Clarification of factors contributing to the low effectiveness of the mating disruption for Asiatic common looper, *Autographa nigrisigna*, and Application of molecular techniques to identify plusiine species

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Aoi Hashiyama

Graduate School of Horticulture
CHIBA UNIVERSITY

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千葉大学大学院 園芸学研究科
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General Introduction

0.1 Pheromones for Integrated Pest Management

The term ‘pheromone’ is derived from the Greek words pherein (to carry) and hormao (to exicite). Just like the name implies, pheromones are the substances secreted to the outside by an individual and received by a second individual of the same species in which they release a specific reaction (Wyatt, 2003). Nowadays, pheromones of many kinds of insects have been identified and some of them are applied for the pest control. A web-site ‘Pherobase’ cites more than 900 genera from nearly 60 families of moths and butterflies which pheromones have been identified (El-Sayed, 2012). In the case of moths, males generally find their mates by following the females’ sex pheromones. During the last few decades, artificial synthesized sex pheromones have been utilized for the control of these moths and have shown successful results in a variety of ways in numerous agricultural systems. They have been used for mass trapping, mating disruption, monitoring and surveying (Mitchell, 1986). Of these uses, mating disruption has offered the greatest potential for integrated pest management (IPM) programs (Pfeiffer et al., 1991; McLaughlin et al., 1994; Shorey et al., 1996). In mating disruption method, male moths are confused by synthetic pheromones permeating through the field, and they cannot find females. The main goal of this method is to prevent adult males from finding females and reduce the number of fertilised eggs on the host crops.

0.2 Use of pheromones in Japan

Since 1970s, it has been reported that mating disruption experiments were successful against several fruit tree and tea pests in Japan (Oyama et al., 1978; Ohtaishi, 1986; Furuno, 1986; Sato, 1986). Subsequently, experiments against vegetable pests started in cultivated fields. The mating disruptant for vegetables has been commercially available since 2004 as the name of Confuser V® (Shin-Etsu Chemical Co., Ltd., Tokyo, Japan) (Fig. 0-1). Although the Confuser V® has been used in vegetable fields in Japan, an increase in the number of plusiine moths (Lepidoptera: Noctuidae) has been observed in IPM lettuce fields (Hashiyama et al., 2011).

0.3 Plusiine species

The subfamily Plusiinae is comprised of moths with a robust body, large scale tufts on the thorax, and a characteristic metallic gold or silver pigmentation pattern in the center of the forewing (Pogue, 2005; Spect et al., 2007). Their larvae, known as “semi loopers”, are characterized by the combination of the two pairs of prolegs which determine their locomotion as “measuring worms” (Eichlin and Cunningham 1978; Lafontaine and Poole, 1991). This subfamily consists of more than 400 species world-wide (Kitching, 1987), and

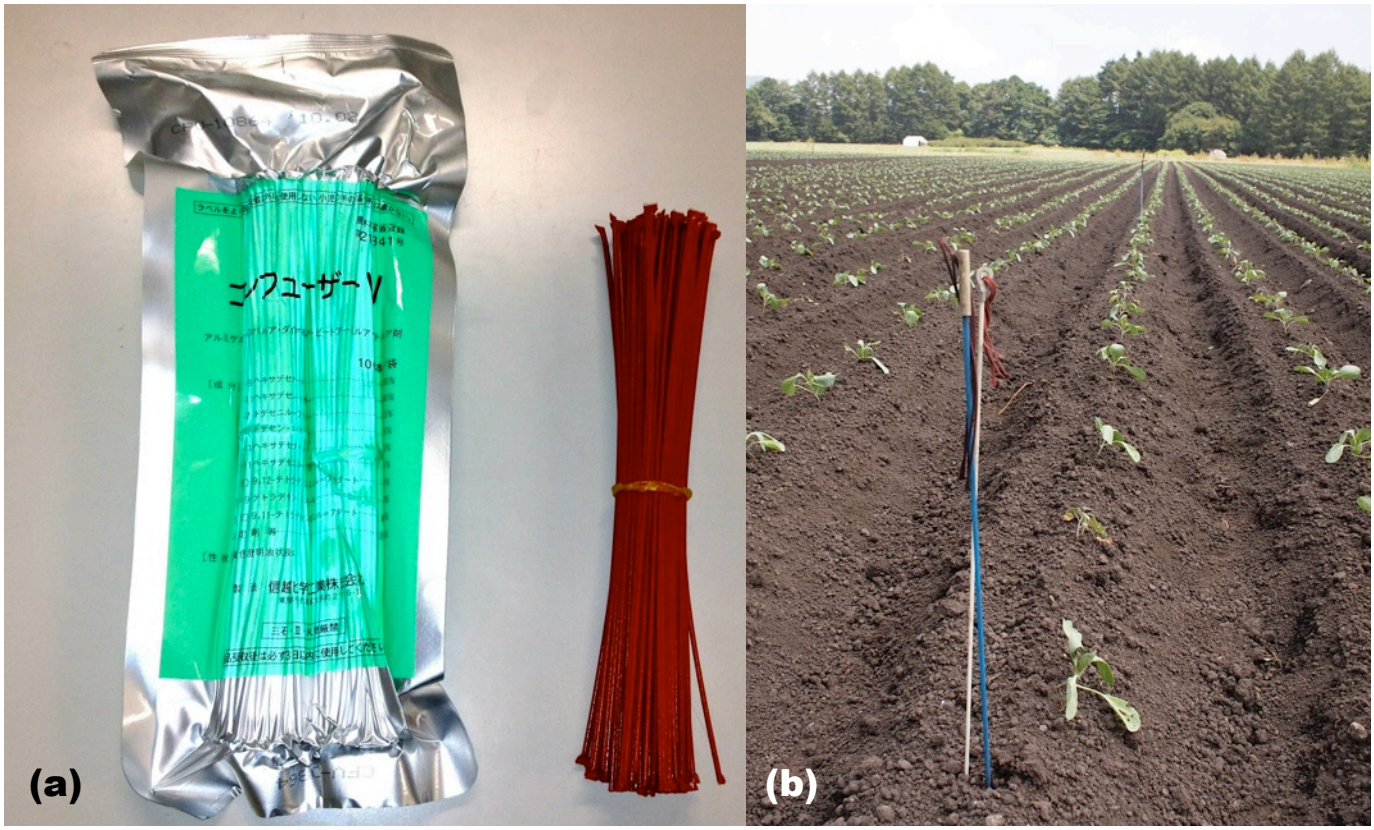

Fig. 0-1. The mating disruptant for vegetables, Confuser V®.
(a) Package and contents. (b) Dispensers distributed in the field.

the Japanese fauna contains 59 species belonging to 20 genera (Kobayashi, 2011).

0.4 *Autographa nigrisigna*

Autographa nigrisigna (Lepidoptera: Noctuidae) is one of the plusiine species and serious pest because of fast-growing, multivoltine and highly polyphagous (Fig. 0-2). Although *A. nigrisigna* is designated as a target species of Confuser V®, the eggs and larvae of *A. nigrisigna* can be found in the Confuser V® treated lettuce field (Hashiyama et al, 2012). This finding showed that mating disruption could not work well for *A. nigrisigna*. In addition, other plusiine species could be also found in the same lettuce field. Because these plusiine species have quite similar morphological characteristics during the immature stages (eggs, larvae and pupae), most researchers who engaged in agriculture do not recognize that various plusiine species occur together in lettuce fields.

0.5 Need for new identification tool

Morphological similarities of plusiine taxa make identification to species difficult. However, understanding the systematics of agricultural pests is important for the effective control and management programs (Parrella and Keil, 1984; Rossman and Miller, 1996). The need, therefore, exists to identify plusiine species.

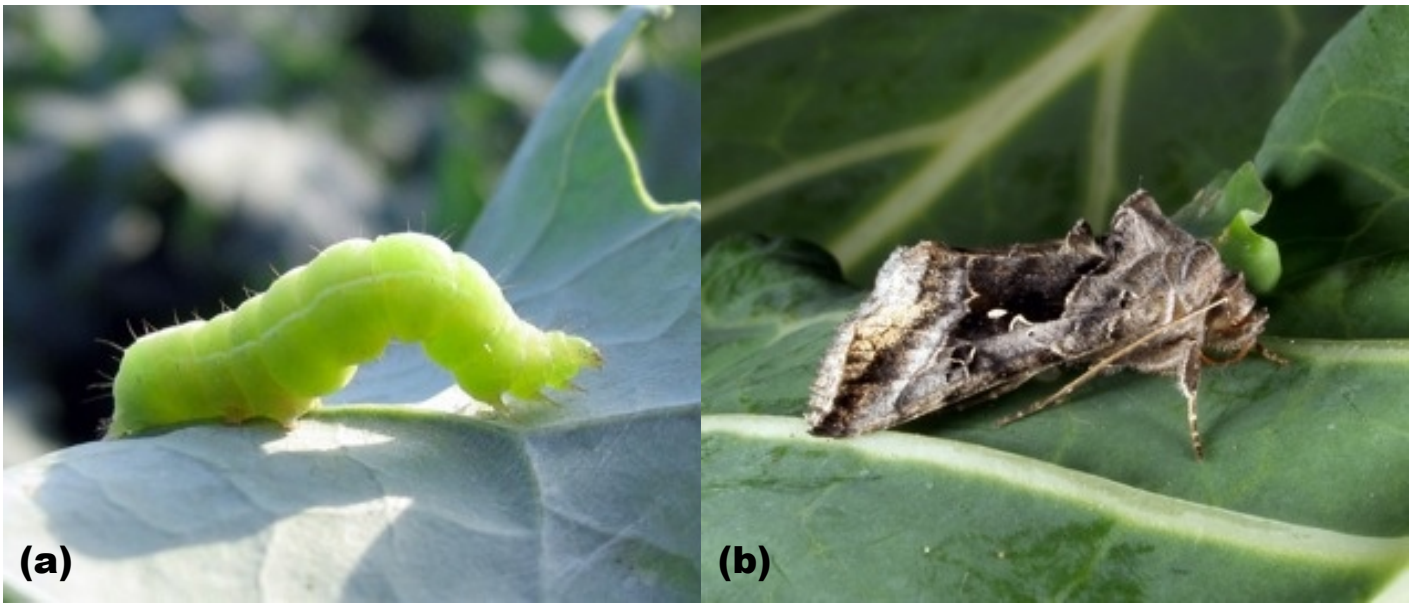

Fig. 0-2. *Autographa nigrisigna*.
(a) Larva. (b) Adult.

0.6 Relationship between moths and plants

Although *A. nigrisigna* is famous as a polyphagous insect (Ichinose, 1962), nothing is known about the females' oviposition preference and larval feeding preference. Control of *A. nigrisigna* in cabbage fields using mating disruptants has produced several good results. On the other hand, The number of positive report on mating disruptants used in lettuce field is very few. Mating disruption is targeting only male moths, which is apparently different from chemical insecticides affecting eggs and larvae. Regardless of mating disruption, females can migrate inside and outside of the pheromone treated field and choose their oviposition site freely. Difference of the attractiveness between cabbage and lettuce as host plants might affect the efficacy of mating disruptant in each crop field.

0.7 Aim of this study

The aim of this study is to reveal the factors contributing to the low effectiveness of the mating disruption for *A. nigrisigna*. To achieve this aim, I considered following four possibilities and examined them respectively.

- Migration of mated females into the pheromone treated area
- Host plant selection by female adults and larvae
- Weak response caused by the absence of minor components in Confuser V®
- False response of males to other lepidopteran pheromones

Through the field investigation, I needed to discriminate *A. nigrisigna* from other plusiine species which existed together. Thus, I established an easy and rapid method to identify plusiine species that are morphologically indistinguishable.

Chapter 1
Development of molecular identification technique

Multiplex PCR method for differentiating plusiine species in lettuce field

1.1 Introduction

Through the field investigation in 2008 and 2009 (Hashiyama et al., 2012), I found that plural plusiine species infested lettuce. In particular, three plusiine species *A. nigrisigna*, *Macdunnoughia confusa*, and *Thysanoplusia intermixta* could be observed in the pheromone treated lettuce field throughout the cropping season. Because these species have morphological similarities and it is difficult to discriminate only by visual characteristics, I established molecular identification techniques. Species identity can be readily determined using DNA sequencing data from any of several mitochondrial and nuclear genes, but this method is somewhat time-consuming and expensive for those not routinely involved with DNA sequencing (Miura et al., 2004). On that point, the DNA polymerase chain reaction (PCR) is an economical and useful technique. Multiplex PCR method - the size and number of PCR products obtained using multiprimer sets - can be used for distinguishing several species (Portillo et al., 1996; Roehrdanz, 2003). Consequently, I applied multiplex PCR to three plusiine species which are the major species in the lettuce field.

1.2 Materials and Methods

1.2.1 DNA extraction

Plusiine species used for this experiments were field-collections from several sites in Japan (Table 1-1). All specimens used in this experiment were identified in adult stage by using the information and keys of Ichinose (1962) and frozen at -20ºC for preservation. Genetic DNA was extracted from the muscles of the thorax using the DNeasy Blood & Tissue Kit® (Qiagen Ltd., Tokyo, Japan). DNeasy Blood & Tissue Kits are designed for rapid purification of total DNA (e.g., genomic, mitochondrial, and pathogen) from a variety of sample sources. The thorax were crushed with dissecting scissors and tissue grinders in 180$\mu\ell$ of PBS (Phosphate-Buffered Salines). The mixture was incubated with 20$\mu\ell$ of proteinase K (20mg/ml) and 180$\mu\ell$ of Buffer AL for 20min at 65°C. After incubation, 200$\mu\ell$ ethanol (96-100%) were added and mixed to inactivate the proteinase K. The mixture was transferred to the DNeasy Mini spin column and centrifuged at ≧ 8000rpm for 1min. Collection tube under the column was discarded. A new collection tube were placed and 500$\mu\ell$ of Buffer AW1 were added for cleaning up impurity. The mixture was centrifuged at ≧ 8000rpm for 1 min and collection tube was discarded again. A new collection tube were placed and 500$\mu\ell$ of Buffer AW2 were added for cleaning up impurity. The mixture was centrifuged at ≧ 14000rpm for 3min and collection tube was discarded. Microcentrifuge tube were placed under the the DNeasy Mini spin column and

Table 1-1. Location locality of species used for DNA sequence analysis in this study.

Species	Location	Stage	Number of specimens
Autographa nigrisigna	Karuizawa, Nagano Pref.	Immature	36
		Adult	15
	Kawakami, Nagano Pref.	Immature	1
		Adult	1
	Komoro, Nagano Pref.	Immature	4
		Adult	3
	Matsudo, Chiba Pref. *	Immature	10
		Adult	10
	Katori, Chiba Pref. *	Adult	3
	Fuchu, Tokyo Pref. *	Adult	4
	Kasai, Hyogo Pref. *	Adult	4
Macdunnoughia confusa	Karuizawa, Nagano Pref.	Immature	27
		Adult	22
	Kawakami, Nagano Pref.	Adult	1
	Komoro, Nagano Pref.	Immature	7
		Adult	2
	Matsudo, Chiba Pref. *	Adult	3
	Katori, Chiba Pref. *	Adult	2
Thysanoplusia intermixta	Karuizawa, Nagano Pref.	Immature	29
		Adult	11
	Kawakami, Nagano Pref.	Immature	1
		Adult	1
	Komoro, Nagano Pref.	Adult	2
	Matsudo, Chiba Pref. *	Adult	15
	Katori, Chiba Pref. *	Adult	2
	Tsukui, Kanagawa Pref. *	Adult	2

* The strains that we only used for multiplex PCR checking.

