed through the glass feeder using a water pump (Sonic®, AP1600, Guangdong, China). Bovine blood was obtained from a fresh market in Nonthaburi Province (x: 13.844757, y: 100.490940). Then engorged gravid females of stable fly were used for the preferred bedding substrates of *S. calcitrans* larvae experiment.

To induce egg laying, adult stable flies were caged and fed under the same conditions as previous described but in the plastic container (12.2×18.2×4.6 cm) containing 200 g of 0–12 hours (Figure 3D). After two days, the plastic container was removed from the cage and placed in the plastic container (15×21×7 cm) for larval residence and closed with a screen lid to prevent other insects. The larvae were reared by cow manure for developing. To perform the larval development, the study was monitored daily until they became to the pupal stage (one day old). The stage of larval development was characterized by posterior spiracles according to Parr (Parr, 1962) (Figures 4A–4F).

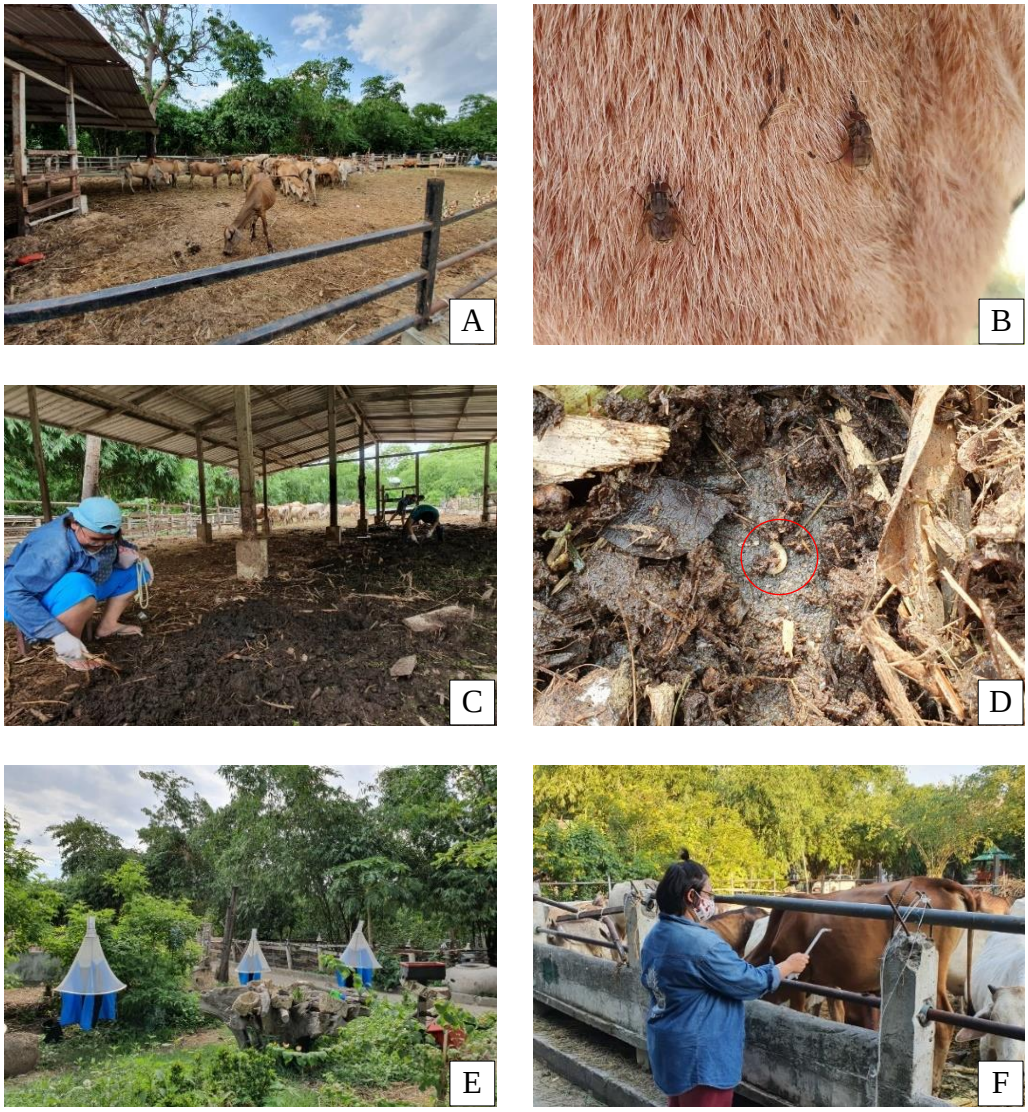

Figure 2 Collection of *Stomoxys calcitrans* (L.), A: a collection site; B: adults of *Stomoxys calcitrans* feeding; C: survey of *Stomoxys calcitrans* larvae in the barn; D: larvae of *Stomoxys calcitrans* on bedding substrate; E: Vavoua traps used to collect adults of *Stomoxys calcitrans*; F: collecting of adults of *Stomoxys calcitrans* by mouth aspirator

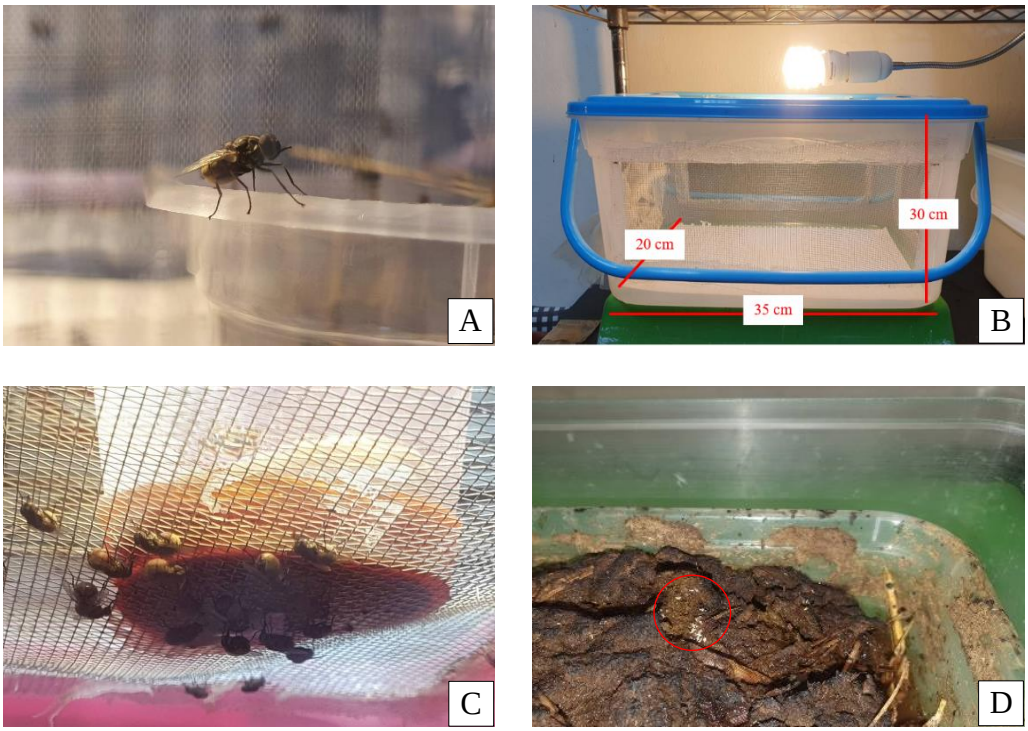

Figure 3 *Stomoxys calcitrans* (L.) rearing technique, A: an adult of *Stomoxys calcitrans* reared in a cage; B: a cage for rearing adults of *Stomoxys calcitrans*; C: adults of *Stomoxys calcitrans* feeding on modified bovine blood; D: a cow dropping tray for laying eggs of *Stomoxys calcitrans*

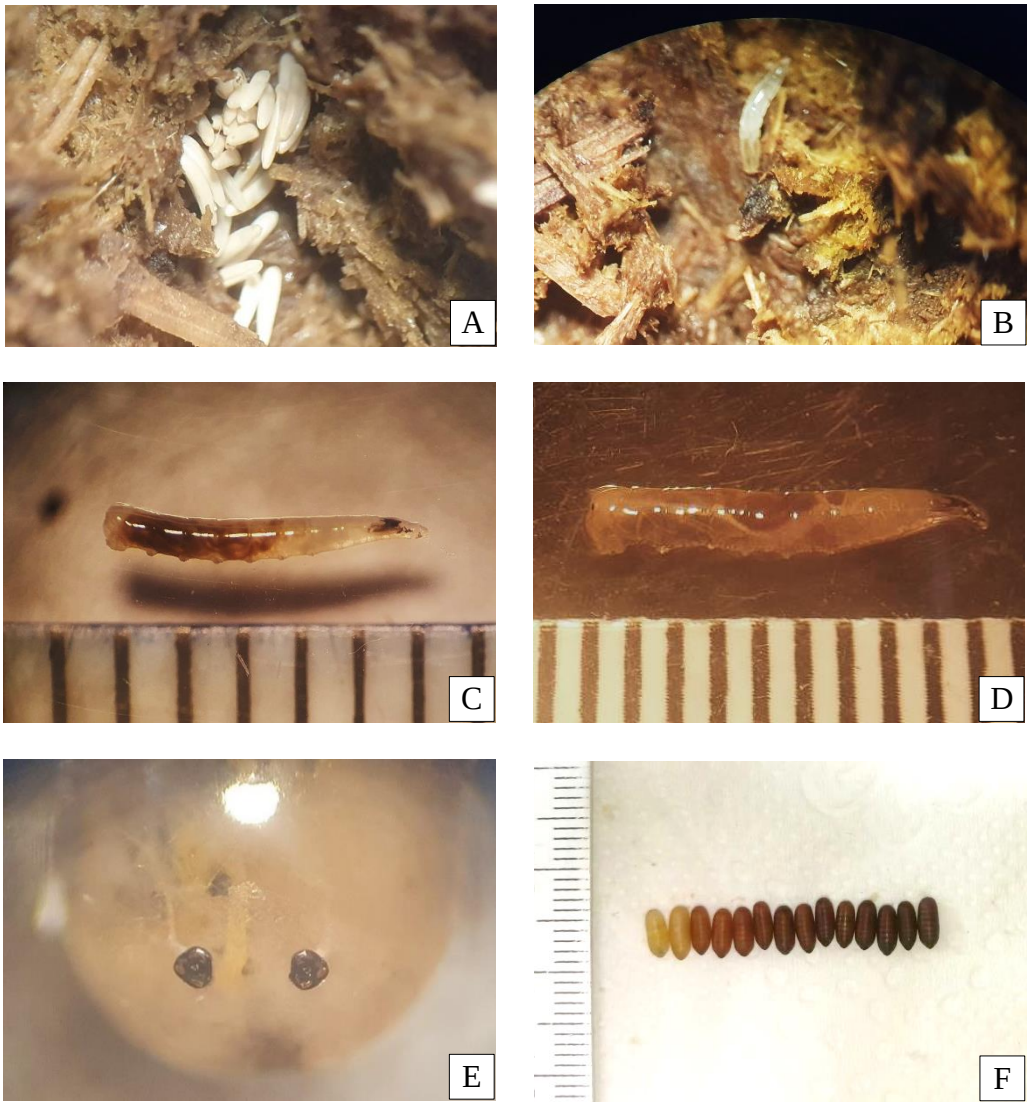

Figure 4 Stages of *Stomoxys calcitrans* (L.), A: eggs of *Stomoxys calcitrans*; B: first instar larva of *Stomoxys calcitrans*; C: second instar larva of *Stomoxys calcitrans*; D: third instar larva of *Stomoxys calcitrans*; E: posterior spiracle of *Stomoxys calcitrans*; F: pupae of *Stomoxys calcitrans*

2. Substrates

Seven substrates related to fly oviposition or living on the cattle were selected to evaluate the preferences for stable fly oviposition and larval development compared to negative control substrate (Figures 5A–5H) (Table 1).

Table 1 Substrate preparations

Substrate (code)	Description
0–12 hours old fresh cow manure (FCM12)	Cow manure was randomly collected after deposition at 0–12 hours.
Seven days old cow manure (CM7)	Fresh cow manure was randomly marked and held in the cattle for seven days before collected.
Seven days old cow bedding (CB7)	Seven days old of bamboo shaving and rice hay combined with urine and manure were randomly collected around the cow cattle.
Manure (MAN)	100% dry natural cow manure (Super Cowboy®) was obtained from gardening store.
Seven days old fermented timothy hay (FT7)	100 g of dry Timothy grass (*Phleum pretense* L.) was incubated with 2,000 ml of distilled water at 250°C for 30 minutes and held at room temperature for seven days (Salem *et al.*, 2012).
Seven days old fermented cow manure (FCM7)	2,000 g of fresh cow dropping with 2,000 ml of distilled water was incubated at 250°C for 30 minutes and held at room temperature for seven days.
Seven days old cow manure and fermented timothy hay (CMFT7)	100 g of dry Timothy grass and 2000 g of fresh cow dropping with 2000 ml of distilled water was incubated at 250°C for 30 minutes and placed at room temperature for seven days.
Water-soaked cotton (WSC)	2 pieces of cotton pad were soaked with 10 ml of tap water as negative control.