200$\mu\ell$ Buffer AE were added directly onto the DNeasy membrane. After 1min incubation at room temperature, the column were centrifuged at ≧ 8000rpm for 1min to elute. The solution in the microcentrifuge tube was used as templates for PCR reaction.

1.2.2 PCR reaction

The PCR was performed in 10$\mu\ell$ reaction volumes. It consisted of 0.5 U Ex Taq (Takara Bio Inc., Shiga, Japan), 1$\mu\ell$ 10× Ex Taq Buffer, 1$\mu\ell$ dNTP mixture, 0.5$\mu\ell$ each of 10μM each primers, 50ng DNA template, and distilled water. The PCR was carried out in a Thermal Cycler Dice (Takara Bio Inc.) with the following program: an initial denaturing step at 94ºC for 2min; 30 cycles of 94ºC for 1min, 52ºC for 2min, 72ºC for 2min; and a final extension step at 72ºC for 7min. The primers which we used included: C1-J-2183(5'-CAACATTTATTTTGATTTTTTGG-3', Simon et al., 1994); PL-3014 (5'-TCCATTACATATAATCTGCCATATT-3'). PL-3014 is a tail primer which was modified TL2-N-3014 (Simon et al., 1994) to fit for plusiine moths. These primers were used to amplify a 875 bp region spanning portion of the mitochondrial cytochrome oxidase I genes (CO I) and the leucine tRNA. Successful amplification was determined by electrophoresing 3$\mu\ell$ of the PCR mixture on a 1.5% agarose gel (1×TBE), staining with ethidium bromide, and observing under an UV transilluminator, Densitograph DT - 20MCP (ATTO

Ltd., Tokyo, Japan). A single band resulted from PCR amplification with this pair of primers.

1.2.3 Purification of PCR products

PCR products were purified using the Min Elute PCR Purification Kit (Qiagen Ltd.). Five volumes of Buffer PB were added to one volume of the PCR reaction and mixed. The sample was applied to the MinElute column and centrifuged at ≧ 8000rpm for 1min. To wash, 750$\mu\ell$ of Buffer PE were added to the MinElute column and centrifuged at ≧ 8000rpm for 1.5min. Flow through in collection tube under the column was discarded. The sample was centrifuged again at ≧ 14000rpm for 1.5min. Residual ethanol from Buffer PE will not be completely removed unless the flow-through is discarded before this additional centrifugation. Microcentrifuge tube was placed in the column instead of the collection tube. To elute DNA, 10$\mu\ell$ of Buffer EB were added to the center of the membrane and incubated at room temperature for 1min. After incubation, the column were centrifuged at ≧ 8000rpm for 1min. The solution in the microcentrifuge tube was used for direct sequencing.

1.2.4 Direct sequencing analysis and alignment

A dye terminator-labeled cycle sequencing reaction was conducted with Big Dye® Terminater v 3.1 Cycle Sequencing Kit (Applied Biosystems Ltd., Tokyo,

Japan). Direct sequencing was performed in 10$\mu\ell$ reaction volumes. It consisted of 2$\mu\ell$ Terminator Ready Reaction Mix, 0.7$\mu\ell$ Sequencing Buffer, 0.5$\mu\ell$ head or tail primer, 1.5$\mu\ell$ DNA template, and 5.3$\mu\ell$ distilled water. Direct sequencing was carried out in a Thermal Cycler Dice (Takara Bio Inc.) with the following program: 25 cycles of 96ºC for 30sec, 50ºC for 30sec, 60ºC for 4min. After direct sequencing, I conducted ethanol precipitation to remove dd NTP residues. Reaction products were transferred to t 1.5ml microcentrifuge tube and added 2$\mu\ell$ of 12mM EDTA and 2$\mu\ell$ of 3 M NaOAC. 50$\mu\ell$ of 100% ethanol were added and mixed. After The mixture was incubated for 15min at the room temperature and centrifuged at ≧ 14000rpm for 5min. After centrifugation, ethanol was discarded and dried. These purified reaction products were analyzed using an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems Ltd.). Sequence alignment was performed using the Clustal W (Thompson et al., 1994). Gen Bank accession numbers for *A. nigrisigna*, *M. confusa* and *T. intermixta* are AB533496, AB533497, and AB533498, respectively.

1.2.5 Design of primers for multiplex PCR

The specific primers for multiplex PCR were designed using Amplify version 3.1 (Engels, 1993) in selected regions of the DNA sequences. Since in a multiplex PCR system a proper design of the primers is essential for a successful DNA amplification, the following aspects were taken into account: (1) that the

size of the amplified fragments should be different enough to be discriminated in agarose gels, (2) that the primers should not have potential matching sequences for nonspecific target sites, and (3) that the optimal DNA-primer annealing temperature should be nearly equal for all templete-primer annealing combinations (Portillo et al., 1996).

1.2.6 Application of multiplex PCR

To gain clear bands, the multiplex PCR was performed in 50$\mu\ell$ volumes. It consisted of 25$\mu\ell$ 2×Multiplex PCR Master Mix (Qiagen Ltd.), 5$\mu\ell$ 10×primer mix (2μM each primers), 100ng DNA template, and RNase free water. The PCR was carried out in a Thermal Cycler Dice (Takara Bio Inc.) with the following program: an initial activation step at 95ºC for 15min; 40 cycles of 94ºC for 30sec, 46ºC for 1.5min, 72ºC for 1.5min; and a final extension step at 72ºC for 10min. The multiplex PCR was performed using the specimens listed in Table 1-1. We used not only adults but also eggs, larvae and pupae to check availability of this method. DNA was extracted from whole body for egg or lower body for larva and pupa.

1.3 Results

Each segment consisting of the 3' portion of the mtDNA cytochrome oxidase I (CO I) and a 5' portion of tRNA leucine from *A. nigrisigna*, *M. confusa* and *T.*

intermixta was amplified and sequenced correctly (Fig. 1-1). These sequences were aligned and found out mismatches between three plusiine species. The mismatches were exploited to design primers for multiplex PCR. I made a head primer and tail primer additionally. Location of four primers used for multiplex PCR was shown in Fig. 1-2. These new primers were tested individually in combination with common primers, C1-J-2183 head primer and PL-3014 tail primer. Based on the sequence, the expected sizes of the amplicons are 875 bp (C1-J-2183 + PL-3014), 577bp (MC-1 + PL-3014) and 273 bp (C1-J-2183 + MC-0). To optimize anealing temperature, I carried out gradient PCR in a 43-52ºC with 1ºC intervals. As a result, 46ºC was chosen as an optimal anealing temperature for all primers. An ethidium bromide-stained gel of DNA amplified by the multiplex PCR with anealing temperature 46ºC is shown in Fig. 1-3. As expected, four bands, corresponding to an ~300 bp fragment, resulting from the amplification for the species-specific *M. confusa*, an ~600 bp fragment form of *M. confusa* and *T. intermixta*, and an ~900 bp fragment form common to three plusiine species, were observed. Because size and number of PCR products are different between each species, the multiplex PCR method enables to distinguish them easily on gel. To check whether the primers reacted differently depending on the geographic origin, we tried >50 specimens from five locations in Chiba, Tokyo, Kanagawa, and Hyogo Prefecture (Table 1-1). All strains could be distinguished by the method mentioned above. Moreover, the individuals among immature stages (eggs, larvae and pupae) could be identified properly as well as

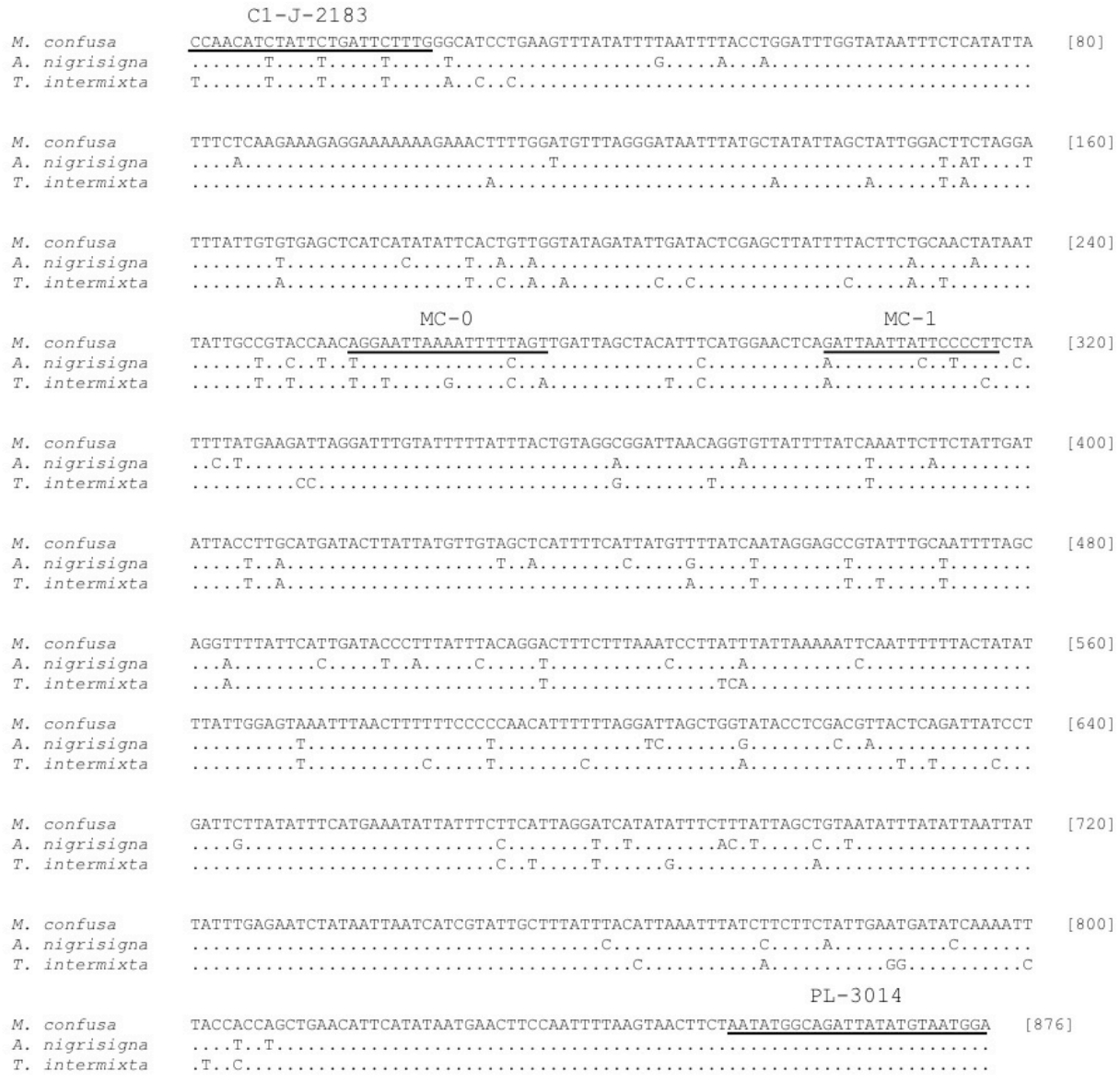

Fig. 1-1. Partial sequences of CO I and tRNA genes of *A. nigrisigna*, *M. confusa* and *T. intermixta*. Underlining shows the region corresponding to the primers.

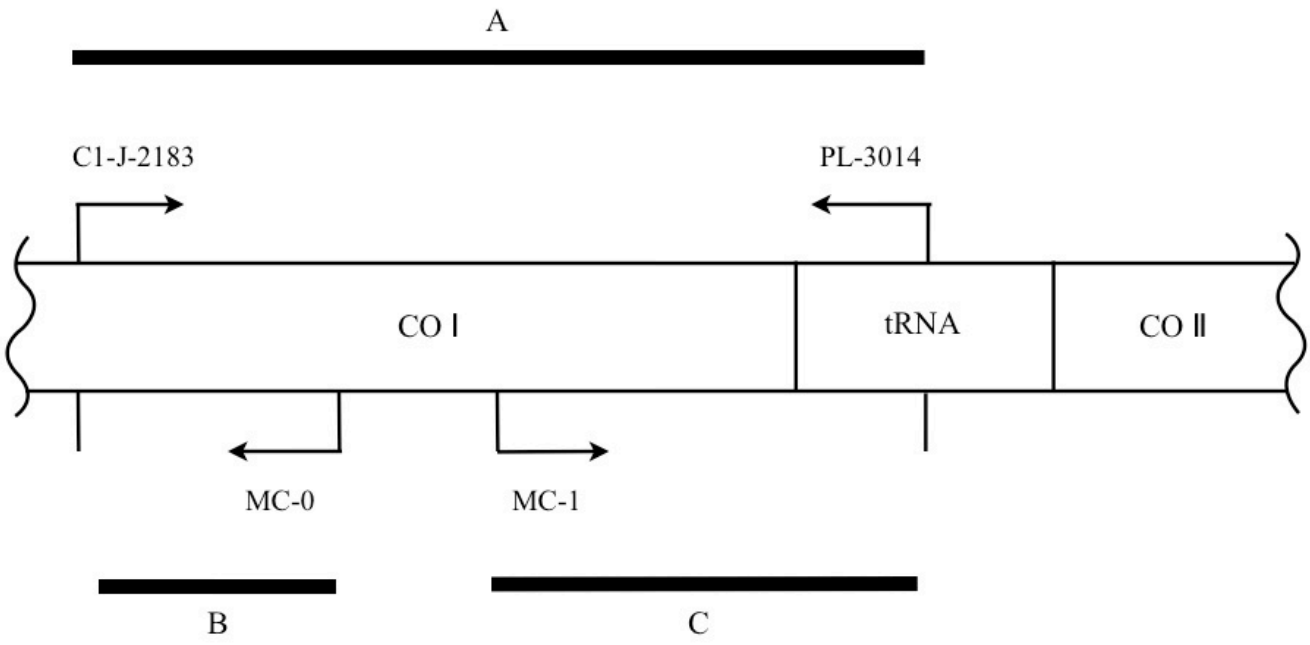

Fig. 1-2. Location of four primers used for multiplex PCR showing the relative positions and directions (arrows) of PCR primers in the mitochondrial COI and tRNA region. Amplification product A (875 bp) is common to all three plusiine species. Product B (273 bp) is obtained from *M. confusa*. Product C (577 bp) is from *M. confusa* and *T. intermixta*.