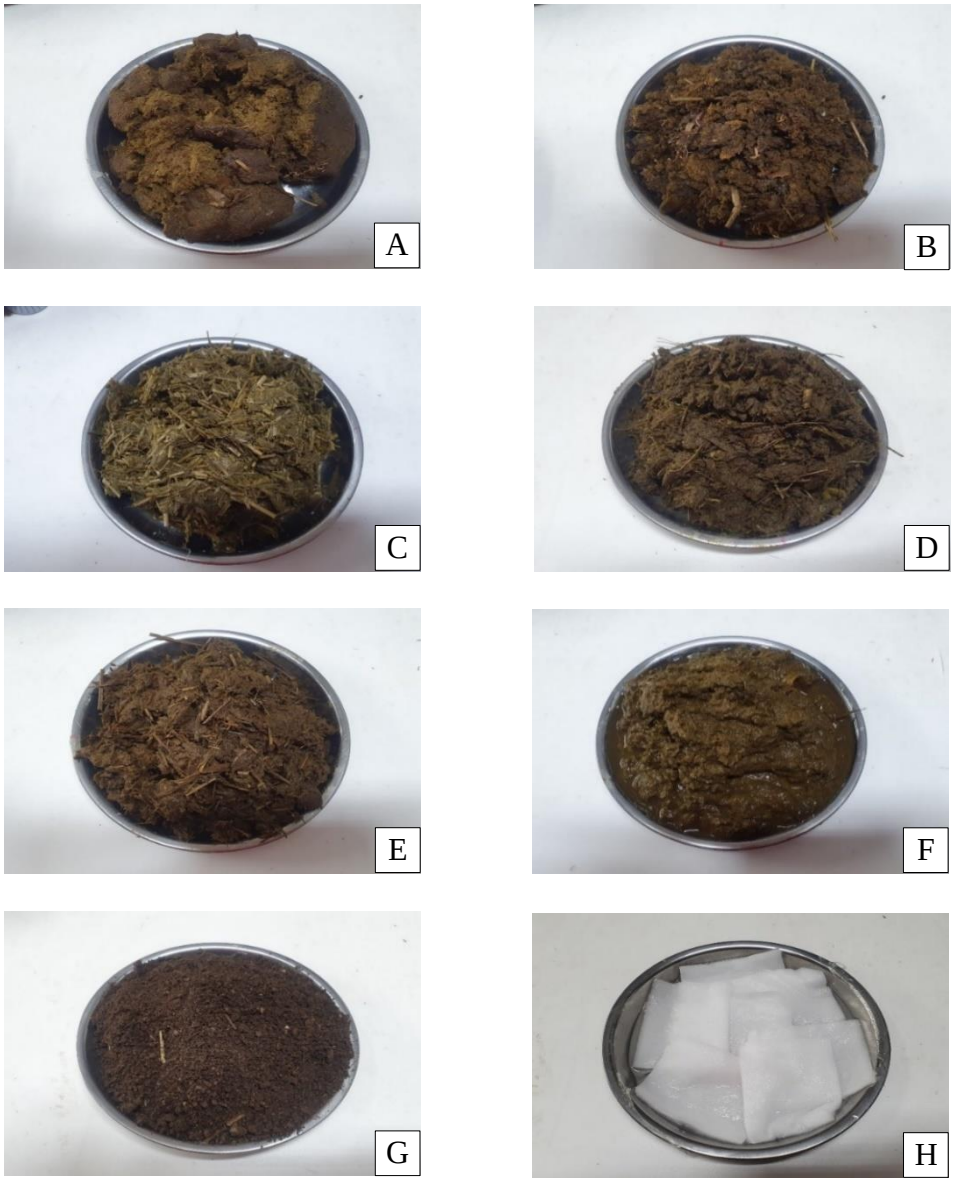

Figure 5 Substrate preparations, A: a plate containing of 0–12 hours old fresh cow manure (FCM12); B: a plate containing seven days old cow manure (CM7); C: a plate containing seven days old fermented timothy hay (FT7); D: a plate containing seven days old cow bedding (CB7); E: a plate containing manure (Man); F: a plate containing seven days old fermented cow manure (FCM7); G: a plate containing seven days old cow manure and fermented timothy hay (CMFT7); H: a plate containing water-soaked cotton (WSC) as negative control

3. Preferred bedding substrates of larvae and adults of *Stomoxys calcitrans* under laboratory conditions

3.1 Preferred bedding substrates of *S. calcitrans* larvae

Fifty ml of 3% boiled liquid agar (Pearl Mermaid Agar Powder®, Patanasin Enterprise Ltd., Thailand) was poured into an aluminum disc (12 cm diameter, 2.5 cm high) and allowed to cool at room temperature (Figures 7A–7B). Then 8 holes with a diameter of 3 cm were punched using a cork. Holes were made close to the edge of disc and equally spaced apart (Figure 7C). Three grams of each substrate was randomly placed in one hole of the agar (Figure 7D). A second instar larva of *S. calcitrans* was carefully placed in the center of the disc. Experiments were designed with completely randomized design (CRD) with 30 replicates and 3 repeats were conducted for each treatment. Bedding substrate that had insect larvae was recorded for 10 minutes after allowed larva at the center of the dish (Figure 7D).

3.2 Preferred bedding substrates of *S. calcitrans* adult

The experimental procedure was modified from the study of (Machtinger *et al.*, 2014). Ten grams of each substrate in 5.5 cm diameter Petri dishes was randomly placed in a cage (17×27×13 cm) covered with 0.28 mm woven aluminum insect screen (Mesh No. 18×16) (Figures 8A–8B). Each Petri dish was approximately 2 cm apart. An engorged gravid female of stable fly was consequently transferred into the cage (Figure 8C). The stable fly was provided with 10% sucrose syrup as an energy source. After three days, the stable fly was taken out by aspirator and number of egg deposition on each substrate was recorded under the microscope (Figure 8D). Experiments were designed using a CRD with and 20 replicates and 2 repeats were conducted for each treatment.

Figure 6 Preferred bedding substrate tests of *Stomoxys calcitrans* larvae under laboratory conditions, A: agar boiler; B: a dish containing cooled agar; C: 8 holes of 3 cm diameter were punch in the agar; D: Releasing a second instar larva in the center of the agar plate

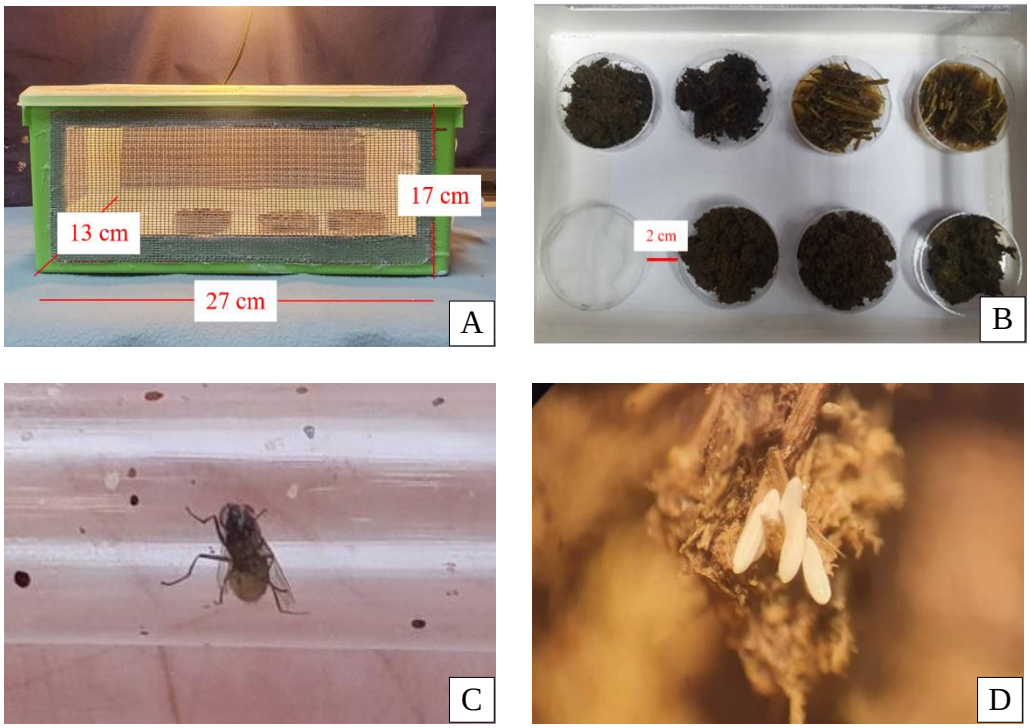

Figure 7 Preferred bedding substrate tests adult of *Stomoxys calcitrans* (L.) adult under laboratory conditions, A and B: Randomly placed substrates in 17×27×13 cm experimental cage; C: gravid female of *Stomoxys calcitrans* for the experiment; D: eggs of *Stomoxys calcitrans* on bedding substrate on day three after treatment

4. Efficacy of EPN against larvae and pupae of *Stomoxys calcitrans* under laboratory conditions

4.1 Greater wax moth rearing for nematode colonization

Greater wax moth, *Galleria mellonella* L. (Lepidoptera: Pyralidae) rearing was modified by Mohamed and Coppel (Mohamed and Coppel, 1983) (Figure 9A). Adults of greater wax moth was placed in a plastic container (15×21×7 cm) and 10% sucrose syrup was provided as a food source on the container lid at 27±2°C, 50±10% relative humidity (RH) and 12:12 hours L:D photoperiod. Then, a 4×21 cm paper was inserted between the lid and the plastic container for egg laying female. A part of the paper containing eggs of wax moth were cut to small and placed onto 200 g of artificial diet located in another plastic container (15×21×7 cm) (Figures 9B–9C). The diet was added every two days for three weeks. Larval development was monitored every two days until they became the last instar larvae (three weeks old). The last instar larvae were used for nematode culture and for rearing the next colony (Figures 9D–9F).

4.2 Nematode culture

Five EPN isolates are native to Thailand; *Steinernema scarabaei* EPNKU60, *Heterorhabditis indica* EPNKU64, *H. indica* EPNKU67, *H. indica* EPNKU82 and *H. bacteriophora* (obtained from the Department of Entomology, Kasetsart University, Thailand) and two commercial EPN isolates; *S. carpocapsae* DOA® and *S. siamkayai* DOA® (obtained from the Department of Agriculture, Ministry of Agriculture and Cooperatives, Thailand) were used in this study. All EPN isolates were maintained on the last instar larvae of greater wax moth (Kaya and Stock, 1997) in the Department of Entomology, Faculty of Agriculture, Kasetsart University, Thailand. EPN suspension (200 IJs/700 µl of H_2O) was dropped onto a 5.5 cm diameter Petri dish lined with two discs of Whatman® No 1 filter paper (Figure 10A). Six last instar larvae of *G. mellonella* were added into the Petri dish and closed the lid. The Petri dish was kept in a dark area at 27±2°C and 50 ± 10% RH.

Larva died, the body color of the insect cadaver was changed to red (Figure 10C) or brown (Figure 10B) (Woodring and Kaya, 1988). The cadavers were soaked

with 0.01% hyamine solution for 1 minute to prevent contamination with other pathogens. The cadavers were transferred to a modified White trap (White, 1927) by placing them in a new 5.5 cm diameter Petri dish lined with a disc of moist Whatman® No 1 filter paper (Figure 10D). The Petri dish was placed in a new 9.0 cm diameter Petri dish containing eight ml of distilled water. After 4–7 days, the IJs emerged from the insect cadaver and migration into the water of a larger Petri dish (Figure 10E). The emerging IJs were cleaned with 0.01% formalin solution. Approximately 200 IJs/700 µl in 60 ml of tap water were collected in a culture flask (225 ml, Corning®) and stored in a refrigerator at 15±3°C for use in all experiments (Figure 10F).

4.3 Filter paper bioassays

The efficacy of six EPN isolates was tested against second instar larvae of *S. calcitrans* using filter paper bioassays. An amount of 400 µL of EPN suspension at the rates of 0, 25, 50, 100, 200 and 400 IJs/cm^2 was applied to a 3.5 cm diameter Petri dish containing two discs of No.1 Whatman® filter paper (Figures 11A–11B). A second instar larva of *S. calcitrans* was placed in a Petri dish (Figure 11C), the lid was closed and wrapped with 3 layers of parafilm (Parafilm M®). The dish was kept in the dark at 27±2°C and 50±10% RH. Insect mortality was recorded every 24 hours until 72 hours. The same protocol was performed for the third instar larvae and pupae of *S. calcitrans* (Figures 11D–11F and Figures 12A–12D). The pupal experiment was specifically observed for 15 days to verify the emergence of the adult stable fly. Experiments were designed using a CRD with 10 replicates and 3 repeats for each treatment.

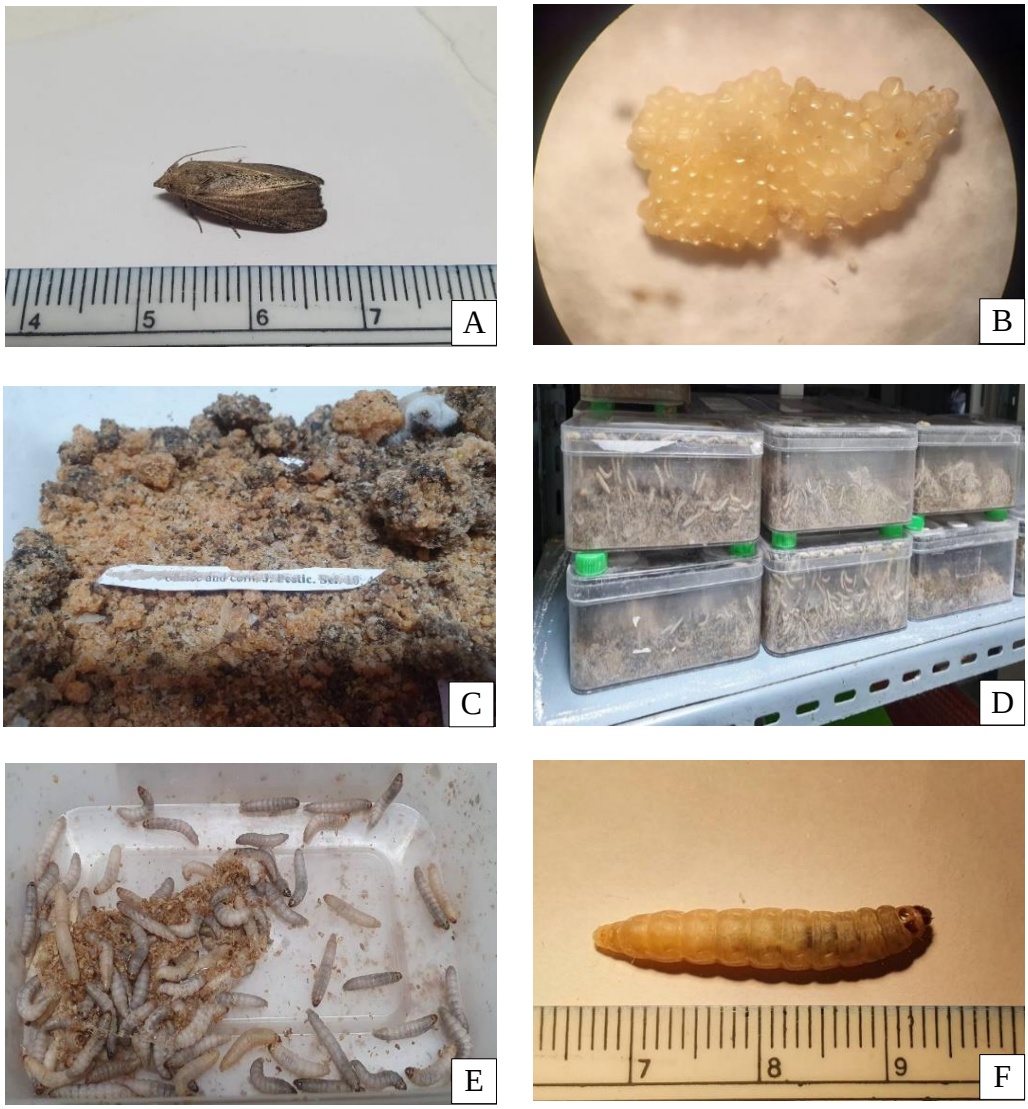

Figure 8 *Galleria mellonella* L. rearing technique, A: an adult of *Galleria mellonella*; B: eggs of *Galleria mellonella* (2X magnification of stereo microscope); C: *Galleria mellonella* egg masses on paper stripes on a diet container; D: *Galleria mellonella* rearing in an insect rearing room; E: Greater wax moth harvesting; F: last instar larva of *Galleria mellonella* for nematode culture

Figure 9 Nematode culture technique, A: nematode infection with 700 µl of EPN suspensions on two discs of Whatman® No 1 filter paper; B: infected larvae by *Steinernema* sp.; C: infected larvae by *Heterorhabditis* sp.; D: insect cadaver in a modified White trap; E: infective juveniles emerge from insect cadavers; F: nematode suspension stored at 14°C in a refrigerator

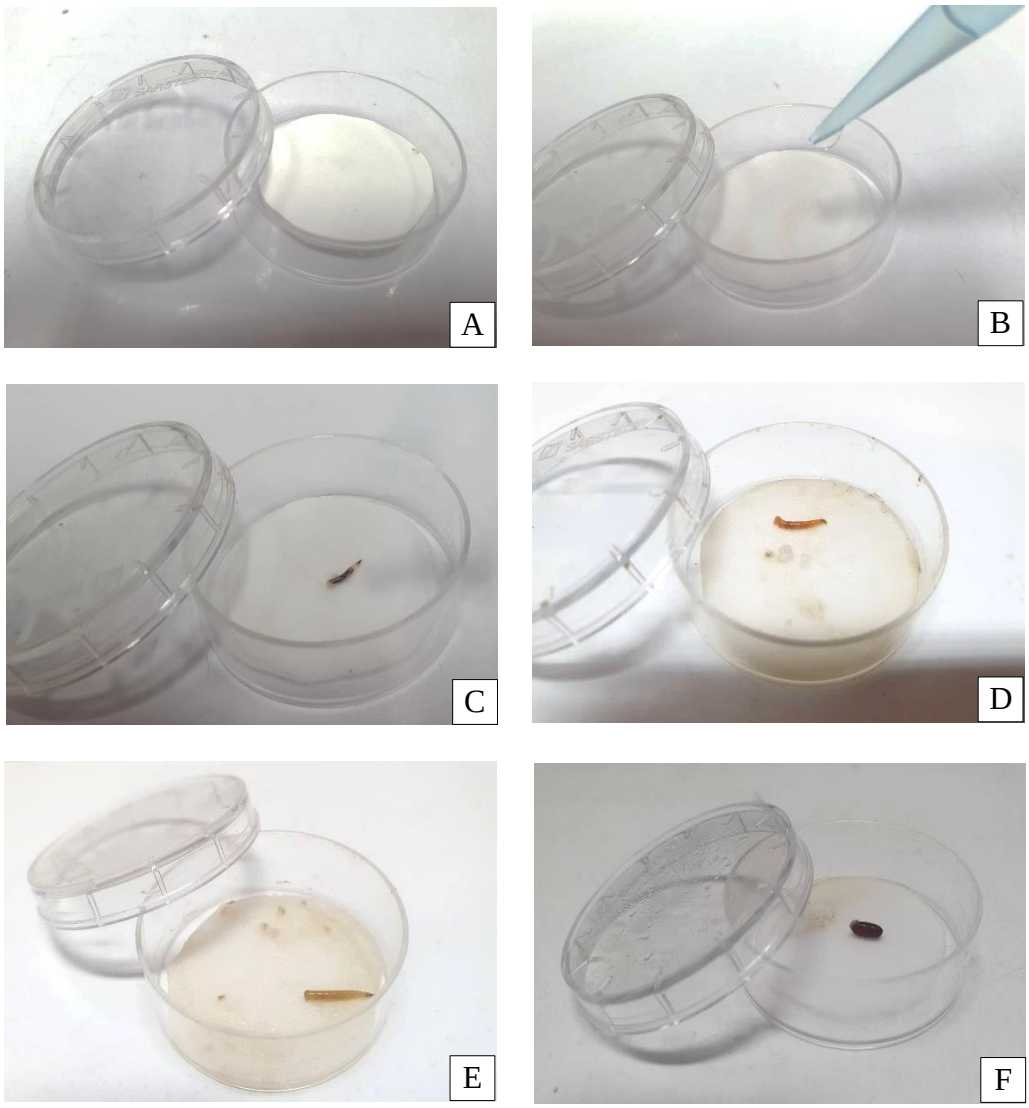

Figure 10 Efficacy of Thai EPNs against larva and pupa of *Stomoxys calcitrans* under laboratory conditions, A, B and C: filter paper bioassays; D: infected second instar larva of *Stomoxys calcitrans*; E: infected third instar larva of *Stomoxys calcitrans*; F: infected pupa of *Stomoxys calcitrans*

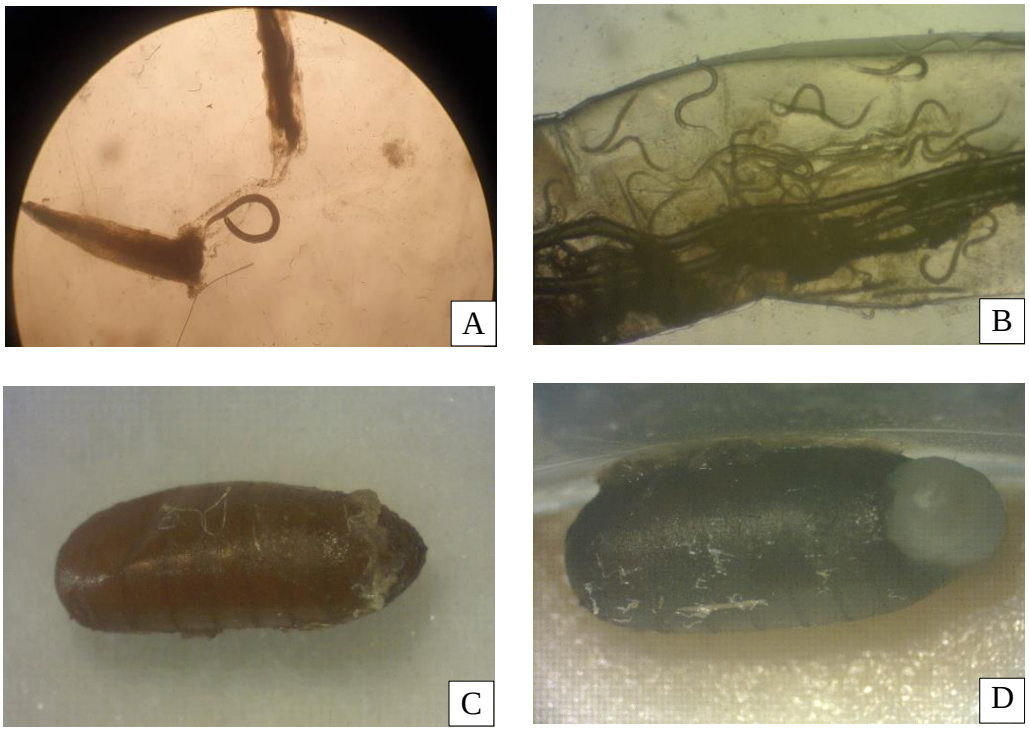

Figure 11 Nematode infection on larva and pupa of *Stomoxys calcitrans*, A: larva of *Stomoxys calcitrans* infected with *Stienernema* sp.; B: larva of *Stomoxys calcitrans* infected by *Heterorhabditis* sp.; C and D: infection of *Stomoxys calcitrans* pupa

5. Persistence of EPN isolates after applied on bedding substrate under laboratory condition

Three EPN isolates; *H. bacteriophora*, *H. indica* EPNKU82 and *S. siamkayai* DOA® which were selected from the previous experiment (experiment 4.3) were used in this experiment. A 5.5 cm diameter Petri dish containing 10 g of the bedding substrates was loaded with 0 and 600 IJs of each EPN in 50 µl of water (25 IJs/cm^2) (Table 1). The water-soaked cotton was replaced with 10% moist sand. Nematode persistence was measured at 1, 3, 5, 7 and 9 days after EPN application using insect baiting technique (Dutky *et al.*, 1964; Kaya and Stock, 1997) (Figure 13A). The last instar larva of greater wax moth was held in a small PVC cage (3×3 cm) (Figure 13B) and placed onto the substrate to bait the alive IJ at different days of exposure. The Petri dishes were transferred to the plastic container (19.2×28×5.6 cm) and kept in the dark at 27±2°C and 50±10% RH. The mortality rate of greater wax moth was recorded every 24 hours until 120 hours after exposure. The cadavers that were infected by *H. bacteriophora* and *H. indica* EPNKU82 presented in red color (Figures 13C–13D) while *S. siamkayai* showed brown color (Figure 13E). The cadaver was dissected (Figure 13F) and the number of adult EPN inside the cadaver was recorded under the microscope. Experiments were designed using 10 replicates and 3 repeats were conducted for each treatment.