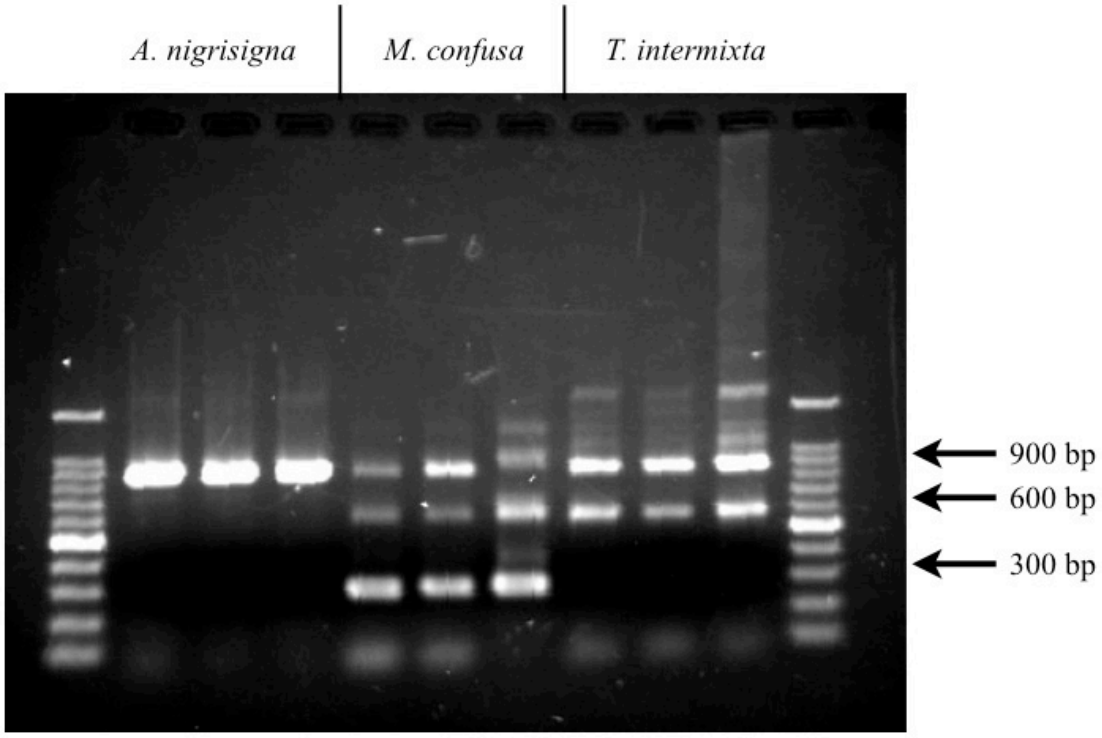

Fig. 1-3. Banding patterns obtained with the multiplex PCR method on 1.5% agarose gel. The size markers on both side lanes are a OneSTEP Ladder 100 (Nippon Gene Inc., Tokyo, Japan).

adults. I utilized this method to the field investigation described in Chapter 3 and 4. In the field investigation, I found many plusiine larvae whose chaetoaxy showed Type-A (Ichinose, 1962). Because *A. nigrisigna* and *M. confusa* larvae showed Type-A chaetoaxy, I could not identify them only by chaetoaxy. Thus, I applied this multiplex PCR method to these individuals and identified them.

1.4 Discussion

In this chapter, I established multiplex PCR method for rapidly and easily identifying the immature stages of three plusiine species commonly found on lettuce crops in Japan. This method has a potential to become a powerful identification tool for agricultural researchers. Without any time-consuming procedure, the researchers can comprehend what kind of plusiine species occurred in their lettuce field.

In the case of Plusiinae, mortality caused by parasitic wasps or diseases varies greatly with their species and stadium. *Copidosoma floridanum* (Hymenoptera: Encyrtidae) is the polyembryonic braconid parasitoid of some plusiine species, *T. intermixta*, *Autographa gamma* and *Ctenoplusia agnata* (Iwabuchi, 1991; Kaneko, 1993; Utsunomiya and Iwabuchi, 2002). In spite of the closely relationship to *A. gamma* (Nomura, 1998), *A. nigrisigna* was not mentioned as a host of *C. floridanum*. And also, susceptibility to diseases differs according to stadium of the hosts. Semel (1956) reported that older plusiine larvae were more susceptible to the virus. However, Hall (1957) reported small

larvae were easier to be killed. This dichotomy was possibly due to the more moderate temperature used in the studies by Hall (1957). It is now generally considered that younger larvae are more susceptible than old (Vail et al., 1999). Because the infection rates to parasitic wasps and diseases are different from species or stadium of the hosts, species-structures could be changed between adult and immature stages.

To carry out integrated pest management effectively, it is essential to understand species interaction of pests. However, it is difficult to distinguish plusiine species among immature stages because of their morphological similarities. Moreover, these species occur together in lettuce fields. To resolve these problems, we applied molecular technique which is not rely on morphological characteristics. Fig. 1-4 ~ 1-6 showed the species-structure of field collection in 2009. In this year, I cultivated 126 lettuce plants in Confuser V® treated and control field for three terms and collected plusiine larvae from 90 plants in each field. After using multiplex PCR method presented here, I detected large number of *A. nigrisigna* occurred in both Confuser V® treated and control treated field. With this molecular identification method, it is possible to identify individuals which died during immature stages. So, I could comprehend accurate and conclusive species-structure of Plusiinae in lettuce field without any time-consuming process. In tandem with the prevalence of PCR technique, our multiplex PCR method will become a useful tool for agricultural researchers to reveal 'true' species-structure and study their interactions in the field.

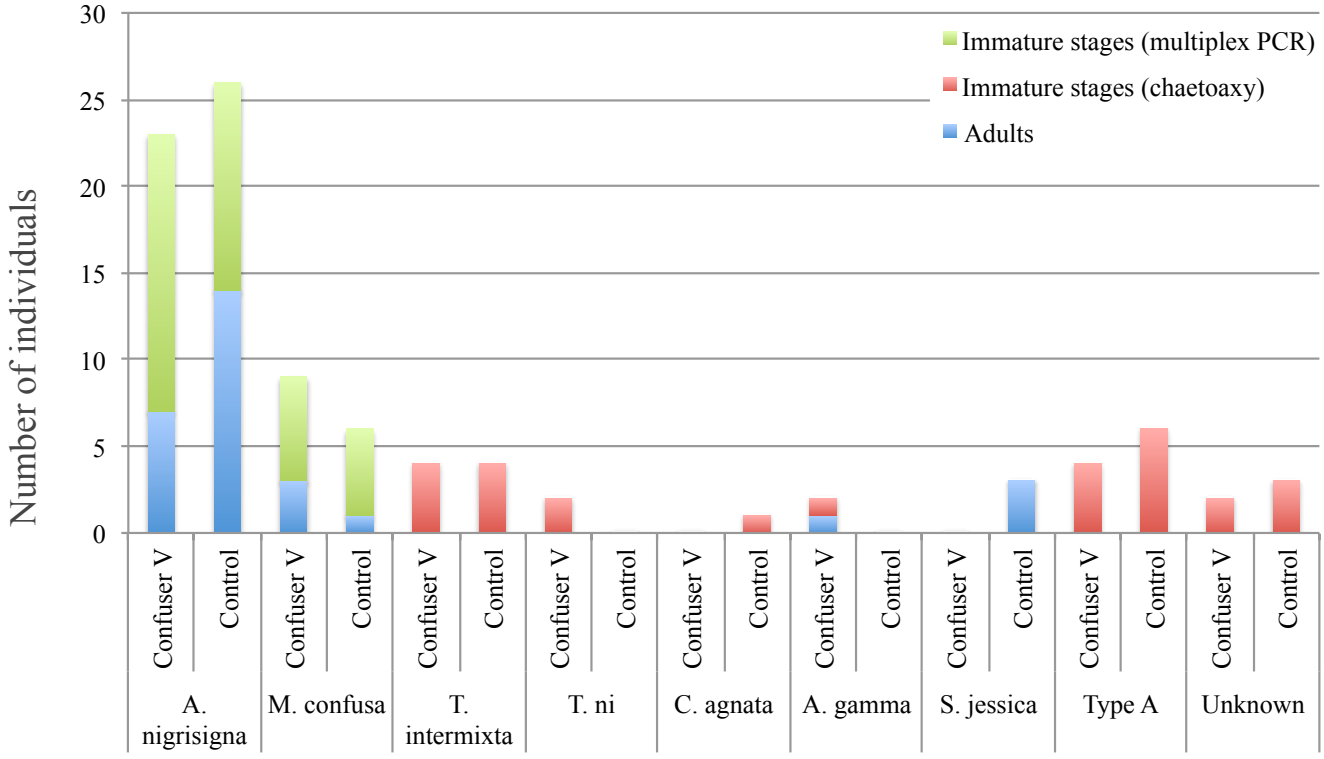

Fig. 1-4. Species-structure of plusiine species in Confuser V® and control field in the first term, 2009. Green bar indicates the number of individuals died before emergence and identified by applying multiplex PCR method. Red bar indicates the number of individuals died before emergence and identified by observing larval setal plan (Ichinose, 1962). Blue bar indicates the number of individuals emerged safely and identified by the colors and pigmentations of wings.

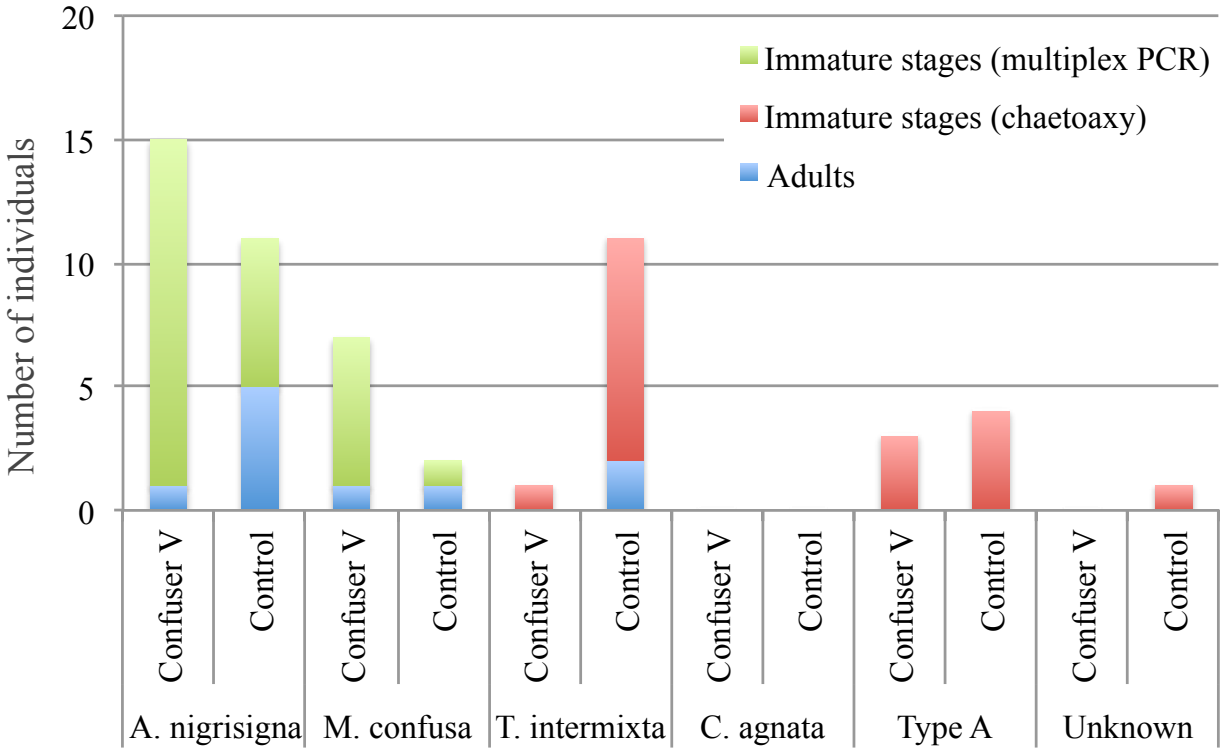

Fig. 1-5. Species-structure of plusiine species in Confuser V® and control field in the second term, 2009. Green bar indicates the number of individuals died before emergence and identified by applying multiplex PCR method. Red bar indicates the number of individuals died before emergence and identified by observing larval setal plan (Ichinose, 1962). Blue bar indicates the number of individuals emerged safely and identified by the colors and pigmentations of wings.

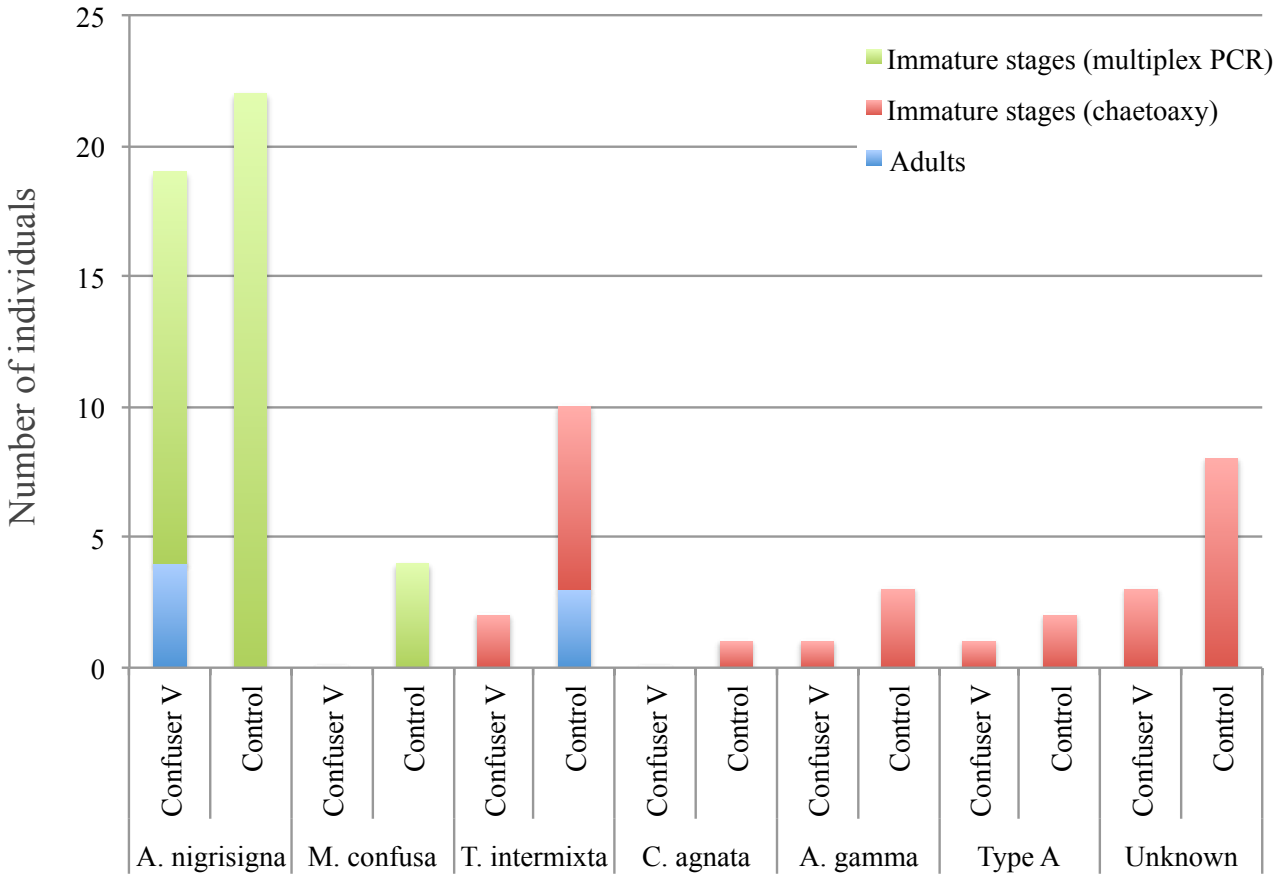

Fig. 1-6. Species-structure of plusiine species in Confuser V® and control field in the third term, 2009. Green bar indicates the number of individuals died before emergence and identified by applying multiplex PCR method. Red bar indicates the number of individuals died before emergence and identified by observing larval setal plan (Ichinose, 1962). Blue bar indicates the number of individuals emerged safely and identified by the colors and pigmentations of wings.