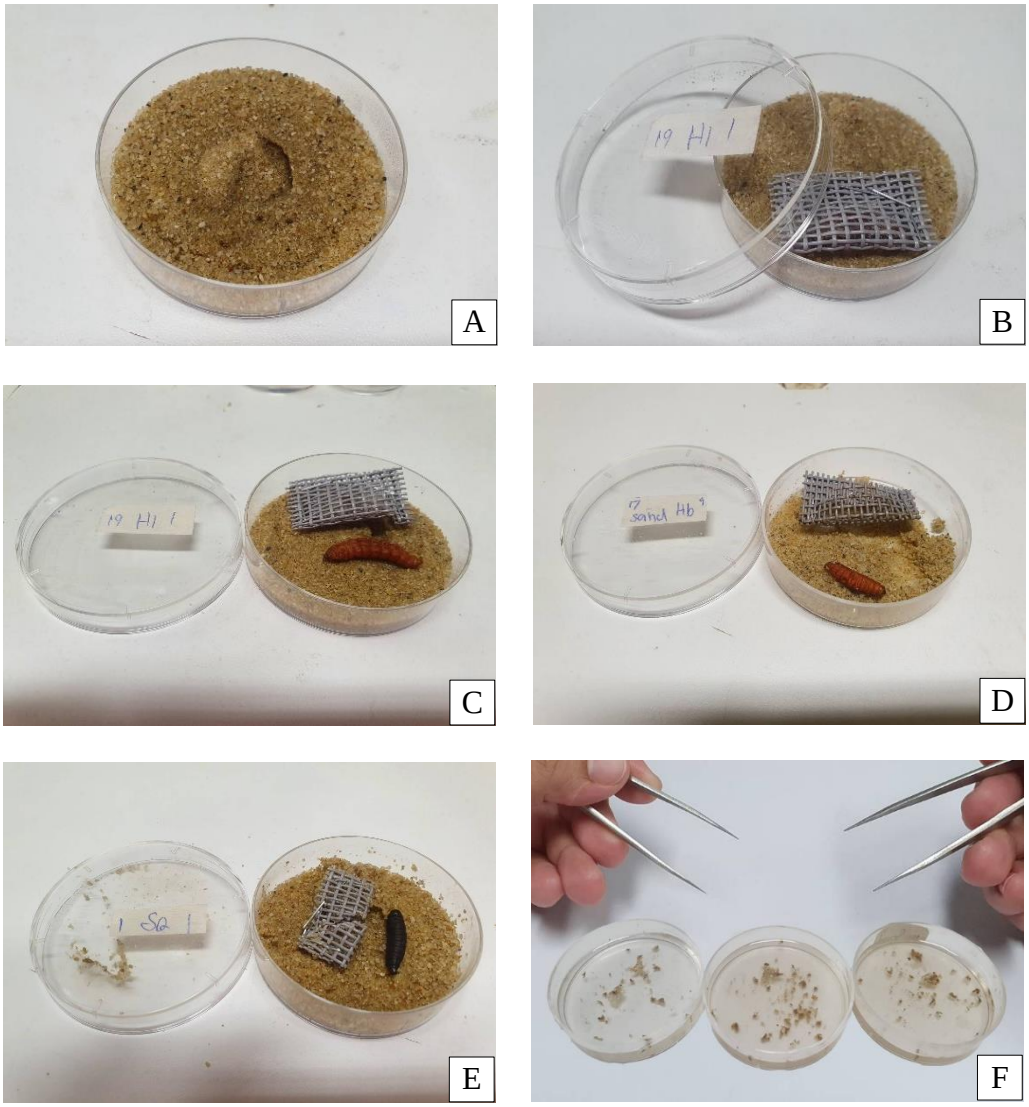

Figure 12 Persistence of Thai EPN applied on bedding substrate under laboratory conditions. A: experimental dish contained 10 g of the bedding substrate; B: the last instar larva of greater wax moth in a small PVC cage was placed onto the bedding substrate; C: a last instar larva of *Galleria mellonella* infected by *Heterorhabditis indica* EPNKU82; D: a last instar larva of *Galleria mellonella* infected with *Heterorhabditis bacteriophora*; E: a last instar larva of *Galleria mellonella* infected with *Steinernema siamkayai*; F: insect cadaver dissection

6. Data analysis

Analyses were performed on the data collected from each set of experiments. The preference of laboratory larvae and oviposition of adult wild-caught of *S. calcitrans* on bedding substrates under laboratory conditions (experiment 3.1 and 3.2) were determined the relationship between the variables by using a two-dimensional crosstabulation and Chi-Square test. Numbers of egg of *S. calcitrans* after released a gravid female for three days was compared by Independent-Samples Kruskal-Wallis Test and asymptotic significances (2-sides tests) were displayed. The efficacy of EPN against larvae and pupae of *S. calcitrans* under laboratory conditions (experiment 4.3) were compared with a one-way analysis of variance (ANOVA) using the Tukey's test. The log transformation of raw data was primality performed to followed normal distribution. Statistical significance was found when a *P*-value less than 0.05. Analysis of LC50 was performed by Probit analysis on mortality data, and obtained together with 95% confidence upper and lower limits. The persistence of EPN isolates inside infected cadavers on bedding substrates (experiment 5) was subjected to one-way analysis of variance (ANOVA) and the Tukey's test (R studio Version 1.2.5001 under the term of version 3).

RESULTS

1. Preferred bedding substrates of larvae and adults of *Stomoxys calcitrans* under laboratory conditions

In the larval experiment after 90 second of exposure, a significant percentage of larval preference was found (χ^2 = 52.165, *df* = 7, 720; *P*<0.001) and the preference ranged from 1.10–26.70%. The CM7 treatment had the highest preference (26.70%) while the preference of the other treatments ranged from 1.10–20.00%, except that FCM12 was absent (Table 2).

For the adult experiments, a significant percentage of preference was also found (χ^2 = 112.000, *df* = 7, 320, *P*<0.001) and the preference ranged from 2.50–55.00%. The FCM12 treatment had the highest preference (55.00%) while the preference of the other treatments ranged from 2.50–35.00%. No preference was observed for FT7, CMFT7, MAN and WSC treatments (Table 3). In addition, eggs of *S. calcitrans* were found in FCM12, CM7, FCM7 and CB7 treatments with the total number of 1,283, 137, 38 and 469, respectively. The average number of eggs was significant difference (χ^2 = 113.817, *df* = 7, 320, *P*<0.001) among the 4 treatments. The highest number of eggs was found on FCM12 (32.07 eggs per Petri dish), followed by CB7 (11.72 eggs per Petri dish) and the other two treatments were less than 3.42 eggs per Petri dish (Table 4).

Table 2 Crosstabulation results of preferred bedding substrates of *Stomoxys calcitrans* larvae under laboratory conditions

Substrates[1/]	FT7	CMFT7	FCM12	CM7	FCM7	MAN	CB7	WSC
Number of Petri dish	6	12	0	24	18	11	18	1
Preference of *S. calcitrans*	6.7%	13.3%	0.0%	26.7%	20.0%	12.2%	20.0%	1.1%
Number of Petri dish	84	78	90	66	72	79	72	89
Disfavor of *S. calcitrans*	13.3%	12.4%	14.3%	10.5%	11.4%	12.5%	11.4%	14.1%

[1/] FT7= Seven days old fermented timothy hay, CMFT7= Seven days old cow manure and fermented timothy hay, FCM12= Less than twelve hours old fresh cow manure, CM7= Seven days old cow manure, FCM7= Seven days old fermented cow manure, MAN= Manure, CB7= Seven days old cow bedding, WSC= Water-soaked cotton

Table 3 Crosstabulation results of preferred bedding substrates of *Stomoxys calcitrans* adults under laboratory conditions

Substrates[1/]	FT7	CMFT7	FCM12	CM7	FCM7	MAN	CB7	WSC
Number of Petri dish	0	0	22	3	1	0	14	0
Preference of *S. calcitrans*	0.0%	0.0%	55.0%	7.5%	2.5%	0.0%	35.0%	0.0%
Number of Petri dish	40	40	18	37	39	40	26	40
Disfavor of *S. calcitrans*	14.3%	14.3%	6.4%	13.2%	13.9%	14.3%	9.3%	14.3%

[1/] FT7= Seven days old fermented timothy hay, CMFT7= Seven days old cow manure and fermented timothy hay, FCM12= 0–12 hours old fresh cow manure, CM7= Seven days old cow manure, FCM7= Seven days old fermented cow manure, MAN= Manure, CB7= Seven days old cow bedding, WSC= Water-soaked cotton

Table 4 Numbers of egg of *Stomoxys calcitrans* after released a gravid female for three days

Bedding substrates[1/]	Number of egg laying (dish)	Egg laying in the substrate (Percentage)	Total	Average±SE
FT7	0	0[2/]	0	0.00±0.00d[3/]
CDFT7	0	0	0	0.00±0.00cd
FCD12	22	55	1283	32.07±5.30a
CD7	3	7.5	137	3.42±1.94c
FCD7	1	2.5	38	0.95±0.95cd
MAN	0	0	0	0.00±0.00cd
CB7	14	35	469	11.72±2.68b
WSC	0	0	0	0.00±0.00cd

[1/] FT7= Seven days old fermented timothy hay, CMFT7= Seven days old cow manure and fermented timothy hay, FCM12= 0–12 hours old fresh cow manure, CM7= Seven days old cow manure, FCM7= Seven days old fermented cow manure, MAN= Manure, CB7= Seven days old cow bedding, WSC= Water-soaked cotton

[2/] Number of egg-laying (dish/40) ×100

[3/] Means followed by different letters in column differs statistically at $P < 0.05$, as determined by Independent-Samples Kruskal-Wallis test.

2. Efficacy of EPNs against larvae and pupae of *Stomoxys calcitrans* under laboratory conditions

Second instar larva test

Susceptibility of second instar larvae of *S. calcitrans* to different EPN isolates increased when increasing of nematode concentration and number of days after exposure. After 3-day exposure, the overall results showed that 50.00% mortality of *S. calcitrans* larvae was found when all EPN isolates reached to 200 IJs/cm^2 (>56.66%). *Heterorhabditis bacteriophora* proved to be the most effective EPN for controlling second instar larvae of *S. calcitrans*. All EPN treatments at rates of 25 and 50 IJs/cm^2 killed less than 80.00% of *S. calcitrans* larvae. Only, *H. indica* EPNKU82 and *S. siamkayai* significantly expressed a high mortality (80.00 and 76.66%, respectively) to *S. calcitrans* larvae at 50 IJs/cm^2 while other EPN treatments killed less than 63% (F = 117.700, df = 7,16; P<0.05). *Heterorhabditis bacteriophora, H. indica* EPNKU82 and *S. siamkayai* (73.33%–86.66%) were significantly effective in killing *S. calcitrans* larvae at the rate of 100 IJs/cm^2 compared to other EPN treatments (F = 117.00, df = 7,16; P<0.05). The high mortality of *S. calcitrans* larvae when applied EPNs at the rate of 200 IJs/cm^2 was significantly observed in *H. bacteriophora, H. indica* EPNKU82 and *S. siamkayai* (73.33–83.33%) (F = 158.80, df = 7,16; P<0.05). All EPN treatments at the rate of 400 IJs/cm^2 killed the second instar larvae of *S. calcitrans* from 70.00% to 100.00% (Table 5).

The lethal concentration that caused 50% insect mortality was calculated by the Probit analysis using data set of the rates of 25, 50, 100, 200 and 400 IJs/cm^2. All seven EPN isolates killed second-instar larvae of *S. calcitrans*. The LC_{50} values, calculated from the bioassay was shown in Table 6. The lowest LC_{50} values was obtained for *S. siamkayai*. *Heterorhabditis indica* EPNKU82, *H. bacteriophora* and *H. indica* EPNKU64 had slightly high values and followed by *S. carpocapsae, H. indica* EPNKU67 and *S. scarabaei* EPNKU60.

Table 5 Mean (±SE) mortality of second instar larvae of *Stomoxys calcitrans* infected with the infective juvenile stage of entomopathogenic nematodes (*Steinernema scarabaei* EPNKU60, *Heterorhabditis indica* EPNKU64, *Heterorhabditis indica* EPNKU67, *Heterorhabditis indica* EPNKU82, *Heterorhabditis bacteriophora*, *Steinernema carpocapsae* and *Steinernema siamkayai*) exposed to different concentrations [Control (0), 25, 50, 100, 200 and 400 IJs/cm^2] after three days of exposure

EPN species	Mortality (%)				
	25 IJ/cm^2	50 IJ/cm^2	100 IJ/cm^2	200 IJ/cm^2	400 IJ/cm^2
Steinernema scarabaei EPNKU60	26.66±3.33Ed[1/]	40.00±0.00Cc	33.33±3.33Dd	56.66±3.33Db	80.00±0.00BCa
Heterorhabditis indica EPNKU64	43.33±3.33CDd	56.66±3.33Bcd	60.00±0.00Cbc	73.33±3.33BCab	86.66±3.33Ba
Heterorhabditis indica EPNKU67	30.00±0.00Ed	40.00±0.00Cd	50.00±0.00Cc	60.00±0.00Db	70.00±0.00Da
Heterorhabditis indica EPNKU82	46.66±3.33BCb	80.00±0.00Aab	83.33±3.33ABab	83.33±3.33Bab	86.66±3.33Ba
Heterorhabditis bacteriophora	56.66±3.33ABc	63.33±3.33Bc	86.66±3.33Ab	100.00±0.00Aa	100.00±0.00Aa
Steinernema carpocapsae	33.33±3.33DEd	43.33±3.33Ccd	53.33±3.33Cbc	63.33±3.33CDab	70.00±0.00Da
Steinernema siamkayai	60.00±0.00Ab	76.66±3.33Aab	73.33±3.33Bab	80.00±0.00Ba	76.66±3.33CDab
Control	00.00±00.00Fa	00.00±00.00Da	00.00±00.00Ea	00.00±00.00Ea	00.00±00.00Ea

[1/] Means followed by the same letters are not significantly different at the 0.05% level as determined by Tukey's test at $P<0.05$. Lowercase letters compare means in the same column and uppercase letters compare means in the same row.

Table 6 Median lethal concentration at 50% (LC_{50}) of seven EPN isolates against second instar larvae of *Stomoxys calcitrans* tested on filter paper bioassays after three days of exposure

EPN species	No	LC_{50} (IJ/cm^2)	95% Confidence Limits for concentration	χ^2	Regression equations
Steinernema scarabaei EPNKU60	180	116 [1/]	32–969	13.267[2/]	y = -2.257+1.093x[3/]
Heterorhabditis indica EPNKU64	180	40	25–54	2.455	y = -1.556+0.973x
Heterorhabditis indica EPNKU67	180	100	72–137	0.008	y = -1.30+0.865x
Heterorhabditis indica EPNKU82	180	15	–	15.351	y = -1.076+0.917x
Heterorhabditis bacteriophora	180	26	3–43	11.576	y = -3.028+2.140x
Steinernema carpocapsae	180	82	56–113	0.130	y = -1.538+0.804x
Steinernema siamkayai	180	2	0–11	5.065	y = -0.108+0.369x

[1/] LC_{50}= lethal concentration that kills 50% of exposed larvae

[2/] Chi square value

[3/] Regression equations= Probit model = Intercept + Bx (Covariates x are transformed using the Log base 10)

Third instar larvae test

Susceptibility of third instar larvae of *S. calcitrans* to different EPN isolates increased when increasing nematode concentration and day after exposure. After three days of exposure, all EPN treatments at a rate of 25 IJs/cm^2 killed less than 40.00% of *S. calcitrans* larvae. The first 50.00% infection to *S. calcitrans* larvae was *H. bacteriophora* at the rate of 50 IJs/cm^2, followed by *H. indica* EPNKU82 at a rate of 100 IJs/cm^2. *Heterorhabditis bacteriophora* tended to be the most effective EPN for controlling third instar larvae of *S. calcitrans* that killing up to 90.00% of larvae at a rate of 200 IJs/cm^2. *H. bacteriophora* and *H. indica* EPNKU82 caused significantly high mortality (100.00 and 80.00%, respectively) to *S. calcitrans* larvae at a rate of 400 IJs/cm^2 while other EPN treatments killed the insect larvae at less than 33.33% (F = 61.01, df = 7,16; P<0.05) (Table 6).