Chapter 2
Migration into the pheromone-treated area

Estimation of flight capacity of *A. nigrisigna* females

2.1 Introduction

Through the field density investigation in 2008 and 2009, I found that more than 50% of all collected individuals were *A. nigrisigna* and their larvae infested lettuce in pheromone treated area (Hashiyama et al., 2012). And more, some male moths were caught by the pheromone traps located in the treated area throughout the lettuce cropping season. This result indicates that some low level of ovipositing may occur within the treated area. As one of the reasons for the low effectiveness of the mating disruption technique, I suggested that mated females fly into the treated area and lay fertile eggs on the crops. To date, many studies on insect migration were generated. For example, *Spodoptera litura* (Lepidoptera: Noctuidae) female moths are able to fly 36km in one night (Tu et al. 2010). As for plusiine species, *Autographa gamma* (L.) are known to long-distance migratory insects (Williams, 1958). However, there is no information on the flight activity of *A. nigrisigna*. Therefore, I assessed the flight ability of *A. nigrisigna* female adults as one of the criteria to decide the possibility of the migration into the pheromone treated area.

2.2 Materials

2.2.1 Insects

A. nigrisigna female adults used in this experiment were the second to fifth offspring generation obtained from he cabbage field in Matsudo City, Chiba Prefecture, Japan in May 2011.

2.2.2 Actograph device

An actograph is a device for recording periodic activity of insects. To determine periodicity of insect behaviors, different types of actographs have been developed based on various physical principles including mechanical vibration, light interference ultrasonic wave and sound. In this study, I used an infrared light type actograph. This actograph device was linked to the data loggar and computer. The insect's movements that interrupt infrared beam were detected and recored by this actograph system (Fig. 2-1). The actograph device used in this study was basically same as Ferguson et al. (1994) and Yoon et al. (2012). Experiments were conducted using 10 actograph containers located inside a room under a 12L-12D photoperiod at 23 ± 1ºC, 70 ± 10% RH. The container (Diameter 14 × Height 20cm) was made out of clear plastic board. Two pairs of type photoelectric switches FU-12 (Keyence Co. Ltd., Osaka, Japan) were attached to the container diagonally at the height of 20cm, the top of container. One pair of switches was consists of infrared sending part and receiving part,

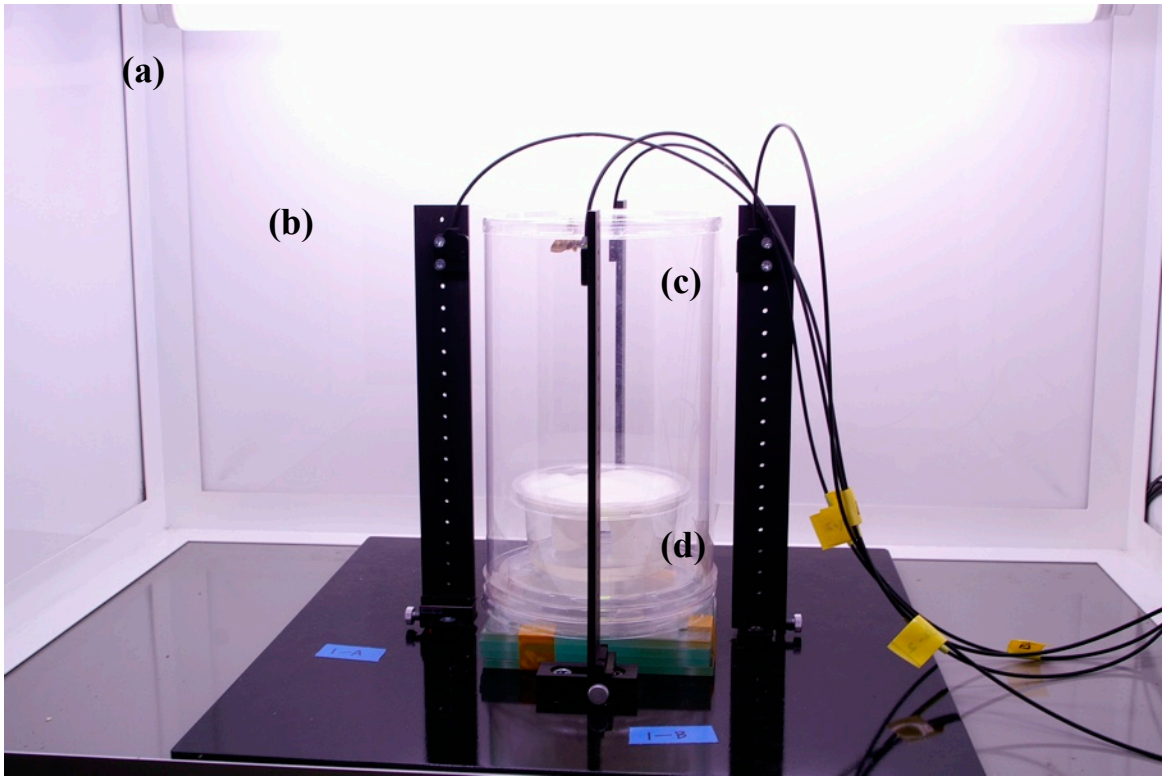

Fig. 2-1. Structure of the actograph device used in this study. (a) Fluorescent light. (b) Infrared sensor. (c) Container. (d) Diet for moths (3% honey solution).

and controlled to count if a moth cut across in front of the sensor. For the nutritional support, plastic cup (Diameter 7 × Height 3.5cm) filled with 3% honey solution was placed in the center of container. The sensor signals were recorded by a datalogger KV-700 (Keyence Co. Ltd.) and saved in a computer. All movements that block the infrared beam can be recorded. Thus, walking and fluttering through the beam were also counted as well as flying. The number of sensor interaction indicates the amount of insect's activity.

2.2.3 Flight mill device

To measure the mobility of *A. nigrisigna* females, I used flight mill device. The flight mill is a tool used for evaluating dispersal capabilities of insects such as moths and beetles. Although this is obviously an artificial situation, the flight mill can be used to measure the impact of various influences on the insect's dispersal when used in paired comparisons such as the effects of age, diet, temperature, wind speed or pesticide exposure. In this study, I referred to the flight mill system of Moriya (1995) and modified it to fit for lepidopteran insects (Fig. 2-2). Same as the actograph, the flight mills were also connected with the computer. A piece of balsawood stick (Height 0.3 × Width 0.3 × Length 20cm) was used as each of flight mill rotor with a box nail and a insect pin (Shiga-konchu-fukyusha Co., Ltd., Tokyo, Japan) as an axle. The insect was attached to the end of the rotor with a small amount of quick-drying adhesive G17 (Konishi Co., Ltd., Osaka, Japan) fastening its pronotum to a balsawood glueblock

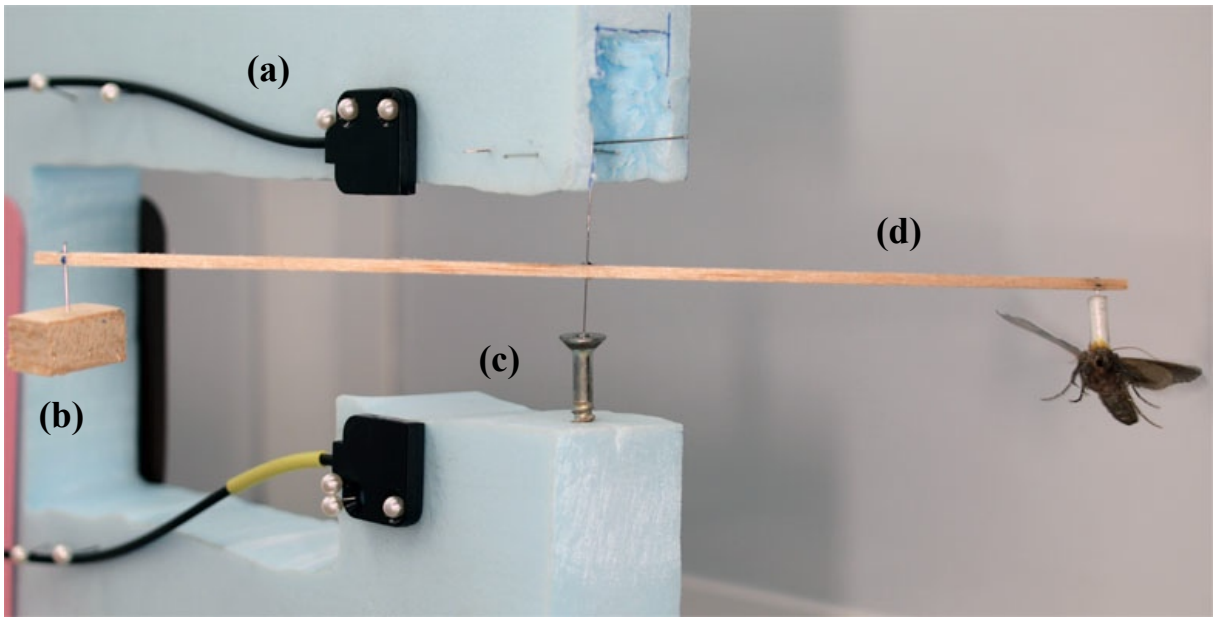

Fig. 2-2. Structure of the flight mill device used in this study. (a) Infrared sensor. (b) Balsawood glueblock. (c) Box nail and insect pin. (d) Balsawood stick.

(Height 0.3 × Width 0.3 × Length 3cm). The glueblock was attached to the end of the rotor using a pin. Before fastening, the crest of pronotum was removed. To counterbalance the weight, the ball of clay was attached to the other end of stick. Same system with actograph was used to record revolutions automatically. The interruption of an infrared beam by rotating arm generated an electrical signal that was recorded by the computer. The flight mill device was positioned in a room under a 12L-12D photoperiod at 23 ± 1ºC, 70 ± 10% RH.

2.3 Methods

2.3.1 Rearing the laboratory colony

The eggs and larvae were placed in plastic containers (Height 14 × Width 21 × Height 7cm). Larvae were reared on an artificial diet (Kawasaki et al., 1987). After pupation, individuals were sexed and kept in plastic cages (Height 16 × Width 22 × Height 12cm). In the center of cages, plastic cups (Diameter 7 × Height 3.5cm) filled with 3% honey solution were set. All procedures were conducted under a 12L-12D photoperiod at 25 ± 1ºC, 70 ± 10% RH.

2.3.2 Preparing the specific aged individuals

In the actograph and flight mill experiments, 4 to 6 day-old mated and unmated females were used because the mating behavior was observed most frequently at 5 days after emergence. Thus, I conducted that the activity for mating was best

represented by 4 to 6 day-old individuals. To obtain the specific aged individuals, newly emerged moths were divided into two groups. In one group, three virgin females and males were reared together in the plastic cage to obtain mated females. In the other group, six virgin females were reared in the cage to obtain unmated females for 3-5 nights. After 3-5 nights, 4-6 day-old mated and unmated females were created.

2.3.3 Actograph assay

Four to six day-old females in mated and unmated group were placed into the actograph container respectively and measured their activity for 24 hours. After measurement, females in mated group were dissected and their mating status was judged by the presence of spermatophore(s) in the bursa copulatorix. In addition, the number of eggs laid inside the actograph container was counted. With each age, 24 mated and unmated females were examined for the actograph assay.

2.3.4 Flight mill assay

Four to six day-old females in mated and unmated group were forced to suck 3% honey solutions for 15 seconds and placed to the flight mill device for 24 hours. After measurement, female moths in the mated group were dissected and their mating status was judged by the presence of spermatophore(s) in the bursa

copulatorix. Twenty-four mated and unmated females of each age were used for the flight mill assay and measured total flight duration and flight range. Flight range were computed by multiplying the number of sensor interaction and the circumferential length of flight mill rotor.

2.3.5 Data analysis

Comparison between mating status was analyzed by Mann-Whitney U-test. Comparison between ages were analyzed using Steel-Dwass test. The computer software JMP release 4.0.3 (JMP Software, SAS Institute, Cary, NC) was used for these statistical analyses.

2.4 Results

2.4.1 Actograph assay

Total number of sensor interruptions of mated and unmated female *A. nigrisigna* is shown in Fig. 2-3. At 4 days old, interruption numbers of mated and unmated females were 547.4 ± 589.0 and 152.7 ± 138.3 (mean ± SD). At 5 days old, interruption numbers of mated and unmated females were 549.7 ± 685.3 and 161.7 ± 192.5 (mean ± SD). At 6 days old, interruption numbers of mated and unmated females were 501.9 ± 589.1 and 181.8 ± 254.3 (mean ± SD). From these interruption numbers, it was revealed that mated females moved 2.8-3.6 times more actively than unmated females. At 4-6 days old, mated females were

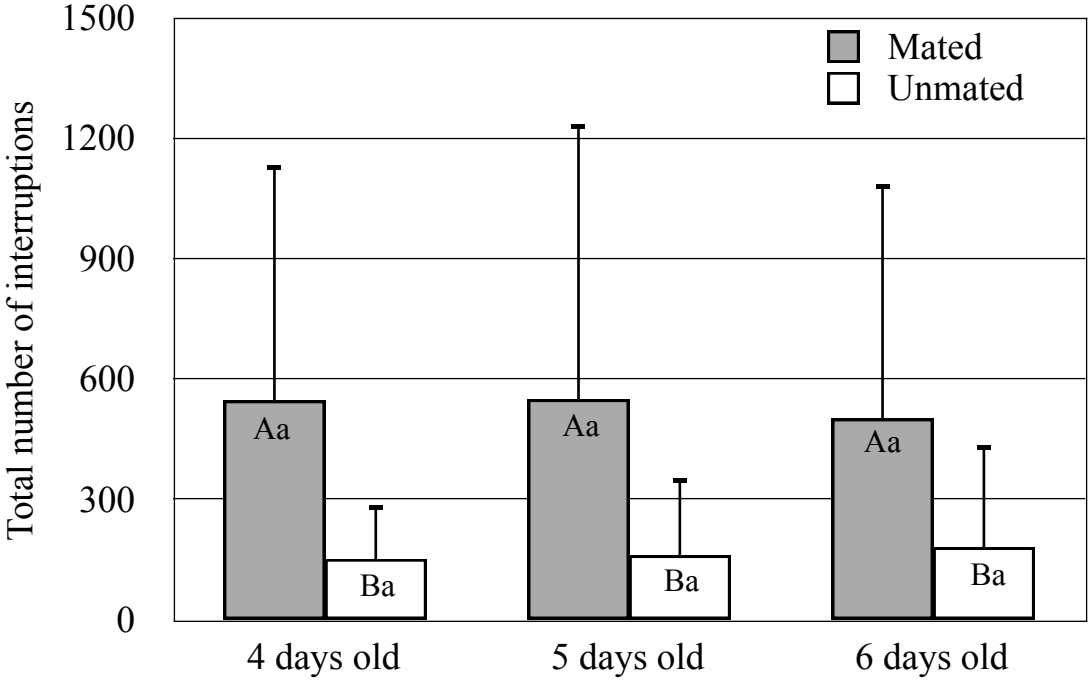

Fig. 2-3. Effect of mating status on the sensor interruptions of females 4 to 6 days old measured by actograph (mean ± SD). Different uppercase letters denote significant difference between mated and unmated females by Mann-Whitney U-test ($P < 0.05$). Different lowercase letters denote significant difference between age groups by Steel-Dwass test ($P < 0.05$). Twenty-four individuals of each mating status and age were tested.

significantly more active than unmated females. However, I found no significant difference in the activity of mated females between age groups. And also, there was no significant difference in the activity of unmated females between age grops. After measurement, the mean number of eggs laid by mated females was significantly more than that of unmated females (Table 2-1).