The lethal concentration that caused 50% insect mortality was calculated by the Probit analysis using a data set of the rates of 25, 50, 100, 200 and 400 IJs/cm^2. All seven EPN isolates killed third-instar larvae of *S. calcitrans*. The LC_{50} values, calculated from the bioassay was shown in Table 8. The lowest LC_{50} values was obtained for *H. bacteriophora*. *H. indica* EPNKU82 and *H. indica* EPNKU64 had slightly high values at 26, 15 and 40 IJs/cm^2, respectively.

Table 7 Mean (±SE) mortality of third instar larvae of *Stomoxys calcitrans* infected with the infective juvenile stage of entomopathogenic nematodes (*Steinernema scarabaei* EPNKU60, *Heterorhabditis indica* EPNKU64, *Heterorhabditis indica* EPNKU67, *Heterorhabditis indica* EPNKU82, *Heterorhabditis bacteriophora*, *Steinernema carpocapsae* and *Steinernema siamkayai*) exposed to different concentrations [Control (0), 25, 50, 100, 200 and 400 IJs/cm^2] after three days of exposure

EPN species	Mortality (%)				
	25 IJ/cm^2	50 IJ/cm^2	100 IJ/cm^2	200 IJ/cm^2	400 IJ/cm^2
Steinernema scarabaei EPNKU60	3.33±3.33BCb	6.66±3.33EFab	13.33±3.33Cab	20.00±0.00Cab	26.66±3.33BCa
Heterorhabditis indica EPNKU64	3.33±3.33BCb	20.00±0.00CDab	33.33±3.33Ba	30.00±0.00Ca	33.33±3.33Ba
Heterorhabditis indica EPNKU67	16.66±3.33Ba	16.66±3.33CDEa	26.66±3.33Ba	26.66±3.33BCa	30.00±0.00BCa
Heterorhabditis indica EPNKU82	33.33±3.33Ab	33.33±3.33Bb	50.00±0.00Aab	73.33±3.33Ba	80.00±10.00Aa
Heterorhabditis bacteriophora	40.00±0.00Ac	53.33±3.33Abc	56.66±3.33Abc	90.00±0.00Aab	100.00±0.00Aa
Steinernema carpocapsae	13.33±3.33BCa	26.66±3.33BCa	30.00±0.00Ba	33.33±3.33Ca	33.33±3.33Ba
Steinernema siamkayai	3.33±3.33BCa	10.00±0.00DEFa	13.33±3.33Ca	20.00±0.00Ca	16.66±3.33BCa
Control	0.00±0.00Ca	0.00±0.00Fa	0.00±0.00Da	0.00±0.00Ca	0.00±0.00Ca

[1/] Means followed by the same letters are not significantly different at the 0.05% level as determined by Tukey's test at $P<0.05$. Lowercase letters compare means in the same column and uppercase letters compare means in the same row.

Table 8 Median lethal concentration at 50% (LC_{50}) of seven EPN isolates against third instar larvae of *Stomoxys calcitrans* tested on filter paper bioassays after three days of exposure

EPN species	No	LC_{50} (IJ/cm^2)	95% Confidence Limits for concentration	χ^2	Regression equations
Steinernema scarabaei EPNKU60	180	1,544[1/]	767–6,488	0.424[2/]	y = -3.153+0.989x[3/]
Heterorhabditis indica EPNKU64	180	842	–	15.154	y = -2.372+0.811x
Heterorhabditis indica EPNKU67	180	6,748	1,117–225,140,757	1.321	y = -1.546+0.404x
Heterorhabditis indica EPNKU82	180	80	40–138	5.924	y = -2.275+1.195x
Heterorhabditis bacteriophora	180	45	2–96	21.656	y = -2.938+1.776x
Steinernema carpocapsae	180	1,915	604–126,735	3.933	y = -1.579+0.480x
Steinernema siamkayai	180	7,657	1,657–1,489,517	4.044	y = -2.439+0.628x

[1/] LC_{50}= lethal concentration that kills 50% of exposed larvae

[2/] Chi square value

[3/] Regression equations= Probit model = Intercept + Bx (Covariates x are transformed using the Log base 10)

Pupae test

Low susceptibility of *S. carpocapsae* pupae to all EPN isolates was observed. After seven days of exposure, the results showed that 50.00% of pupae were killed at the rate of 400 IJs/cm^2 (56.66%) for *S. carpocapsae* treatment, while other EPN treatments were killed less than 33.33% of *S. calcitrans* pupae (F = 102.10, df = 7,16; P<0.05) (Table 9).

Table 9 Mean (±SE) mortality of pupa of *Stomoxys calcitrans* infected with the infective juvenile stage of entomopathogenic nematodes (*Steinernema scarabaei* EPNKU60, *Heterorhabditis indica* EPNKU64, *Heterorhabditis indica* EPNKU67, *Heterorhabditis indica* EPNKU82, *Heterorhabditis bacteriophora*, *Steinernema carpocapsae* and *Steinernema siamkayai*) exposed to different concentrations [Control (0), 25, 50, 100, 200 and 400 IJs/cm^2] after seven days of exposure

EPN species	Mortality (%)				
	25 IJ/cm^2	50 IJ/cm^2	100 IJ/cm^2	200 IJ/cm^2	400 IJ/cm^2
Steinernema scarabaei EPNKU60	0.00±0.00Aa[1/]	3.33±3.33Ca	0.00±0.00Ca	0.00±0.00Ca	0.00±0.00Da
Heterorhabditis indica EPNKU64	0.00±0.00Ab	0.00±0.00Cb	0.00±0.00Cb	23.33±3.33Ba	20.00±0.00Ca
Heterorhabditis indica EPNKU67	0.00±0.00Aa	0.00±0.00Ca	6.66±3.33BCa	0.00±0.00Ca	0.00±0.00Da
Heterorhabditis indica EPNKU82	0.00±0.00Aa	0.00±0.00Ca	0.00±0.00Ca	0.00±0.00Ca	6.66±3.33Da
Heterorhabditis bacteriophora	0.00±0.00Ac	0.00±0.00Cc	13.33±3.33Bb	33.33±3.33ABa	33.33±3.33Ba
Steinernema carpocapsae	0.00±0.00Ad	13.33±3.33Bc	0.00±0.00Cd	26.66±3.33Bb	56.66±3.33Aa
Steinernema siamkayai	0.00±0.00Ac	23.33±3.33Ab	30.00±0.00Aab	43.33±6.66Aa	30.00±0.00Bab
Control	0.00±0.00Aa	0.00±0.00Ca	0.00±0.00Ca	0.00±0.00Ca	0.00±0.00Da

[1/] Means followed by the same letters are not significantly different at the 0.05% level as determined by Tukey's test at $P<0.05$. Lowercase letters compare means in the same column and uppercase letters compare means in the same row.

4. Persistence of EPN isolates applied on bedding substrate under laboratory condition

Survival of three selected EPN isolates including 1. *H. indica* EPNKU82, 2. *H. bacteriophora* and 3. *S. siamkayai* in different substrates was evaluated by measuring the infection and penetration of the three EPN isolates to the last instar larvae of *G. mellonella* after three days of exposure under laboratory conditions.

The EPN infection (mortality of *G. mellonella* larvae) was detected in all treatments from 1 day to 9 days of exposure, except all MAN treatments. Significant differences were observed in mortality of *G. mellonella* larvae in different substrates of *H. indica* EPNKU82 (F = 137.20, df = 40,82; P<0.001), *H. bacteriophora* (F = 189.60, df = 40,82; P<0.001) and *S. siamkayai* (F = 144.10, df = 40,82; P<0.001).

Heterorhabditis indica EPNKU82 applied in SAN and FT7 substrates persistently showed 83.00–100.00% mortality of *G. mellonella* larvae after 9 days of exposure. The similar result was found in FCM12, CB7 and CM7 substrates after 7 days of exposure. The CMFT7 substrate showed over 90.00% mortality of *G. mellonella* larvae after 5 days of exposure, while the FCM7 substrate showed 66.66% mortality of *G. mellonella* larvae after 3 days of exposure.

The high mortality of *G. mellonella* larvae (93.33–100.00%) infected with *H. bacteriophora* were found on SAN and CB7 substrates after 9 days of exposure. FCM12 and CM7 substrates were effective to kill the insect larvae after 7 days of exposure whereas FT7, CMFT7 and FCM7 substrates killed the insect larvae at 70.00%, 46.66% and 46.66% respectively, after 1 day of exposure.

Steinernema siamkayai was more effective when applied in FCM7 substrate than others as the mortality of *G. mellonella* larvae reached to 100.00% after 7 days of exposure. CMFT7 had high efficacy followed by CB7, CM7, SAN and FCM12 substrates with ranging from 90.00 to 100.00% mortality of *G. mellonella* larvae at 5, 3, 3, 1 and 1 day of exposure respectively.

The number of EPN found inside *G. mellonella* cadaver of three selected EPN isolates including *H. indica* EPNKU82, *H. bacteriophora* and *S. siamkayai* in

different substrates was evaluated by counting under dissecting microscope. The number of EPN was found in all treatments from 1 day to 9 days of exposure, except in all MAN treatments. Significant differences were observed in the number of EPN in different substrates of *H. indica* EPNKU82 (F = 53.50, df = 34,834; P<0.001), *H. bacteriophora* (F = 66.01, df = 34,683; P<0.001) and *S. siamkayai* (F = 66.01, df = 34,683; P<0.001).

Overall results *H. bacteriophora* showed the most ability to penetrate and had a greater number of IJ inside *G. mellonella* larvae than the other two EPN isolates. *H. indica* EPNKU82, *H. bacteriophora* and *S. siamkayai* applied in SAN expressed high numbers of EPN inside the *G. mellonella* cadaver on the first day of exposure and the number of EPN were 59, 114 and 60 EPNs/insect cadaver, respectively. CB7 and FCM12 treatments showed over 67 and 52 EPNs/insect cadaver inside *G. mellonella* on the first day of exposure and consequently when decreased increasing day of exposure. All other substrates showed numbers of EPNs inside the cadaver between 30–35 EPNs.

Table 10 Mortality rate of wax moth (mean±SE) treated with EPNs on difference substrate three days of exposure under laboratory condition.

Substrates[1/]	Day	Mortality rate (%)		
		H. indica EPNKU82	*H. bacteriophora*	*S. siamkayai*
SAN	1	100.00±0.00Aa[2/]	100.00±0.00Aa	100.00±0.00Aa
	3	100.00±0.00Aa	96.66±3.33Aa	60.00±30.55GHb
	5	100.00±0.00Aa	100.00±0.00Aa	70.00±30.00EFGb
	7	76.66±3.33BCDEa	96.66±3.33Ab	66.66±33.33FGb
	9	83.33±3.33ABCDa	93.33±6.66ABa	50.00±28.86HIb
FCM12	1	100.00±0.00Aa	80.00±11.54BCb	96.66±3.33ABa
	3	90.00±10.00ABb	100.00±0.00Aa	76.66±12.01DEFc
	5	93.33±3.33ABa	80.00±10.00BCab	73.33±6.66EFGb
	7	96.66±3.33Aa	93.33±3.33ABa	66.66±3.33FGb
	9	50.00±5.77GHIab	70.00±10.00CDa	36.66±8.81IJb
CB7	1	100.00±0.00Aa	90.00±5.77ABb	93.33±3.33ABCab
	3	100.00±0.00Aa	93.33±3.33ABab	90.00±5.77ABCDb
	5	100.00±0.00Aa	100.00±0.00Aa	80.00±5.77CDEFb
	7	86.66±8.81ABCab	93.33±6.66ABa	76.66±3.33DEFb
	9	46.66±8.81HIc	100.00±0.00Aa	70.00±5.77EFGb
FT7	1	100.00±0.00Aa	70.00±25.16CDb	73.33±21.85EFGb
	3	90.00±10.00ABa	60.00±30.55DEb	46.66±21.85HIc
	5	96.66±3.33Aa	53.33±29.05EFb	46.66±8.81HIb
	7	100.00±0.00Aa	46.66±26.03EFGb	36.66±17.63IJb
	9	100.00±0.00Aa	33.33±17.63GHIb	36.66±20.27IJb
CMFT7	1	90.00±0.00ABb	46.66±29.05EFGc	100.00±0.00Aa
	3	93.33±3.33ABa	26.66±14.52HIJb	90.00±10.00ABCDa
	5	93.33±6.66ABa	26.66±26.66HIJb	90.00±0.00ABCDa
	7	70.00±5.77CDEFa	20.00±20.00IJc	46.66±8.81HIb
	9	66.66±24.03DEFGa	20.00±20.00IJb	23.33±23.33Jb

Table 10 (Continued)