2.4.2 Flight mill assay

Flight duration of mated and unmated female *A. nigrisigna* is shown in Fig. 2-4. At 4 days old, flight durations of mated and unmated females were 222.5 ± 59.9 and 245.0 ± 58.5 (min, mean ± SD). At 5 days old, flight durations of mated and unmated females were 195.0 ± 64.4 and 220.0 ± 67.8 (min, mean ± SD). At 6 days old, flight durations of mated and unmated females were 175.0 ± 49.8 and 195.0 ± 56.7 (min, mean ± SD). At 4-6 days old, no significance difference was found in the flight duration between mated and unmated females. A significant difference in flight duration was found between 4- and 6-day-old mated females, and between 4- and 6-day old unmated females. Flight range of mated and unmated female *A. nigrisigna* is shown in Fig. 2-5. At 4 days old, flight ranges of mated and unmated females were 4.1 ± 1.1 and 4.5 ± 1.6 (km, mean ± SD). At 5 days old, flight ranges of mated and unmated females were 3.7 ± 0.7 and 4.1 ± 1.3 (km, mean ± SD). At 6 days old, flight ranges of mated and unmated females were 3.0 ± 1.0 and 3.4 ± 0.7 (km, mean ± SD). The maximum flight range of mated and unmated females reached 6.3 and 6.5 km. The flight range of females

Table 2-1. Comparison of egg production between mated and unmated female *A. nigrisigna.*

Age of adult moths (days old)	Mating status	Number of moths examined	Total number of eggs (mean ± SD)
4	Mated	24	61.2 ± 23.5 *
	Unmated	24	8.8 ± 4.7
5	Mated	24	72.1 ± 25.7 *
	Unmated	24	9.6 ± 5.0
6	Mated	24	81.5 ± 25.0 *
	Unmated	24	9.0 ± 5.1

* shows a significant difference at the 0.1 % level using Mann-Whitney U-test.

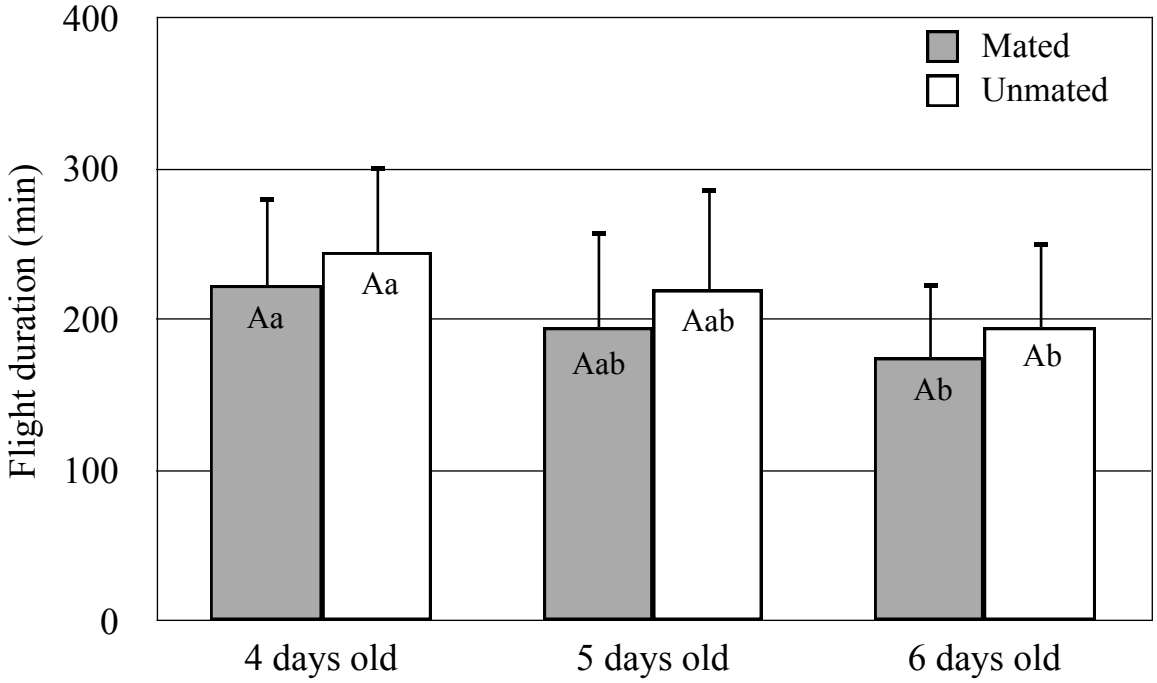

Fig. 2-4. Effect of mating status on the flight duration of females 4 to 6 days old measured by flight mill (mean ± SD). Different uppercase letters denote significant difference between mated and unmated females by Mann-Whitney U-test ($P < 0.05$). Different lowercase letters denote significant difference between age groups by Steel-Dwass test ($P < 0.05$). Twenty-four individuals of each mating status and age were tested.

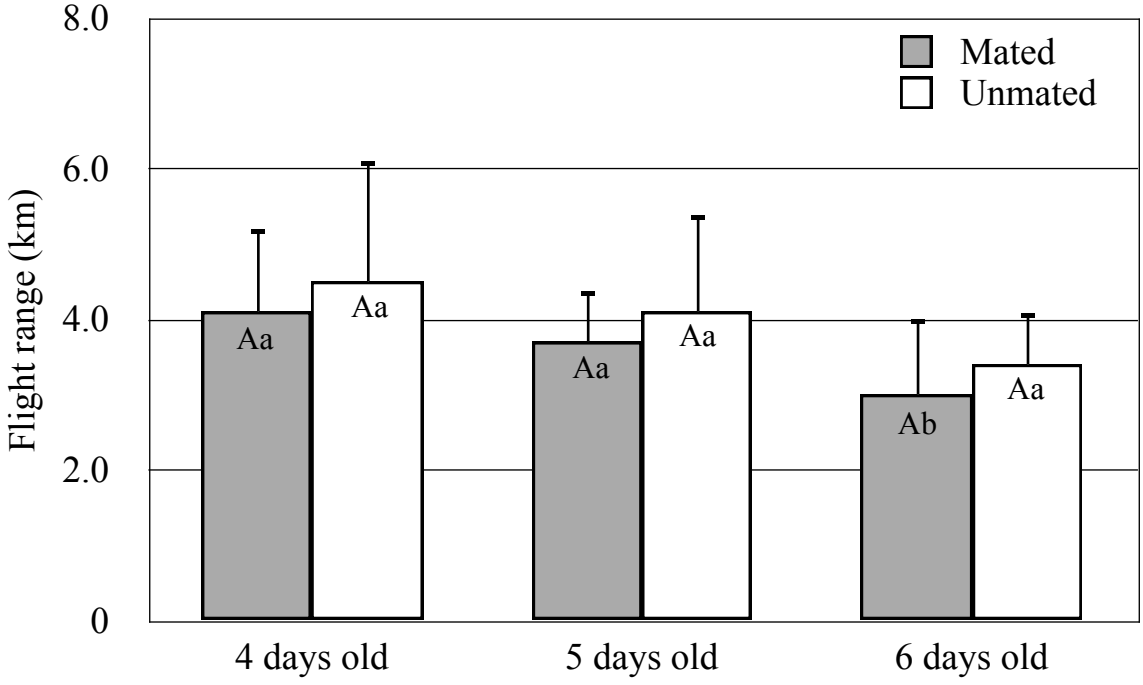

Fig. 2-5. Effect of mating status on the flight range of females 4 to 6 days old measured by flight mill (mean ± SD). Different uppercase letters denote significant difference between mated and unmated females by Mann-Whitney U-test ($P < 0.05$). Different lowercase letters denote significant difference between age groups by Steel-Dwass test ($P < 0.05$). Twenty-four individuals of each mating status and age were tested.

4-6 days old did not differ between mated and unmated individuals. I found no significant difference in the flight range of unmated females between each age. In contrast, 6-day-old mated females showed a significantly lower range compared to 4- and 5-day-old mated females.

2.5 Discussion

In this experiment, I utilized the actograph and the flight mill as means to evaluate the flight ability of *A. nigrisigna* females. In the actograoh assay, mated females 4 to 6 days old were significantly more active than unmated females of the same age. According to the mean number of sensor interruptions for each age, the activity of mated females were 2.8-3.6 times higher than unmated females. The same tendency was detected by Bloem et al. (2006), who reported that mated females of *Cydia pomonella* (Lepidoptera: Tortricidae) were significantly more mobile than virgin females. In addition, a significantly higher number of eggs were laid by mated females compared to unmated females inside the plastic container used for the actograph. The activity of mated females might be due to this oviposition behavior. Saito (2000) also mentioned the relationship between flight activity and oviposition behavior in his research on *Helicoverpa armigera* (Lepidoptera: Noctuidae), and indicated that the percentage of frequent flyers among the copulated females did not decrease and frequent flyer status was achieved based on the honey-sucking or ovipositing movement.

Although I suggested that the activity of mated females was the result of their oviposition behavior, I could not find a clear separation between mating status and flight duration or range in the flight mill experiments. In the flight mill assay, the mating status of females did not affect their mobility. This finding conforms with that of other studies such as Ishiguri and Shirai (2004), and Shirai (2006). Among the previously studied migratory lepidopterous insects, none of the species showed a clear temporal discrimination or negative correlation between reproductive and flight traits (Gatehouse and Zhang, 1995). *A. nigrisigna* appears to be included this theory.

From these experiments, mated females can fly a maximum of 6.3 km in a night. It was suggested that females maintain physical energy after mating at the same level as before mating and then mated females locomote more actively around the host plants for oviposition. I can guess easily that mated females move into the pheromone treated field for oviposition on cultivated vegetables. To block out their migration, the placement of trap plants around the pheromone treated area might be effective. However, there are few studies presented the effectiveness of trap plants with combination of the mating disruptant. Further investigations must be needed.

In this chapter, I considered the possibility that female *A. nigrisigna* adults migrate into the pheromone treated area and this could be one of the reasons for non-decreasing number of *A. nigrisgna*. From the experiments reported here, it

was suggested that this possibility might happen. In the next chapter, I made other hypothesis based on host selection of *A. nigrisigna* and verified it.

Chapter 3
Host plant selection by female adults and larvae

Oviposition and feeding preference of *A. nigrisigna*

3.1 Introduction

Development of effective strategies for IPM will require a knowledge of the biological interaction of insects and its host plants. An important aspect of such interactions is the host preference for female oviposition. Additionally, economic damage of crops is the result of larval feeding on the leaves. Consequently, this stage has been also focused on the host plant studies as well as female oviposition behavior (Ulmer et al., 2002).

The pheromone treated field where I investigated in 2008 and 2009 was surrounded by forests and agricultural areas which mostly cabbage (*Brassica oleracea* L.) and lettuce (*Lactuca sativa* L.) are cultivated by local farmars. Although both crops can become host plants for *A. nigrisigna*, emergence of their larvae in lettuce fields were frequently reported by farmers. Thus, I suggested that *A. nigrisigna* preferred lettuce to cabbage and this could be one of the reasons for the low effectiveness of the mating disruption for *A. nigrisigna*. To consider this possibility, I compared the oviposition and feeding preferences of *A. nigrisigna* on cabbage and lettuce.

3.2 Materials and methods

3.2.1 Oviposition preference

This experiment were carried out in Karuizawa, Nagano Prefecture, Japan (Lat: 36.31N, Long: 138.56E) during summer season in 2012. In 144m^2 of farmland, I planted 132 cabbage seedlings (cv. Greenball) and 192 lettuce seedlings (cv. Souther) on June 27th. Layout of experimental site was shown in Fig. 3-1. Four replicates of each crop were set up. Each replicate was composed of three rows. In each row, 11 cabbage seedlings and 16 lettuce seedlings were planted. The distance between seedlings was 27cm for lettuce and 40cm for cabbage. No insecticides were applied until the investigations were finished. The need for fungicides against gray mold and stem rot disease was decided independently by a licensed professional crop adviser. Field density of *A. nigrisigna* was estimated by counting the number of eggs, small larvae (1st, 2nd and 3rd instar), large larvae (4th and 5th instar) and pupae on each crop. To distinguish *A. nigrisigna* from other plusiine species, collected individuals were identified by observing larval setal plan (Ichinose, 1962) and applying molecular technique (Hashiyama et al., 2011). Field density investigations were carried out two times on July 16th and August 1st. On July 16th, I counted the number of *A. nigrisigna* eggs, larvae and pupae from all plants. On August 1st, five plants per row were randomly selected and counted the number of *A. nigrisigna* eggs, larvae and pupae. The larvae were divided into two categories based on their instars, i.e., small (1st,

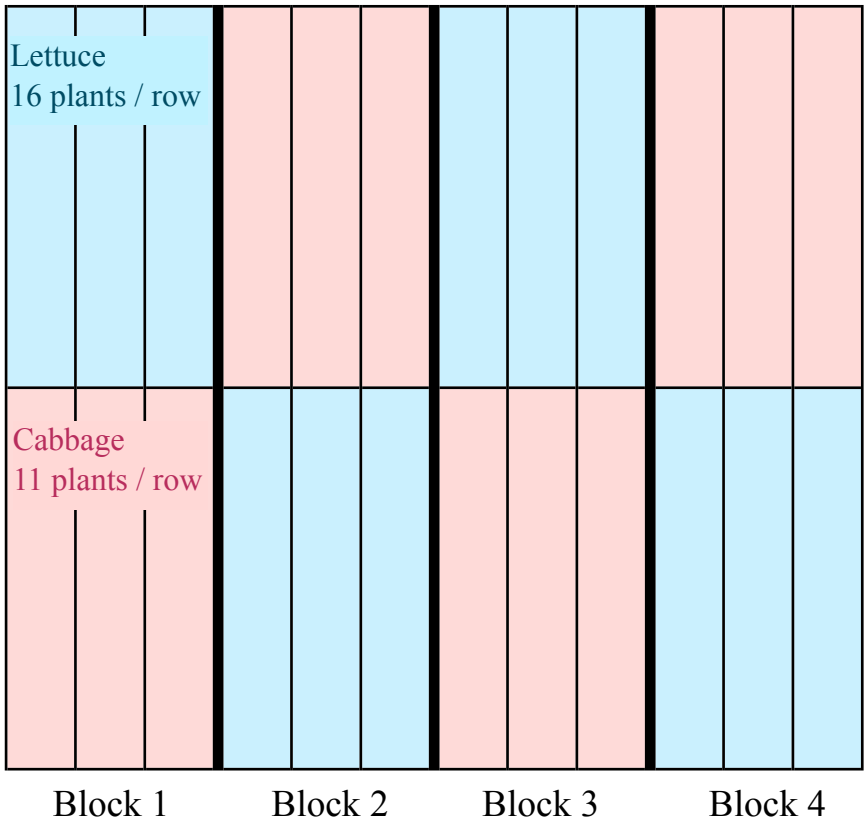

Fig. 3-1. Field layout of experimental site. The site consists of four blocks (four replicates). Each block involves 48 (16×3) lettuce plants and 33 (11×3) cabbage plants.

2nd and 3rd instar) or large (4th, 5th and 6th instar). A comparison of *A. nigrisigna* population between cabbage and lettuce was made with a two-tailed t-test by the software JMP release 4.0.3 (JMP Software, SAS Institute, Cary, NC).

3.2.2 Feeding preference

Time of development from eggs to adults was investigated on cabbage, lettuce and artificial diet (Kawasaki et al., 1987). Thirty replicates of each food were set up. Eggs were placed in plastic cups (Diameter 7 × Height 3.5cm) individually and sufficient amounts of foods were provided every day until pupation. As for cabbage and lettuce, I used same cultivars with field investigation described above. The larvae pupated on the walls of plastic cups. All procedures were conducted under a 16L-8D photoperiod at 20 ± 1ºC, 70 ± 10% RH. Larval period, pupal period, and sex-ratio were recorded and analyzed using ANOVA and Tukey's HSD test by using JMP release 4.0.3 (JMP Software, SAS Institute, Cary, NC).

3.3 Results

3.3.1 Oviposition preference

As for the field investigation on July 16th, populations of eggs and small larvae on cabbage were higher than lettuce (Table 3-1).The same tendency was also

Table 3-1. Mean ± SD number of *A. nigrisigna* eggs, larvae and pupae on cabbage and lettuce per 10 plants on July 16th, 2012.

Crop	Eggs	Small larvae	Large larvae	Pupae
Cabbage	25.68 ± 5.70*	7.96 ± 2.03*	0.08 ± 0.15	0.08 ± 0.15
Lettuce	3.39 ± 1.05	0.84 ± 0.61	0.00 ± 0.00	0.00 ± 0.00

* Significant difference within stages at the 0.1% level using two tailed t-test.

observed in the second investigation on August 1st (Table 3-2). Additionally, a significant difference could be detected in this term's large larvae. The number of pupae was very few and significant difference could not be detected in both two investigations.

3.3.2 Feeding preference

The insects took significantly longer to develop from eggs to pupation on artificial diet than cabbage and lettuce leaves (Table 3-3). Compared to the insects fed on artificial diet, larval period of the insects on cabbage and lettuce was increased by 4.2 days and 4.4 days, respectively. However, there were no significant differences between cabbage and lettuce leaves. And more, pupal period was not changed by the kind of food.

3.4 Discussion

In the field investigations of *A. nigrisigna*'s oviposition preference, I found a large number of eggs and early instar larvae on cabbage leaves (Table 3-1, 3-2). This result was very clear and the possibility "superiority of lettuce as host plant" was denied. However, lettuce has the potentials to become their main host plant because lettuce leaves were suitable for larval development at the same level of cabbage leaves(Table 3-3).

Table 3-2. Mean ± SD number of *A. nigrisigna* eggs, larvae and pupae on cabbage and lettuce per 15 plants on August 1st, 2012.

Crop	Eggs	Small larvae	Large larvae	Pupae
Cabbage	11.50 ± 4.93*	31.00 ± 7.48**	3.25 ± 0.50**	0.00 ± 0.00
Lettuce	1.25 ± 1.0	0.00 ± 0.00	0.00 ± 0.00	0.50 ± 1.00

*, ** Significant difference within stages at the 0.5 % and 0.1% level using two tailed t-test.

Table 3-3. Performance of *A. nigrisigna* on cabbage, lettuce and the artificial diet.

	Developmental period (days)						
Food	1st instar	2nd instar	3rd instar	4th instar	5th instar	Larval period	Pupal period
Cabbage	4.5 ± 0.1a	4.7 ± 0.2a	4.4 ± 0.1a	5.0 ± 0.2a	8.0 ± 0.2a	26.5 ± 0.4a	16.7 ± 0.1a
Lettuce	4.4 ± 0.1a	4.6 ± 0.2a	4.3 ± 0.1a	4.9 ± 0.2a	8.2 ± 0.2a	26.3 ± 0.3a	16.3 ± 0.3a
Artificial diet (Kawasaki et al., 1987)	6.3 ± 0.1b	3.7 ± 0.2b	5.5 ± 0.1b	5.5 ± 0.2b	9.8 ± 0.5b	30.7 ± 0.5b	16.6 ± 0.2a

Mean ± SEM followed by different letters within instars differed significantly ($P<0.05$) by using ANOVA and Tukey's HSD test for larval period, pupal period.

As for the relationship of oviposition and feeding preference, Chow et al. (2005) showed an interesting finding. They suggested that cabbage looper, *Trichoplusia ni* (Lepidoptera: Noctuidae) may be acquiring oviposition preferences from larval feeding experience. In their experiment, three groups of *T. ni* were used: naive, experienced and the offspring (F_1) of experienced. The experienced insects were reared on an artificial diet treated with a latex from *Hoodia gordonii*, South African milkweed (Asclepiadaceae). Naive and the offspring of experienced insects were reared on untreated diet. After eclosion, male and female pairs moths were maintained with a cabbage leaf treated with *H. gordonii* latex and an untreated cabbage leaf. The results showed that naive groups of *T. ni* moths are deterred to feed and oviposit on cabbage leaves with *H. gordonii* latex, and that larval feeding experience can lessen or reverse this deterrence. The offspring of experienced moths were deterred from laying eggs on treated leaves, unlike the response of their experienced parents. The reduced feeding deterrence exhibited by experienced larvae can be explained by habituation (Akhtar et al., 2003). This mechanism, the change in oviposition preference resulting from larval experience, may give some indication of how host plant range expansion might occur in the polyphagous insects like *T. ni* and also *A. nigrisigna*.

In this chapter, I considered the possibility that *A. nigrisigna* preferred lettuce to cabbage. From my investigations mentioned above, it is suggested that this possibility might not happen. As the reasons for non-decreasing number of

A. nigrisgna in the pheromone treated field, I made other possibilities and verified them in next chapter.

Chapter 4
Weak response caused by the absence of minor components in Confuser V®

The effect of mating disruptant specialized for *A. nigrisigna*

4.1 Introduction

The components of *A. nigrisigna* sex pheromones are identified as a 100: 62: 4: 2 blend of (Z)-7-Dodecenyl acetate (Z7-12Ac), (Z)-7-Dodecen-1-ol (Z7-12OH), (Z)-7-Tetradecenyl acetate (Z7-14Ac) and (Z)-5-Dodecenyl acetate (Z5-12Ac) (Sugie et al., 1991). From the ratio and the field trials by Sugie et al. (1991), it is considered that Z7-12Ac and Z7-12 OH are major components, and Z7-14Ac and Z5-12Ac are minor components for the attraction of *A. nigrisigna*. Of these components, Z7-12Ac and Z7-12OH are contained in Confuser V® as a lure for *A. nigrisigna* (Table 4-1). Its ratio is 100: 71, which is approximately equal to that of natural sex pheromone produced by *A. nigrisigna* females. Minks and Cardé (1988) suggested that the natural pheromone blend should be the best disruptant at the lowest application rate because it would probably elicit the greatest number of disruptive mechanisms (Bartell, 1982; Cardé, 1990). I sought to test this hypothesis on *A. nigrisigna.* According to this hypothesis, the synthetic chemicals used in a mating disruption should be a natural blend of target pest, because sex pheromones in lepidopterans are species specific. Thus,

I suggested that absence of minor components in Confuser V® could be one of the reasons for the low effectiveness of Confuser V®. To consider this possibility, I prepared new type of mating disruptant which contains all four pheromone components of *A. nigrisigna* and compared the effects to Confuser V®. The effects were estimated from three perspectives; (1) mate location by male moths, (2) field density of immature stages, and (3) mating frequency by female moths.

4.2 Materials

4.2.1 Pheromone dispenser

For the field study, I prepared two types of the mating disruptant, major-components and four-components (Table 4-1). Major-components disruptant contains only Z7-12Ac and Z7-12OH, totaling 41mg per dispenser. These were major sex pheromone components of *A. nigrisigna* females and its ratio is coincident with Confuser V®. Four-components disruptant contains all of *A. nigrisigna* sex pheromone components, i.e., Z7-12Ac, Z7-12OH, Z7-14Ac and Z5-12Ac. Total amount is 41mg per dispenser and its ratio is same as natural sex pheromone. All kinds of disruptants are 20cm long dispenser, consisting of polyethylene tubes with an aluminium wire. Synthetic sex pheromones diffuse through the polyethylene layer and permeate from the surface into the air. These dispensers were supplied by Shin-Etsu Chemical Co., Ltd.

Table 4-1. Components and target pests of three mating disruptants, Confuser V®, Major components and Four components.

Disruptant	Confuser V®	Major components	Four components
Component	(Z)-11-Hexadecenal	(Z)-7-Dodecenyl acetate	(Z)-7-Dodecenyl acetate
	(Z,E)-9,11-Tetradecadienyl acetate	(Z)-7-Dodecen-1-ol	(Z)-7-Dodecen-1-ol
	(Z)-11-Hexadecenil acetate		(Z)-7-Tetradecenyl acetate
	(Z,E)-9,12-Tetradecadienyl acetate		(Z)-5-Dodecenyl acetate
	(Z)-7-Dodecenyl acetate		
	(Z)−9−Tetradecen−1-ol		
	(Z)-7-Dodecen-1-ol		
	(Z)−9−Hexadecenal		
	(Z)-11-Hexadecene-1-ol		
Target pest	*Plutella xylostella*	*Autographa nigrisigna*	*Autographa nigrisigna*
	Helicoverpa armigera		
	Spodoptera exigua		
	Mamestra brassicae		
	Autographa nigrisigna		
	Trichoplusia ni		
	Spodoptera litura		

Three mating disruptants share same components and target pest (red underlines).

4.2.2 Experimental site

The experimental site used in this study is located in Karuizawa, Nagano Prefecture, Japan (N36°19′55.2″ E138°35′24″). A total 10.2 ha farmland was divide into three areas (Fig. 4-1). Two areas were treated with major-components (treated-M area) and four-components (treated-F area), respectively. Treated-M and treated-F area were 4.4 and 3.8ha. The remaining 2.9ha area is control area. The experimental site was surrounded by forests, public roads and agricultural areas which mostly cabbage and lettuce are cultivated by local farmars. These agricultural areas were not treated with any mating disruptant, but insecticides were applied as conventional chemical control.

4.2.3 Lettuce

To determine the population density of *A. nigrisigna* on lettuce cultivation, I planted lettuce in the experimental sites. I used major crisphead lettuce varieties in Nagano Prefecture, var. Souther (Takii Seed Ltd., Kyoto, Japan).

4.2.4 Pheromone trap

To determine whether male moths locate a pheromone point source in the pheromone treated and the untreated areas, pheromone traps for *A. nigirisigna* were placed in each area. I used sticky-type traps (Width 27 × Length 30cm

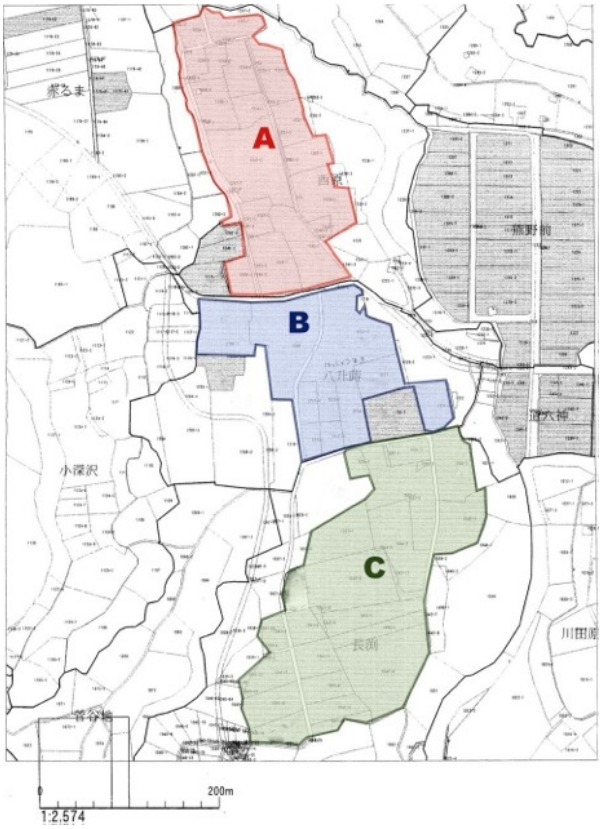

Fig. 4-1. Field layout of three areas in the experimental site. (A) Treated-F area. (B) Control area. (C) Treated-M area.

bottom plate with a roof; Shin-Etsu Chemical Co., Ltd.). Each trap was baited with a rubber septum impregnated with synthetic pheromone components as a lure (Shin-Etsu Chemical Co., Ltd.) (Fig. 4-2).

4.2.5 Mating table

To determine whether mating disruptants can confuse males and prevent the mating with females, the examination of mating rate was carried out. For this survey, I prepared the mating tables constructed from white plastic trays (Height 15 × Width 39 × Length 52cm) (Fig. 4-3). The table was attached to the container (Height 30 × Width 25 × Length 40cm) by the plastic rope. To prevent from climbing out of the table and running away, a fluoropolymer resin were painted onto the upper half of walls. After it has dried, it forms a slick barrier that prevents moths from obtaining a foothold. Sometimes this product is sold as 'Fluon'. On the lower half of the wall, packing tape were wrapped. A cylinder (Diameter 8 × Height 18cm) made of fine mesh hardware cloth and capped with a white styrofoam plate (Diameter 20 cm) was positioned in the center of table to provide shade and protection of the moth from attacking of birds. For the construction of mating tables, I referred to the report by Mitchell et al. (1997).

4.3 Methods

4.3.1 Pheromone treatment

Fig. 4-2. Pheromone trap baited with a rubber septum (inside).

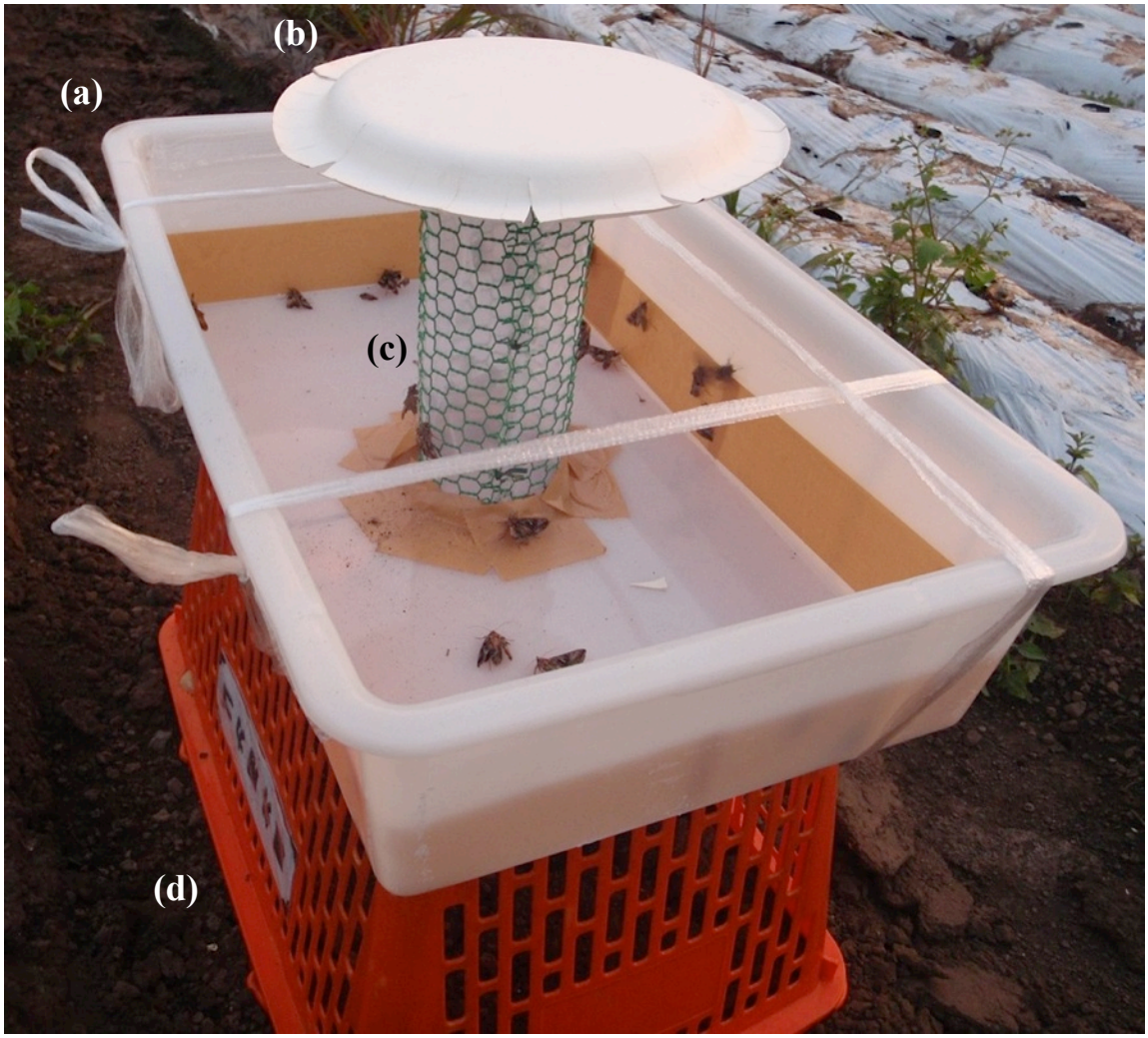

Fig. 4-3. Structure of the mating table used in this study.
(a) Plastic tray. (b) Styrofoam plate. (c) Cylinder. (d) Container.