Substrates[1/]	Day	Mortality rate (%)		
		H. indica EPNKU82	*H. bacteriophora*	*S. siamkayai*
FCM7	1	63.33±20.27EFGHb[2/]	46.66±3.33EFGc	100.00±0.00Aa
	3	66.66±33.33DEFGb	46.66±12.01EFGc	96.66±3.33ABa
	5	46.66±23.33HIb	40.00±17.32FGHb	73.33±8.81EFGa
	7	56.66±28.48FGHIb	36.66±17.63GHc	100.00±0.00Aa
	9	46.66±24.03HIb	16.66±3.33Jc	70.00±15.27EFGa
CM7	1	93.33±3.33ABa	100.00±0.00Aa	100.00±0.00Aa
	3	76.66±3.33BCDEb	86.66±3.33ABab	93.33±3.33ABCa
	5	96.66±3.33Aa	90.00±10.00ABab	83.33±3.33BCDEb
	7	83.33±3.33ABCDab	100.00±0.00Aa	80.00±5.77CDEFb
	9	43.33±13.33Ib	40.00±5.77FGHb	80.00±5.77CDEFa
MAN	1	0.00±0.00Ja	0.00±0.00Ka	0.00±0.00Ka
	3	0.00±0.00Ja	0.00±0.00Ka	0.00±0.00Ka
	5	0.00±0.00Ja	0.00±0.00Ka	0.00±0.00Ka
	7	0.00±0.00Ja	0.00±0.00Ka	0.00±0.00Ka
	9	0.00±0.00Ja	0.00±0.00Ka	0.00±0.00Ka
control		0.00±0.00Ja	0.00±0.00Ka	0.00±0.00Ka

[1/] SAN= 10% humidity sand, FCM12= 0–12 hours old fresh cow manure, CB7= Seven days old cow bedding, FT7= Seven days old fermented timothy hay, CMFT7= Seven days old cow manure and fermented timothy hay, FCM7= Seven days old fermented cow manure, CM7= Seven days old cow manure, MAN= Manure

[2/] Means followed by different uppercase letters in the same column and by different lowercase on the same line differs statistically at $P<0.05$, as determined by Tukey's test

Table 11 Number of adult EPN inside wax moth cadavers (mean±SE) treated with EPNs on difference substrate three days of exposure under laboratory condition

Substrates[1/]	Day	Number of adult EPN inside wax moth cadavers (IJs/larva)		
		H. indica EPNKU82	*H. bacteriophora*	*S. siamkayai*
SAN	1	59.23±2.77Ab[2/]	114.90±5.32Aa	60.63±2.47Ab
	3	22.43±1.46GHIJb	59.34±5.51BCa	26.72±3.88BCDb
	5	13.76±0.86JKLMb	37.23±2.30EFGa	11.47±1.02FGHIb
	7	10.78±0.45KLMb	24.62±1.36GHIa	1.25±0.12Ic
	9	7.84±0.46KLMb	23.21±1.12GHIa	1.13±0.09Ic
FCM12	1	35.96±2.21CDb	52.25±3.77CDa	31.34±1.21BCb
	3	25.25±1.21EFGHb	33.06±2.57EFGa	34.30±1.26Ba
	5	12.03±0.54KLMc	27.79±1.69FGHa	18.36±1.02DEFb
	7	11.93±0.48KLMb	21.28±1.17GHIa	20.05±0.79DEFa
	9	10.60±0.54KLMc	14.04±0.97HIb	18.18±0.92DEFGa
CB7	1	32.53±1.20DEFa	67.51±3.24Ba	33.89±2.00Bb
	3	23.76±1.33FGHIb	44.57±1.75DEa	26.00±1.09BCDc
	5	14.70±1.24IJKLa	26.83±1.17FGHa	20.62±2.14DEFa
	7	17.65±0.78HIJKLa	17.92±0.86HIa	19.78±1.16DEFa
	9	8.28±0.60KLMb	7.43±0.44Ia	14.28±0.94EFGb
FT7	1	31.86±3.20DEFGab	38.61±4.05DEFGa	26.50±1.38BCDb
	3	35.22±2.88CDEa	35.16±2.01EFGa	23.50±1.13CDEb
	5	13.51±1.60JKLMb	23.50±1.54GHIa	15.50±0.62DEFGb
	7	12.00±1.25KLMb	28.42±2.42FGHa	16.45±1.34DEFGb
	9	9.70±1.05KLMb	21.30±2.05GHIa	10.27±1.14FGHIb
CMFT7	1	32.92±5.89DEFab	41.28±4.07DEFa	21.73±3.01DEb
	3	7.67±1.63LMb	39.12±5.42DEFGa	12.51±2.12FGHb
	5	4.50±0.82Mb	26.00±4.50FGHIa	8.70±1.35GHIb
	7	3.14±0.64Mc	21.66±2.92GHIa	11.07±1.86FGHIb
	9	2.45±0.55Mb	16.33±1.02HIa	3.85±1.42GHIb

Table 11 (Continued)

Substrates[1/]	Day	Number of adult EPN inside wax moth cadavers (IJs/larva)		
		H. indica EPNKU82	*H. bacteriophora*	*S. siamkayai*
FCM7	1	47.57±1.98Ba[2/]	19.92±1.35GHIb	24.36±2.64CDb
	3	45.55±2.27BCa	17.78±1.83HIb	14.82±1.78EFGb
	5	20.00±1.53GHIJKa	12.50±0.76HIa	16.40±2.35DEFGa
	7	13.94±1.48IJKLMa	12.36±0.43HIab	8.30±1.51GHIb
	9	11.21±1.44KLMa	10.60±1.07HIa	3.66±1.34HIb
CM7	1	45.39±2.03BCa	50.03±2.12CDa	17.70±1.08DEFGb
	3	24.73±1.43FGHIb	33.92±1.16EFGa	19.78±0.75DEFc
	5	19.10±1.12HIJKa	18.07±1.50HIa	18.44±1.10DEFa
	7	14.92±0.68IJKLa	17.80±0.97HIa	15.37±0.91EFGa
	9	12.84±0.77JKLMb	15.91±1.35HIa	13.00±0.53EFGHb
MAN	1	NA[3/]	NA	NA
	3	NA	NA	NA
	5	NA	NA	NA
	7	NA	NA	NA
	9	NA	NA	NA
control		NA	NA	NA

[1/] SAN= 10% humidity sand, FCM12= 0–12 hours old fresh cow manure, CB7= Seven days old cow bedding, FT7= Seven days old fermented timothy hay, CMFT7= Seven days old cow manure and fermented timothy hay, FCM7= Seven days old fermented cow manure, CM7= Seven days old cow manure, MAN= Manure

[2/] Means followed by different uppercase letters in the same column and by different lowercase on the same line differs statistically at $P<0.05$, as determined by Tukey's test

[3/] NA= Not Available data

DISCUSSION

The top three most preference substrates of stable fly larvae were shown on seven days old cow dropping (CM7), seven days old cow bedding (CB7) and seven days old fermented cow dropping (FCM7). This result could explained that these second instar larvae (5 days old) were reared in FCM12 prior to testing, which was consistent with previous work by Baleba *et al.* (2019), suggesting that third instar larvae of *S. calcitrans* rather preferred the substrate in which they had developed than another substrates. In this study, 10 minutes was set to allow the stable fly larvae to select the bedding substrate that may not be sufficient for the larvae. Likewise, other study showed that the preference was interrupted after 5 hours (Baleba *et al.*, 2019). Larvae selected substrates for pupation and discriminated soil organic matter concentrations related to the amount of air available for insect respiration, as well as soil texture, structure, moisture and porosity (Edwards *et al.*, 1973; McColloch and Hayes, 1922; Pietrantuono *et al.*, 2015).

The results of this study showed that gravid females of *S. calcitrans* preferred to lay their eggs on less than twelve hours old fresh cow manure (FCM12), 7 days old cow bedding (CB7), 7 days old cow manure (CM7) and 7 days old fermented cow manure (FCM7). This result exhibited their behavior by olfactory cues from odors of the substrates. This agreed with Birkett *et al.* (2004) who reported that the volatile odors associated with rumen compounds and cattle urine were attractive to gravid females of stable fly. Large numbers of stable fly larvae were also observed in the cow bedding and aged cow manure. These results were similar to Baleba *et al.* (2019) who reported that gravid females of *S. calcitrans* used vertebrate herbivore dung such as cattle, camels and horses for oviposition, as well as the material used on in the farm consists of decaying plant material, such as silage, hay, grass clippings, and garden compost (Cook *et al.*, 2017).

The observation of preference of gravid females stable fly on carboxylic acids suggested aged cow manure produces twice the concentration of volatiles than fresh manure (Broce and Haas, 1999; Miller and Varel, 2001). Other compounds were consisted of short-chain aliphatic alcohols, phenols, indoles, sulfides, terpenes and CO_2 (Jeanbourquin and Guerin, 2007). Moreover, Baleba *et al.* (2019) reported that stable fly used a single semiochemical to select an oviposition site. Because, the gravid female stable fly examined the quality of the substrate for their fitness progeny development (Baleba *et al.*, 2020).

In this study, most of the substrates which founding eggs was consistent with the chemical legacy hypothesis. The relationship between larval chemosensory environment and adult chemosensory responsiveness were observed in relation to life stages of stable fly, accordingly found on the substrates that they lived during larvae development (Corbet, 1985). Moreover, the stable fly uses visual, olfactory, gustatory, and physical stimuli for host search (Zhu *et al.*, 2008). Gravid females of the stable fly searched and preferentially selected an oviposition site based on microbial stimuli suitable for larval development (Romero *et al.*, 2006), since gravid females preferred cow dropping compound. Moreover, several cow urine, manure and rumen-associated odorants attractive to stable flies were investigated (Jeanbourquin and Guerin, 2007; Logan and Birkett, 2007).

The increasing of mortality rate of second instar larvae were observed when increased with increasing in the nematode concentration and exposure time in all species tested. The previous research by Pierce (2007) found that *S. feltiae, Steinernema* spp. and *Heterohabditis* spp. caused stable fly larvae death in hay substrate at the rate of 7500 IJs/l were 55.59%, 38.25% and 20.35% mortality, mortality rate of respectively. In this study, *Heterorhabditis* nematode showed greater efficacy in killing second and third instar larvae of *S. calcitrans* than other strains due to its ability to use its dorsal tooth for cuticle penetration (Griffin *et al.*, 2005). *Steinernema scarabaei* EPNKU60 was a new Thai EPN isolate that had never been used to control stable fly larvae before, resulting in *S. scarabaei* EPNKU60 exposed low efficacy in against to *S.*

calcitrans larvae at low concentrations. Other study indicated that *S. scarabaei* EPNKU60 was suitable for the control of Japanese beetle, (*Popillia japonica* Newman) as showed 80.00–100.00% mortality (Stock and Koppenhöfer, 2003). However, *S. scarabaei* EPNKU60 was be possible to control other insect pests in Thailand.

In this experiment, lower susceptibility of third instar larvae to entomopathogenic nematodes was observed than second instar larvae. The lowest rate of EPN showed only 6.66% and the highest mortality at 100.00%, which caused by *H. bacteriophora*. However, the efficacy of EPN in this study was low compared to other studies. For example, Leal *et al.* (2017) showed that *H. bacteriophora*, isolate HP88 caused 90.00% mortality of third instar larvae of stable fly (eight days old) at the rate of 25 IJs/larva (2 IJs/cm^2) after two days of exposure. In addition,(Mahmoud *et al.*, 2007) reported that *S. feltiae* caused mortality of third instar larvae of stable fly ranging from 33.33% at the rate of 50 IJs/ml (1.31 IJs/cm^2) to 100.00% at the highest rate (500 IJs/ml, 13 IJs/cm^2) after 72 hours of exposure.

In another pest, the virulence of *H. bacteriophora*, *H. indica, S. carpocapsae* in third instar maggots of *Bactrocera dorsalis* at 100 IJs/ml (1.57 IJs/cm^2) was 69.42%, 37.15%, and 71.12%, respectively (Aatif *et al.*, 2019). The LC_{50} of this experiment showed different mortality rate that could be characteristic of nematode foraging strategy. *Heterorhabditis indica* and *H. bacteriophora* showed low LC_{50} as they are cruiser foraging strategy (Lortkipanidze *et al.*, 2016) whereas *S. siamkayai* belongs to ambush predators foraging strategy (Campbell and Kaya, 2002).

These experiments showed that EPNs were less effective in controlling *S. calcitrans* pupae than second and third instar larvae. The mortality rate of *S. calcitrans* pupae showed low efficacy of EPN after seven days of exposure. The low mortality rate of pupae could be due to the hardness of the puparium which made it difficult for the nematode to penetrate through the pupal spiracles (Beavers and Calkins, 1984; Toledo *et al.*, 2005). Compared to other recent studies, low mortality ranging from 3.33%–56.66% was observed in this experiment when pupae were exported for seven days. Batalla-Carrera *et al.* (2010) reported that *S. feltiae, S. carpocapsae* and *H.*

bacteriophora showed low efficacy (<10% mortality) to tomato leaf miner pupae in Petri dishes with filter paper bioassay at two rates (25 and 50 IJs/cm^2) after 72 h of nematode application. Mahmoud *et al.* (2007) tested the efficacy of *S. feltiae* to pupae of four fly species and found that 5.00%–52.50% mortality was caused in soil bioassays at the rates of 500–3000 IJs/ml (7.87–47.20 IJs/cm^2) and 22.50% mortality was caused in manure bioassays at the rate of 5000 IJs/ml (78.70 IJs/cm^2). Petri dishes with filter paper bioassay at two rates (25 and 50 IJs/cm^2) after 72 h of nematode application. Mahmoud *et al.* (2007) tested the efficacy of *S. feltiae* to pupae of four fly species and found that 5.00%–52.50% mortality was caused in soil bioassays at the rates of 500–3000 IJs/ml (7.87–47.20 IJs/cm^2) and 22.50% mortality was caused in manure bioassays at the rate of 5000 IJs/ml (78.70 IJs/cm^2).

As the size of insects, larval and pupal stage was probably not large enough to be parasitized that produced the low efficacy of EPNs. This agrees with previous findings by Jess and Bingham (2004); (Jess *et al.*, 2005) who reported that a longer period between nematode (*S. feltiae*) application would help the nematode to find larger larvae (third and fourth stage of sciarid flies). *Steinernema carpocapsae* showed lower susceptibility to control first instars compared to second and third instars of western corn rootworm (*Diabrotica virgifera virgifera* LeConte) (Jackson and Brooks, 1995; Journey and Ostlie, 2000).

Entomopathogenic nematode were tested to investigate nematode persistence in different substrates using the EPNs recommended rate (25 IJs/cm^2) in the field (Duncan *et al.*, 2003). Persistence of EPNs was exhibited by the mortality of greater wax moth, the color of an insect cadaver, and cadaver dissection. Three days after exposure of all EPN isolates and all the day of exposure showed a significant decreasing with the day after treatment. The difference in nematode persistence was depended on many abiotic environmental factors, including soil moisture, light (Fujiie and Yokoyama, 1998) temperature (Grewal *et al.*, 1994; Kung *et al.*, 1991) soil texture and bulk density (Barbercheck and Kaya, 1991) and relative humidity (Smits, 1996).

Moreover, the nematode requires high relative humidity and a film of free water for its movement to survive (Grant and Villani, 2003; Wallace, 1958).

Thai EPN has high persistence in sand (SAN) substrate at 1 day of exposure. This result was similar to Barbercheck and Kaya (1991) who reported that *H. bacteriophora* was more mobile than *S. carpocapsae* in organic and fine sandy loam soils. In addition, some EPN species are not effective in soil texture and the pathogenicity of nematodes increased with soil sand content (Choo and Kaya, 1991; Koppenhöfer and Fuzy, 2006; Kung *et al.*, 1990; Molyneux and Bedding, 1984) These results correspond to previous works which reported that nematodes applied on sandy soil showed high nematode mobility and oxygen level (Kaya, 1990; Kung *et al.*, 1990; Molyneux and Bedding, 1984).

The application of nematodes on substrates in cattle farm in this study showed the difference in survival of nematodes. Belton *et al.* (1987) found that *H. heliothidis* Poinar and *Steinernema* spp. survived only a few days in moist manure, giving these nematodes had low potential for controlling *M. domestica* and *H. heliothidis* that found in chicken manure than *Steinernema* spp. In addition, Shapiro *et al.* (1996) reported that the addition of manure to soil as fertilizer reduced the pathogenicity of nematodes. However, (Bednarek and Gaugler, 1997) reported that after the addition of manure to soil, the population of *S. feltiae* increased and reproduced successfully within all the fly species. In the same way as Taylor *et al.* (1998) suggesting that the mortality of *M. domestica* caused by *S. feltiae* was as high as in a filter paper bioassay.

This study showed the low number of EPN inside the insect cadaver (10.00–19.00%). However, low number of nematodes can lead to mortality. Similar to Ehlers *et al.* (1997) who reported that 1 monoxenic IJ/insect resulted in 80.00% mortality after two days injection.

In addition, Archana *et al.* (2017) reported that mortality of *M. domestica* in the second instar larvae by *H. indica* was 60.00% after 3 days treatment at the rate of 50 IJs/maggot (2.5 IJs/cm^2). Converse and Miller (1999); (Grewal *et al.*, 2002)

reported that one or few IJs of *S. carpocapsae, S. feltiae* and *S. riobrave* killed 50.00% of greater wax moth larvae. The previous finding by Raja *et al.* (2011) showed that the number of penetrated EPN on sand was 22.6±1.7 IJs per larva when 50 IJs/60 µl were delivered and caused 100.00% mortality. *H. bacteriophora* established 23.00% of infective juveniles (100 IJs per insect) while *S. carpocapsae* established less than 5.00% of infective juveniles in cadaver on the bottom of the sand column bioassays (Lortkipanidze *et al.*, 2016). *Heterorhabditis indica* and *H. bacteriophora* showed high persistence rate (>80.00%) which supported previous experiment by *H. bacteriophora* nematodes. This nematode species survived almost four months of exposure in sandy loam soil and almost five months of exposure in silty loam soil (Babendreier *et al.*, 2015; Kurtz *et al.*, 2007; Toepfer *et al.*, 2010).

CONCLUSION AND RECOMMENDATION

The substrates most preferred by the larvae of *S. calcitrans* was CM7, followed by CB7 and FCM7 while gravid females of *S. calcitrans* preferred oviposition on FCM12. The number of eggs was found on 4 substrates consisting of FCM12, CM7, FCM7 and CB7. The highest number of eggs was found on FCM12.

The susceptibility of second instar larvae and third instar larvae of *S. calcitrans* to the different EPN isolates increased with the increase in nematode concentration and day exposure. The second instar larvae of *S. calcitrans* were highly susceptible to *S. siamkayai* followed by *H. indica* EPNKU82, *H. bacteriophora*, *H. indica* EPNKU64, *S. carpocapsae*, *H. indica* EPNKU67 and *S. scarabaei* EPNKU60. Third instar larvae of *S. calcitrans* were highly susceptible to *H. bacteriophora* and *H. indica* EPNKU82. All EPN isolates caused very low efficacy to pupae of *S. calcitrans*. These studies confirmed that EPN are natural enemies of stable fly larvae.

Persistence of three selected EPN isolates showed that *H. indica* EPNKU82 expressed high persistence on SAN and FT7 up to nine days after EPN application. Similarly, *H. bacteriophora* showed high survival of EPN on SAN and CB7 up to nine days after EPN application. In contrast, *S. siankayai* can persist only on one day of exposure.

In conclusion, this study demonstrated the susceptibility of second and third instar larvae of *S. calcitrans* to the commercial and indigenous EPN isolates (*H. bacteriophora* and *H. indica* EPNKU82). Also, the two EPN isolates were able to persist well on the substrates associated with *S. calcitrans* larvae up to nine days after EPN application.

Therefore, control of *S. calcitrans* larvae would be possible with EPN application in the field. However, EPN application in the field must consider the stage of the stable fly, substrate, EPN species and environmental factors. Field application should survey the information before application

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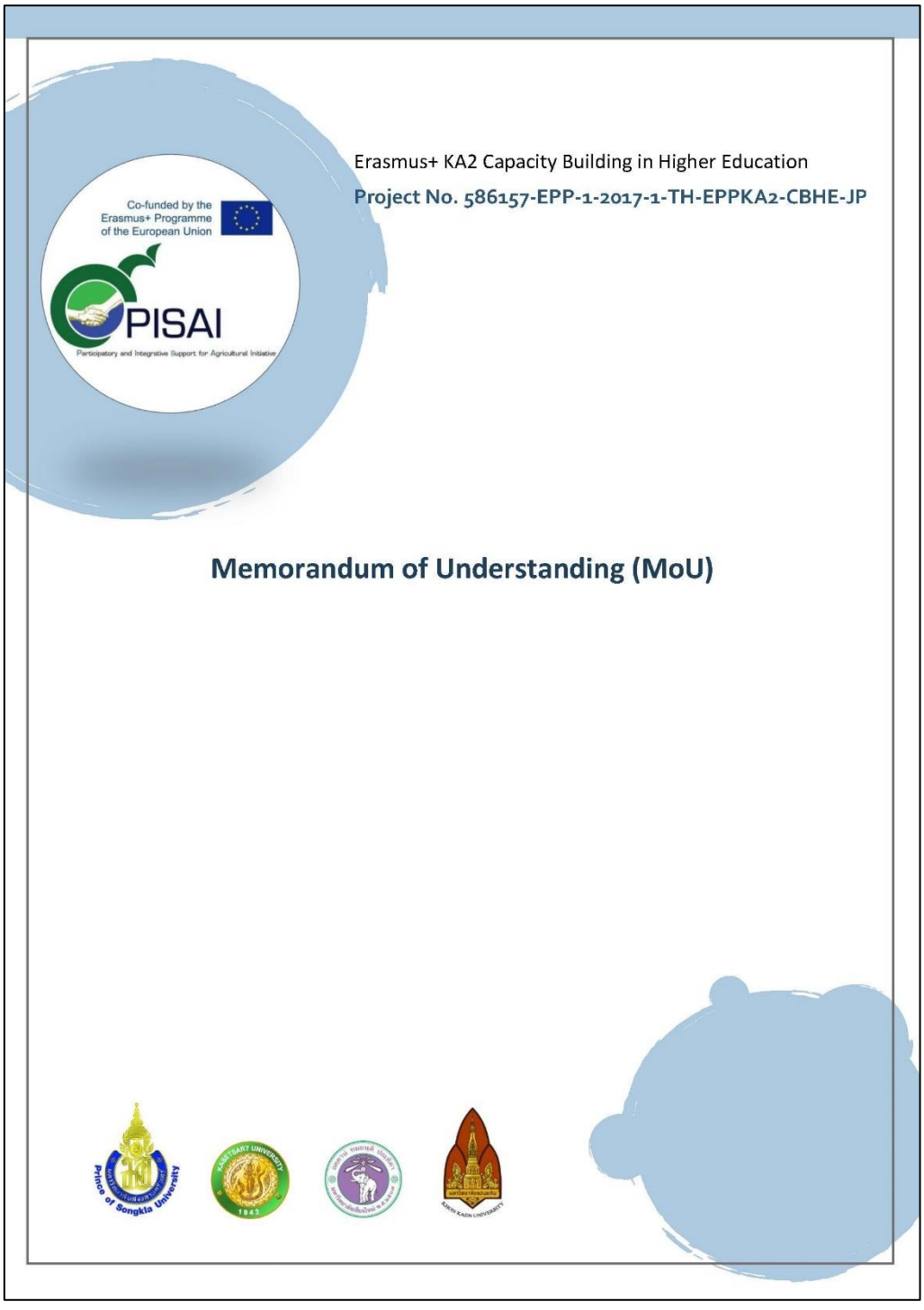
APPENDICES

APPENDIX A

Memorandum Of Understanding (MOU)

between Thai partners for Double Degree Master's program


Co-funded by the
Erasmus+ Programme
of the European Union
PISAI
Participatory and Integrative Support for Agricultural Initiative
Erasmus+ KA2 Capacity Building in Higher Education
Project No. 586157-EPP-1-2017-1-TH-EPPKA2-CBHE-JP
Memorandum of Understanding (MoU)

MEMORANDUM OF UNDERSTANDING
FOR INSTITUTIONAL COOPERATION
AMONG

MEMBERS OF THE THAI UNIVERSITIES FOR GRADUATE EDUCATION UNDER PISAI PROJECT (ERASMUS PLUS CAPACITY BUILDING PROJECT)

This Memorandum of Understanding is entered into this 20th day of July 2018, by and between the following Thai University Project Partners for Graduate Education in Agriculture and Natural Resources under PISAI - Erasmus Plus Capacity Building Project:

PRINCE OF SONGKLA UNIVERSITY, with official address at 15 Karnjanawanich Road, Hat Yai, Songkhla, Thailand and represented herein by its President, Asst. Prof. Dr. Niwat Keawpradub,

KASETSART UNIVERSITY, with official address at 50 Ngam Wong Wan Rd, Lat Yao Chatuchak Bangkok, Thailand and represented herein by its President,

CHIANG MAI UNIVERSITY, with official address at 239 Huay Kaew Road, Muang District, Chiang Mai, Thailand and represented herein by its President,

KHON KAEN UNIVERSITY, with official address at 123 Mittapap Road, Moo 16, Nai-Muang, Muang District, Khon Kaen, Thailand and represented herein by its President, Dr. Kittichai Triratanasirichai,

WITNESS:

WHEREAS, the partner university members have complementary objective and a mutual desire to collaborate in a number of areas to pursue common education, training, and research objective, and

WHEREAS, the partner university members recognize the comparative advantage of joining forces to improve the quality and relevance of graduate education in sufficiency and sustainable agriculture and related fields to make these programs globally competitive and easily accessible to the clients in the region.

Now, THEREFORE, and in consideration of the foregoing premises, the parties hereto have agreed and do hereby agree as follows:

1. To collaborate in undertaking joint activities such as joint graduate modules/ courses and dual/double degree programs in agriculture, natural resources and other fields relevant to the PISAI project,

2. To execute separate agreements in writing for any particular undertaking jointly implemented, wherein a sharing of responsibilities shall be specified, such case as exemption of tuition fee for the students under the PISAI Project.

This Memorandum shall remain in force for a period of 5 years from the date of signing by the authorized representatives of the Parties hereto and may not be modified except upon mutual written agreement of the parties herein, It shall be extended for another 5 years, unless either party gives written notice to terminate the agreement at least six (6) months prior to the expiration date.

IN WITNESS WHEREOF, the parties hereinto have affixed their signatures this 20th day of July 2018 at Office of the Higher Education Commission, Bangkok, Thailand.

PRINCE OF SONGKLA UNIVERSITY BY:

N. Keawpradub

Asst. Prof. Dr. Niwat Keawpradub
President

KASETSART UNIVERSITY BY:

Chongrak

Dr. Chongrak Wachrinrat
Acting President

CHIANG MAI UNIVERSITY BY:

N. Nantachit

Clin. Prof. Niwes Nantachit, M.D
President

KHON KAEN UNIVERSITY BY:

Kittichai T.