To prevent the pheromone dispensers from coming into contact with the ground or the plants, they were attached to 70cm long sticks, by twisting two pheromone dispensers onto the top of a stick (Fig. 4-4) inserted 10cm into the soil. The sticks with dispensers were uniformly distributed in the fields at a rate of 100 dispensers per 0.1ha on June 15th, 2010.

4.3.2 Cultivation of lettuce

From June to September in 2010, I cultivated lettuce for three terms in each area. Two hundred and eighty eight lettuce seedlings were planted in 64m^2. Fifty five cm wide beds were set up and one seed line was created per bed. No insecticides were applied until the surveys were finished. The need for fungicides spraying against gray mold and stem rot disease was decided independently by a licensed professional crop adviser.

4.3.3 Placement of pheromone traps

Pheromone traps of *A. nigrisigna* and *T. ni* were placed in each field on June 15th, 2010. *T. ni* is one of the plusiine species (same subfamily as *A. nigrisigna*) and one of the target species of Confuser V®. The lures were replaced every month. The number of captured males were counted every seven days throughout the cropping season (from June 22nd to September 29th).

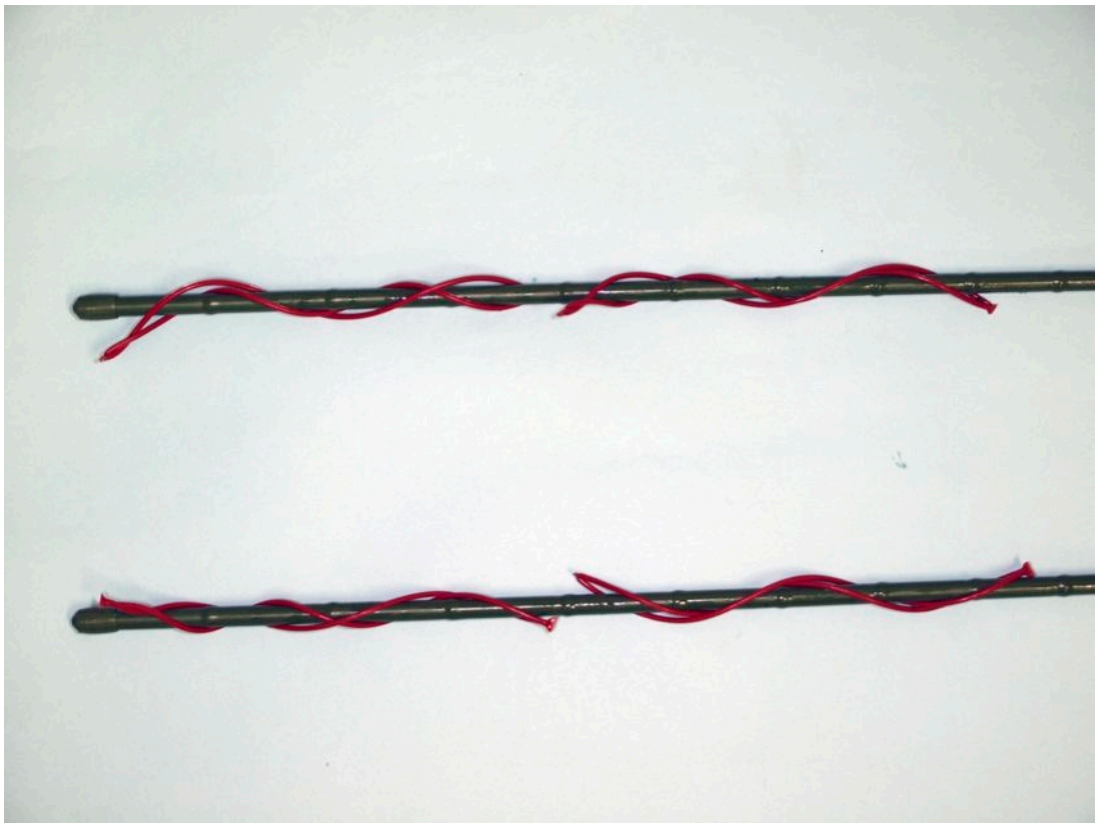

Fig. 4-4. Pheromone dispensers attached to a stick.

4.3.4 Survey of field population

Date

The survey schedule in this study were shown in Table 4-2. The survey was conducted during the harvest period (35-40 days after planting) in each cropping season.

Collecting of insects

I selected 100 from 288 plants of each area and collected eggs, larvae and pupae of plusiine species. After collecting, I brought back individuals to the laboratory and reared them until they emerged. Because plusiine species have morphological similarities among the immature stages, I waited for their emergence and identified species in adult stage.

Rearing of collected insects

Eggs and larvae were placed in petridishes (Diameter 9 × Height 1.5cm) individually. Larvae were reared on an artificial diet (Kawasaki et al., 1987). Pupae were placed in plastic cups (Diameter 7 × Height 3.5cm). All procedures were conducted under a 16L-8D photoperiod at 25 ± 1ºC.

Identification of collected insects

Individuals that emerged successfully were identified by colors and pigmentations of wings (Ichinose, 1962). Individuals that died before emergence

Table 4-2. Schedule of the field surveys in 2010.

	Cropping season		
Month Day	First term	Second term	Third term
June 10th	Planting		
June 15th	Installation of mating disruptants		
July 1st		Planting	
July 23rd	Survey		Planting
August 4th		Survey	
August 23rd			Survey

were identified by observing larval setal plan (Ichinose, 1962) and applying molecular technique (Hashiyama et al., 2011).

4.3.5 Placement of mating tables

To verify the mating rate of females on the table, two mating tables were positioned 50cm above the ground in each area. Newly emerged females of laboratory colony were held under ambient light at 20 ± 1 ºC and were fed a 10% honey-water solution. Twenty virgin females of 2-3 days old were placed on each mating table 2-3 hours before sunset (Fig. 4-5). One forewing of each moth was clipped to prevent escape by flight. The moths were collected in the following morning and returned to the laboratory where they were dissected to establish mating status as determined by the presence or absence of a spermatophore in the bursa copulatrix. A comparison of mating rates between pheromone treated and untreated area were made with χ^2 test by the software JMP release 4.0.3 (JMP Software, SAS Institute, Cary, NC).

4.4 Results

4.4.1 Mate location by male moths

Pheromone trap is one of the common tools to evaluate the effects of mating disruption. If mating disruption operates properly, it will prevent moths from locating traps. Trap catches of males from June to September were shown in Fig.

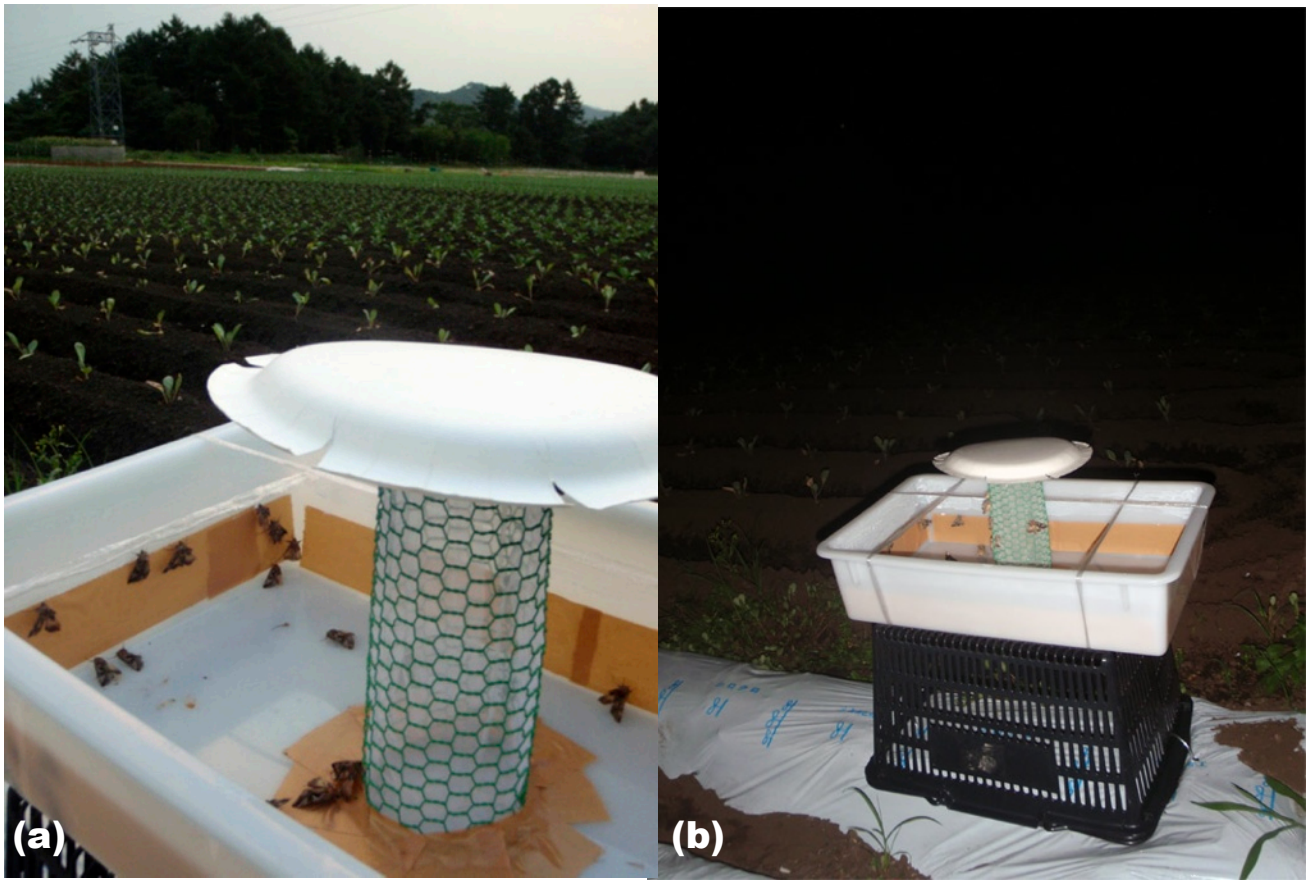

Fig. 4-5. Mating table placed in the experimental site. (a) Three hours before sunset. (b) Three hours after sunset.

4-6 and 4-7. For reference, trap catches in the area treated with Confuser V® were also showed in Fig. 4-6. Confuser V® area is in Karuizawa and 5km northeast from the experimental site used in this study. *A. nigrisigna* male adults were captured in control area throughout the investigation period (Fig. 4-6). Conversely, trap catches in Confuser V®, Treated-F and Treated-M area were reduced in the number considerably. However, some males were captured in these pheromone treated areas. In total, 26 males were caught in Confuser V® area from June to September. Such males were also captured in Treated-F and Treated-M area. Three and two males were captured in Treated-F and Treated-M area, respectively. These males were trapped during August and September. Trap counts of *T. ni* in the pheromone treated areas were lower than control area (Fig. 4-7). However, a few males were captured in both Treated-F and Treated-M areas.

4.4.2 Population density of immature stages

Throughout the three cropping seasons, Treated-F area maintained the smallest populations of *A. nigrisigna* (Table 4-3). In the first and third term, population density of Treated-M area was lower than control area. In the second term, same number of *A. nigrisigna* immature stages were detected in Treated-M and control area. Any larvae of *T. ni* were not detected in the pheromone treated and control area.

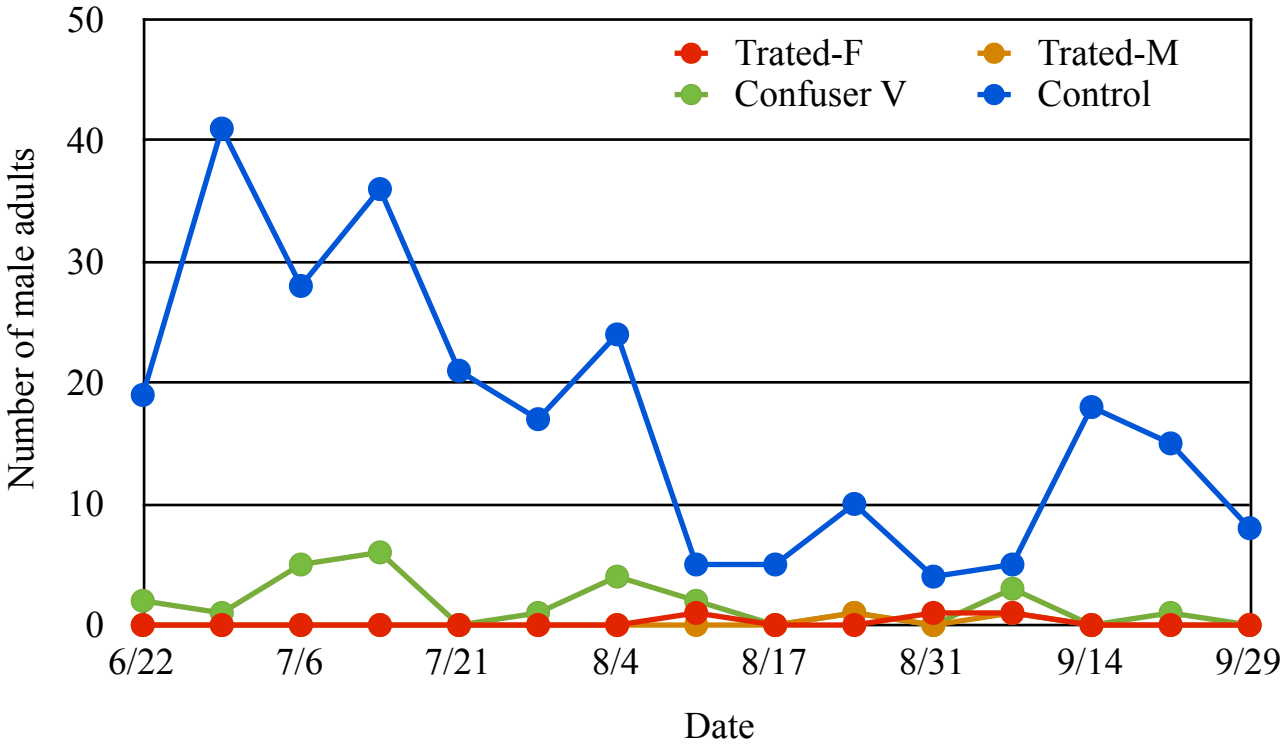

Fig. 4-6. Number of *A. nigrisigna* males captured by sex pheromone trap after installation of mating disruptants.