Assoc. Prof. Dr. Kittichai Triratanasirichai
President

APPENDIX B

Memorandum Of Agreement (MOA)

Bilateral Agreement between

Kasetsart University and Price of Songkla University

Co-funded by the Erasmus+ Programme of the European Union

PISAI

Participatory and Integrative Support for Agricultural Initiative

Erasmus+ KA2 Capacity Building in Higher Education

Project No. 586157-EPP-1-2017-1-TH-EPPKA2-CBHE-JP

Memorandum of Agreement (MoA)

Bilateral Agreement between

Kasetsart University and Prince of Songkla University

บันทึกข้อตกลง (MEMORANDUM OF AGREEMENT)
ในการดำเนินการโครงการจัดการเรียนการสอนหลักสูตรสองปริญญา
ระดับบัณฑิตศึกษา

ชื่อมหาวิทยาลัยคู่สัญญา: มหาวิทยาลัยเกษตรศาสตร์ และ มหาวิทยาลัยสงขลานครินทร์
หลักสูตรคู่สัญญา:

- ระดับมหาบัณฑิต ที่มหาวิทยาลัยเกษตรศาสตร์ และ มหาวิทยาลัยสงขลานครินทร์

ข้อตกลงความร่วมมือนี้ ทำขึ้น
ระหว่าง

มหาวิทยาลัยเกษตรศาสตร์ เป็นมหาวิทยาลัยในกำกับของรัฐ มีที่ตั้งสำนักงานใหญ่อยู่ที่เลขที่ 50 ถนนงามวงศ์วาน แขวงลาดยาว เขตจตุจักร กรุงเทพมหานคร 10900

ในที่นี้ ชื่อว่า “มก.”

และ

มหาวิทยาลัยสงขลานครินทร์ เป็นมหาวิทยาลัยในกำกับของรัฐ มีที่ตั้งสำนักงานใหญ่อยู่ที่เลขที่ 15 ถนนกาญจนวณิชย์ อำเภอหาดใหญ่ จังหวัดสงขลา 90110

ในที่นี้ ชื่อว่า “ม.อ.”

ข้อตกลงนี้ทำขึ้นโดยเป็นไปตามระเบียบและ/หรือข้อบังคับว่าด้วยการศึกษาระดับบัณฑิตศึกษาของบัณฑิตวิทยาลัย มหาวิทยาลัยสงขลานครินทร์ มหาวิทยาลัยเกษตรศาสตร์ มหาวิทยาลัยเชียงใหม่ และมหาวิทยาลัยขอนแก่น ที่บังคับใช้อยู่ในปัจจุบัน โดยมีข้อตกลงในด้านต่าง ๆ ของคู่สัญญาทั้งสี่ฝ่าย ดังนี้

1. ขอบข่ายความร่วมมือ

1.1 เป็นความร่วมมือเป็นลายลักษณ์อักษรระหว่าง มหาวิทยาลัยสงขลานครินทร์ มหาวิทยาลัยเกษตรศาสตร์ มหาวิทยาลัยเชียงใหม่ และมหาวิทยาลัยขอนแก่น ซึ่งเป็นคู่สัญญา ในการจัดทำโครงการจัดการเรียนการสอนหลักสูตรสองปริญญา ระดับบัณฑิตศึกษา

1.2 โครงการนี้จะนำไปสู่การได้รับปริญญาโทจากมหาวิทยาลัยคู่สัญญา

1.3 ความร่วมมือนี้ จำกัดเฉพาะกับหลักสูตรที่เข้าร่วมโครงการจัดการเรียนการสอนสองปริญญา โดยมีความร่วมมือในการดำเนินการโครงการในด้านต่าง ๆ ของคู่สัญญา

2. การคัดเลือกผู้เข้าศึกษา

2.1 เป็นไปตามข้อบังคับว่าด้วยการศึกษาระดับบัณฑิตศึกษา ของมหาวิทยาลัยคู่สัญญา และข้อกำหนดเพิ่มเติมของหลักสูตร

2.2 มหาวิทยาลัยคู่สัญญาต้องยอมรับคุณสมบัติของผู้เข้าศึกษาร่วมกัน

2.3 การรับนิสิต/นักศึกษาเข้าศึกษาต้องได้รับความเห็นชอบจากมหาวิทยาลัยคู่สัญญา

3. การลงทะเบียนเรียน

3.1 นิสิต/นักศึกษาต้องลงทะเบียนเป็นนักศึกษาของทั้งสองมหาวิทยาลัยคู่สัญญา

3.2 นิสิต/นักศึกษาต้องลงทะเบียนเรียนเป็นนักศึกษาแบบเต็มเวลา (Full time student) ทั้งสองมหาวิทยาลัยคู่สัญญา

3.3 มหาวิทยาลัยคู่สัญญาจะต้องทำการบันทึกข้อมูลเกี่ยวกับนิสิต/นักศึกษาตามขั้นตอนมาตรฐานของแต่ละหลักสูตรในแต่ละมหาวิทยาลัย

3.4 มหาวิทยาลัยคู่สัญญาจะให้ข้อมูลเกี่ยวกับประวัติการศึกษาของนิสิต/นักศึกษาเมื่อได้รับการร้องขอ

3.5 ระยะเวลาการศึกษามีดังนี้

โครงการจัดการเรียนการสอนหลักสูตรสองปริญญา	**ระยะเวลาดังระบุในระเบียบของ สกอ.**	**ระยะเวลาขั้นต่ำที่ต้องเรียนในแต่ละสถาบัน**
ปริญญาโท	2 ปี	อย่างน้อย 1 ปีการศึกษา

4. การจัดการเรียนการสอน

4.1 การจัดการเรียนการสอนให้เป็นไปตามข้อตกลงของมหาวิทยาลัยคู่สัญญา

4.2 นิสิต/นักศึกษา ต้องสามารถเข้าถึงข้อมูลของหลักสูตรที่เข้าศึกษาของแต่ละสถาบันคู่สัญญา

4.3 การแต่งตั้งคณะกรรมการที่ปรึกษาประจำตัวนิสิต/นักศึกษา ต้องประกอบด้วยอาจารย์ที่ปรึกษาวิทยานิพนธ์ หลักจากสถาบันต้นสังกัด และ อาจารย์ที่ปรึกษาวิทยานิพนธ์ ร่วมจากสถาบันคู่สัญญา ทั้งนี้ต้องเป็นไปตามระเบียบและข้อบังคับของมหาวิทยาลัยคู่สัญญา

5. กฎระเบียบและข้อบังคับ

5.1 นิสิต/นักศึกษาจะต้องปฏิบัติตามกฎและข้อบังคับว่าด้วยการศึกษาระดับบัณฑิตศึกษาของมหาวิทยาลัยคู่สัญญา

6. วิทยานิพนธ์

นิสิต/นักศึกษาต้องทำวิทยานิพนธ์และปฏิบัติตามข้อกำหนดดังต่อไปนี้

6.1 จัดทำวิทยานิพนธ์เป็นภาษาไทยหรือภาษาอังกฤษ โดยจัดทำเป็นฉบับเดียว ตามมหาวิทยาลัยต้นสังกัด โดยระบุข้อความถึงความร่วมมือ

6.2 การจัดทำรูปเล่มวิทยานิพนธ์ ต้องเป็นไปตามข้อบังคับของมหาวิทยาลัยต้นสังกัด

6.3 ในการแต่งตั้งกรรมการสอบวิทยานิพนธ์ ให้เป็นไปตามข้อบังคับว่าด้วยการศึกษาระดับบัณฑิตศึกษาของมหาวิทยาลัยต้นสังกัด โดยมหาวิทยาลัยต้นสังกัดต้องรับผิดชอบค่าใช้จ่ายในการสอบ

6.4 การส่งรูปเล่มวิทยานิพนธ์ต้องส่งที่มหาวิทยาลัยต้นสังกัด และมหาวิทยาลัยคู่สัญญา หลังจากได้รับการอนุมัติขั้นสุดท้าย ต้องจัดทำวิทยานิพนธ์ในรูปแบบอิเล็กทรอนิกส์ส่งให้กับทั้งสองมหาวิทยาลัย

7. ข้อร้องเรียน การร้องทุกข์และการกระทำผิด

7.1 การร้องเรียนและการอุทธรณ์จะต้องดำเนินการตามขั้นตอนการร้องเรียนและการอุทธรณ์ของทั้งสองมหาวิทยาลัย โดยทางมหาวิทยาลัยทั้งสองต้องให้ข้อมูลดังกล่าวแก่นิสิต นักศึกษา

8. การสำเร็จการศึกษา

8.1 นิสิต/นักศึกษาจะได้รับปริญญาจากมหาวิทยาลัยต้นสังกัดและมหาวิทยาลัยคู่สัญญา เมื่อปฏิบัติตามข้อบังคับและตามที่ระบุในหลักสูตร (มคอ. 2) ของมหาวิทยาลัยต้นสังกัดและมหาวิทยาลัยคู่สัญญาครบถ้วน

8.2 การขอสำเร็จการศึกษาปริญญาเดียว อาจกระทำได้ในกรณีที่มีเหตุอันสมควร ทั้งนี้โดยความเห็นชอบของมหาวิทยาลัยต้นสังกัดและมหาวิทยาลัยคู่สัญญา

9. การประกันคุณภาพการศึกษา

9.1 การประกันคุณภาพให้เป็นไปตามนโยบายและขั้นตอนการประกันคุณภาพตามปกติของแต่ละหลักสูตรและมหาวิทยาลัยคู่สัญญา

10. สิทธิในทรัพย์สินทางปัญญา

10.1 ทรัพย์สินทางปัญญาหรือผลประโยชน์อันเนื่องมาจาก วิทยานิพนธ์หรืองานอื่นๆ ที่ได้รับมอบหมายในระหว่างการศึกษาที่ไม่เกี่ยวข้องกับวิทยานิพนธ์ของนิสิต/นักศึกษาภายใต้ความร่วมมือ ให้เป็นของมหาวิทยาลัยต้นสังกัดและมหาวิทยาลัยคู่สัญญาร่วมกันตามสัดส่วนที่เห็นชอบร่วมกัน การจัดสรรสิทธิประโยชน์ให้กับนิสิตที่เป็นผู้ประดิษฐ์หรือผู้สร้างสรรค์ผลงานให้เป็นไปตามระเบียบหรือข้อบังคับของมหาวิทยาลัยต้นสังกัดและมหาวิทยาลัยคู่สัญญา เว้นแต่ได้จัดทำข้อตกลงเฉพาะกรณีเป็นลายลักษณ์อักษร

10.2 การละเมิด หากมหาวิทยาลัยใดทราบถึงการละเมิดสิทธิในทรัพย์สินทางปัญญาของมหาวิทยาลัยคู่สัญญา จะต้องแจ้งเป็นลายลักษณ์อักษรให้มหาวิทยาลัยคู่สัญญาทราบ โดยไม่สงวนสิทธิ์ในการดำเนินการทางกฎหมายที่เกี่ยวข้อง

10.3 การจดทะเบียนทรัพย์สินทางปัญญาร่วมกันให้เป็นไปตามข้อตกลงเป็นลายลักษณ์อักษร

11. ข้อตกลงทางการเงิน

11.1 นิสิต/นักศึกษาต้องจ่ายค่าธรรมเนียมการศึกษาแก่มหาวิทยาลัยในขณะที่ลงทะเบียนที่มหาวิทยาลัยนั้น ๆ

11.2 นิสิต/นักศึกษาต้องรับผิดชอบค่าใช้จ่ายในการเดินทาง ค่าที่พักและค่าครองชีพทั้งหมด

12. การเปลี่ยนแปลงความร่วมมือ

12.1 ความร่วมมือนี้รวมถึงข้อตกลงที่เกิดขึ้นภายใต้ความร่วมมือฉบับนี้ อาจมีการแก้ไขหรือเปลี่ยนแปลงได้หลังจากการลงนามโดยผู้ที่มีอำนาจ โดยต้องกระทำเป็นลายลักษณ์อักษรและลงนามโดยผู้มีอำนาจในทุกคู่สัญญา

12.2 มหาวิทยาลัยคู่สัญญามีสิทธิ์ที่จะถอนตัวออกจากโครงการ หากฝ่ายใดฝ่ายหนึ่งต้องการที่จะถอนตัวออกจากความร่วมมือนี้ ต้องแจ้งเป็นลายลักษณ์อักษรไม่น้อยกว่า 12 เดือน ก่อนวันที่ต้องการถอนตัว อย่างไรก็ตาม มหาวิทยาลัยคู่สัญญาทั้งที่ยังคงอยู่และที่ต้องการจะถอนตัวต้องร่วมรับผิดชอบนิสิต/นักศึกษาที่ยังคงค้างให้ได้รับการสนับสนุนตามที่ระบุไว้ในความร่วมมือฉบับนี้จนกว่าจะสำเร็จการศึกษา การบอกเลิกความร่วมมือจะต้องไม่ส่งผลกระทบใดๆ ต่อการศึกษาวิจัยของนิสิต/นักศึกษาที่คงค้างอยู่

13. ระยะเวลาของความร่วมมือ

ความร่วมมือนี้มีระยะเวลา 5 ปี นับจากวันที่ลงนามด้านล่างและอาจมีการตกลงเพื่อต่ออายุสัญญาภายใน 12 เดือนก่อนวันหมดอายุ

ให้ความเห็นชอบโดย วันที่ ๒๕ ก.ค. 62

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List of Publication and Proceeding

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