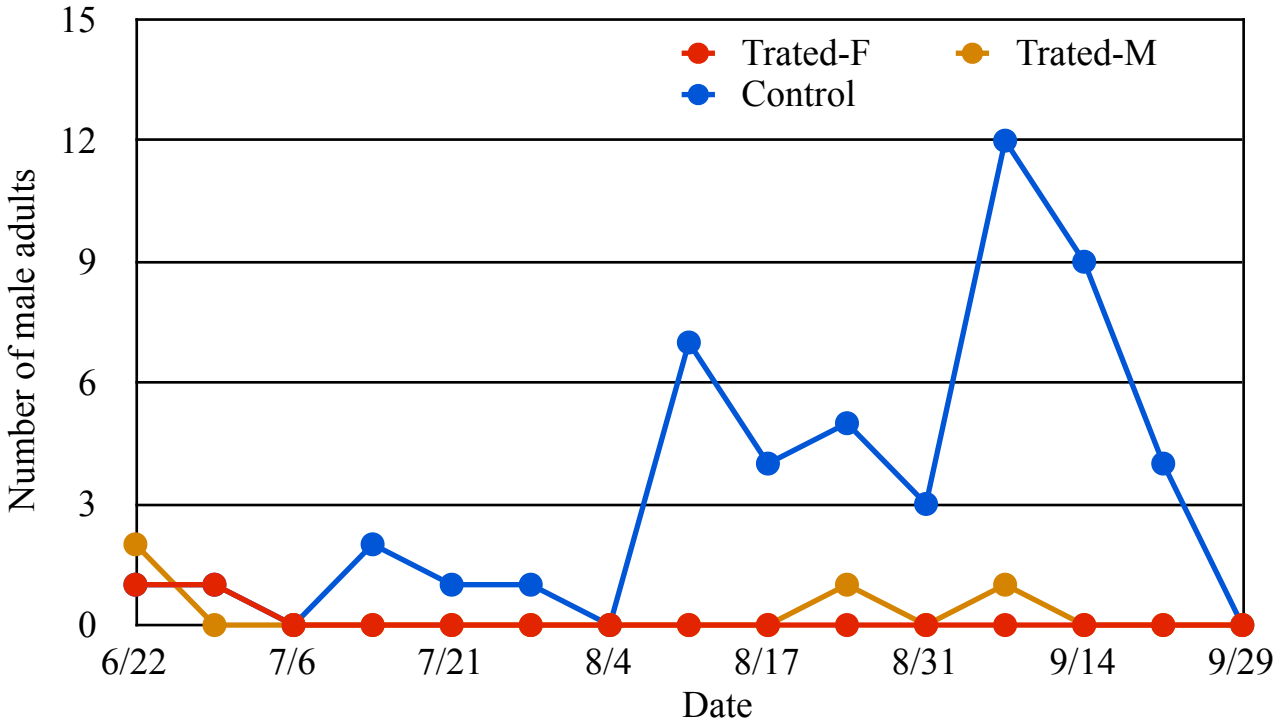

Fig. 4-7. Number of *T. ni* males captured by sex pheromone trap after installation of mating disruptants.

Table 4-3. Population density of *A. nigrisigna* immature stages (eggs, larvae and pupae) per 10 plants in pheromone treated and untreated field.

	Cropping season		
Area	First term	Second term	Third term
Treated-F	0.5	0	0.1
Treated-M	3.5	1.0	1.0
Control	3.8	1.0	1.5

4.4.3 Mating frequency by female moths

Although most females in control area got a chance to mate, any females in Treated-F area could not mate with males (Table 4-4). The mating rate in Treated-F area was significantly lower than Treated-M and control area. In Treated-M area, 30% of females reached mating in spite of the pheromone treatment.

4.5 Discussion

Trap data showed that both major and four components were effective equally in *A. nigrisigna* male adults from finding a point of pheromone source. This results suggested that mating within treated-M and treated-F area might be disrupted. Confuser V® was also able to confuse male moths, but some males were trapped constantly. The number of trapped males in the Confuser V® treated area was larger than treated-M area. This fact suggested that the components for other lepidopteran species in Confuser V® influenced the attractiveness of *A. nigrisigna* males. In Confuser V®, nine pheromone components were contained for six target pest species (Table 4-1). Of these components, Z7-12Ac and Z7-12OH are for *A. nigrisigna* and other seven components are for other lepidopterans (Table 4-1). The ratio and amount of Z7-12Ac and Z7-12OH in Confuser V® were same as Major components disruptant. Thus, the increased number of trapped males in the Confuser V® treated area might be resulted from

Table 4-4. Mating rate of *A. nigrisigna* females set out in pheromone treated and untreated field.

Area	Number of examined females	Mated	Unmated	Escape	Mating rate (%)
Treated-F	44	0	44	0	0 a
Treated-M	44	13	30	1	30.2 b
Control	44	35	7	2	83.3 c

Values followed by different letters are significantly different (χ^2 test, $p<0.01$).

the presence of other seven pheromone components in Confuser V®. This possibility was examined in the chapter 5.

Population density of *A. nigrisigna* was reduced by application of mating disruptant (Table 4-3). Treated-F area indicated the lowest population throughout the three cropping seasons. And more, mating rate of females in Treated-F area was significantly lower than Treated-M area (Table 4-4). Although Major components disruptant could prevent 70% of the mating activity of *A. nigrisigna* females, the remaining 30% of the females could complete the mating activity and produce offspring. These findings might suggest that four pheromone component blend was more effective than major pheromone component only.

Trichoplusia ni is one of the target pests of Confuser V® and shares one pheromone component (Z7-12Ac) with *A. nigrisigna.* As expected, trap catches of *T. ni* were reduced by the use of pheromone including Z7-12Ac (Fig. 4-7). Eggs, larvae and pupae of *T. ni* could not detected in any areas. This might be because *T. ni* is basically a tropical or subtropical insect and Nagano prefecture is one of the limited population area. I need other experimental site where the population is high enough to assess the effect of mating disruption.

While mating disruption technique can be very effective, how it works is still not understood in detail. The mechanism of mating disruption varies according to species. The potential mechanism include: (1) sensory adaptation of the olfactory sensory neurons (2) false-trail following (competition between natural and synthetic sources) (3) camouflage of natural plumes by ubiquitous

high levels of synthetic pheromone (4) imbalance in sensory input by massive release of a partial pheromone blend (5) the effects of pheromone antagonists and mimics (Cardé and Minks, 1995; Sanders, 1997). These five hypothesis have been proposed as the mechanisms of mating disruption. Analysis of 30 years of dosage–response profiles for moth mating disruption (Miller et al., 2006) documented that competitive attraction was involved in 11 of 13 available cases. Remaining cases were noncompetitive disruption such as camouflage of traps and females by pheromone dispensers and desensitization of responder sensory systems without first requiring attraction (Miller et al., 2006).

It has been widely assumed that a natural-type blend of sex pheromone components, which usually showed optimum attraction activity, has been selected to disrupt the mating behavior of male moths in many cases (Cardé & Minks, 1995). However, there is only limited evidence supporting this assumption (Minks and Cardé, 1988). As one of the handful cases, Trimble and Marshall (2008) showed the attractiveness of incomplete and complete blends of *Choristoneura rosaceana* (Lepidoptera: Tortricidae) synthetic pheromone by using a flight tunnel and field trapping experiments. In the fight tunnel, there was an increase in the proportion of moths that were activated when minor component was combined with the main component. The time required for activation was twice longer when using the major component than when using the major component and minor component. In the field experiment, the major and minor component blend was 17-55 times more attractive than the main

component only. On the other hand, Fitzpatrick et al. (1995) demonstrated that the major pheromone component disrupted mating as effectively as a blend close to the natural pheromone blend. Other researchers showed that off-blend pheromone components whose ratio was different from natural pheromone were effective for mating disruption (Minks and Cardé, 1988; Bengtsson et al., 1994; Evenden et al., 1999; McCormick et al., 2012). Whether the complete pheromone blend is required to achieve habituation and subsequent reduction in response to a pheromone source appears to vary among species and correlate with the mechanisms of mating disruption. The five mechanisms mentioned above may act individually or in combination, and may vary according to pheromone blend and dispenser density, and in relation to the pheromone-mediated behavior of a target species and its population biology (Bengtsson et al., 1994; Knight, 2007; Jones et al., 2008). It is unknown that which mechanisms contribute to mating disruption for *A. nigrisigna.*

In this chapter, I considered the possibility that absence of minor components in mating disruptant could be one of the reasons for the low effectiveness of the mating disruption. From the results reported here, it was revealed that full pheromone component blend was more effective for reducing mating rate and field population than partial pheromone component blend. To reveal the importance of minor components, I need to show the clear data of antennal response in GC-EAD and attractiveness in a wind tunnel.

Chapter 5
False response of males to other lepidopteran pheromones

Inhibition of response in *A. nigrisigna* males by the pheromones of sympatric insects

5.1 Introduction

While there are many examples of species discrimination by odour by vertebrates, for example in salamanders (Dawley, 1987), moths provide the best understood examples. In the chemical communication of moths, sex pheromones are generally crucial for ensuring reproductive isolation. In many cases, the precise blend produced by females is responded to by conspecific males. Species-specific pheromone blends not only facilitate mate location by conspecifics, but may also inhibit responses by heterospecific adults, serving as a reproductive isolating mechanism and reducing costly mating mistakes. For example, Eizaguirre et al. (2007) found that the two principal components of the European corn borer, *Ostrinia nubilalis* (Lepidoptera: Crambidae) pheromone inhibited the response of the *Sesamia nonagrioides* (Lepidoptera: Noctuidae) males to their conspecific pheromone. Similarly, when one component of the *S. nonagrioides* pheromone was added to the *O. nubilalis* pheromone, it inhibited the response of *O. nubilalis* males. Males have specialized olfactory sensilla for

these ‘behavioral antagonists’-key components that distinguish the blends of other species similar to their own- and they turn back if they detect antagonists. A responding male would benefit from early rejection of a female not from his species. Males would then save the energy cost of flight and predation risks to find a mating partner.

Although Z7-12Ac and Z7-12OH are contained in Confuser V® as a *A. nigrisigna* lure, these amounts loaded in Confuser V® are very small (3.5 and 2.5%, respectively). Other three components, (Z)-11-Hexadecenal (Z11-16Al), (Z,E)-9,11-Tetradecadienyl acetate (Z9E11-14Ac) and (Z)-11-Hexadecenil acetate (Z11-16Ac), account for over half percentage of Confuser V® (19.0, 16.0 and 15.5%, respectively). These main components are for other lepidopteran pest species that share the habitat and host plant with *A. nigrisigna*. Inhibition by sex pheromone of sympatric lepidopteran species were already reported by some researchers (e.g. Hendrikse, 1986; Eizaguirre et al., 2009). Therefore, the large amount of pheromone components for other lepidopteran pest species may affect mating disruption of *A. nigrisigna* males and contribute to ineffectiveness of Confuser V®. To consider this possibility, I undertook both laboratory and field studies. The effects of other lepidopteran pheromones to *A. nigirisigna* were estimated from three perspectives; (1) GC-MS/EAD analysis, (2) field trap test, and (3) mating disruption test.

5.2 Materials and Methods

5.2.1 GC-MS/EAD analysis

By using electroantennography technique, I sought to determine which pheromone components in Confuser V® led to antennal response of *A. nigrisigna* males.

Insects

A. nigrisigna adults used in GC-EAD analysis were the second to fifth offspring generation obtained from the lettuce field in Karuizawa, Nagano Prefecture, Japan in June 2010.

Chemicals

In this experiment, diluted Confuser V® solution exposed to *A. nigrisigna* male antennae. To obtain diluted Confuser V® solution, 1$\mu\ell$ of the content of Confuser V® dispenser was dilute by adding 10ml distilled water.

GC-MS/EAD device

GC-MS/EAD is gas chromatograph mass spectrometer coupled with electroantennographic detecter. It is an analytical device that permits the rapid identification of components in complex mixtures that stimulate the olfactory sensilla of an insect. This information can be used to discover potentially useful components that alter the behavior of insects. In this study, I used an apparatus, 7890GC/ 5975MSD (Agilent Technologies Japan Ltd., Tokyo, Japan) equipped

with a HP-5MS column (Agilent Technologies Japan Ltd.). This device can record GC, MS and EAD trace at a time. Diluted Confuser V® solution, 1μℓ were applied to the injector and FID and EAD responses were recorded. The oven temperature was 80°C, 10°C/min to 190°C, 20°C/min to 230°C, and then it was held for 10min. For other settings, I referred to Struble and Arn (1984).

A. nigrisigna male antenna is 9mm long and 0.22mm wide at the base. In antennal preparations, live insects were used. Two day-old males were taken out of the rearing chamber during the scotophase shortly before being tested. They were immobilised with CO_2 for 30 seconds. The antenna was severed at the base and one or two segments from the tip were also cut off. The excised antenna was then fixed between two glass electrodes. Short coaxial cables connected the electrodes to a high impedance DC amplifier, the amplified signal (100×) was filtered and fed into an oscilloscope. The treatment was replicated on 10 antennae using 10 different individuals.

5.2.2 Field trap test

In GC-EAD analysis, two components, Z11-16Al and Z9E11-14Ac, induced antennal stimulation of *A. nigrisigna* males. To determine the effect of these pheromone components, four types of lure were created and examined in the field.

Experimental site

This experiment was carried out at 120m^2 cabbage farmland in Yagiri area, Chiba Prefecture, Japan (N35°76′82″ E139°89′20″). The farmland was surrounded by agricultural areas which several vegetables (cabbage, broccoli and green onion) are cultivated by local farmars. The agricultural areas were not treated with any pheromones, but insecticides were applied as conventional chemical control.

Pheromone trap and lure

For the field test, sticky-type traps (Width 27 × Length 30cm bottom plate with a roof; Shin-Etsu Chemical Co., Ltd.) were prepared. Each trap was baited with rubber septum impregnated with synthetic pheromone components as a lure. In this study, four types of lure were created. The number of males attracted by each type of lure was investigated.

(A) Treatment with *A. nigrisigna* pheromone components only (1063.6μg).

(B) Treatment with Z11-16Al (3497.2μg) and *A. nigrisigna* pheromone components (1063.6μg).

(C) Treatment with Z9E11-14Ac (3036.0μg) and *A. nigrisigna* pheromone components (1063.6μg).

(D) Treatment with Z11-16Al (3497.2μg), Z9E11-14Ac (3036.0μg) and *A. nigrisigna* pheromone components (1063.6μg).

The ratio of Z11-16Al and Z9E11-14Ac added in lures conformed to the ratios in Confuser V®. The rubber septa lure (Shin-Etsu Chemical Co., Ltd.) received the treatment dissolved in hexane (1000$\mu\ell$) and left to dry for over 30 minutes in a laboratory hood. The septa of each treatment were put in zip-lock aluminum foil bags and stored until used. Trap catches were recorded during the summers of 2011. Treatments were tested in a randomized block design of four blocks and traps in each block were rotated weekly. The number of *A. nigrisigna* males caught in the traps were recorded once a week from July 4 to August 8 in 2011. Lures were not replaced during this period. Three replicates were created for each type of lure. The influence of the different treatments on catches was analysed by two-way ANOVA and Tukey-Kramer's HSD test. This statistical analysis were performed with transformed data (square root [x + 1]).

5.2.3 Mating disruption test

In order to determine the effect of pheromone components that led to antennal response in GC-EAD on mating disruption of *A. nigrisigna* males, I conducted laboratory experiment and calculated mating disruption rate.

Insects

A. nigrisigna adults used in GC-EAD analysis were the second to fifth offspring generation obtained from the lettuce field in Karuizawa, Nagano Prefecture, Japan in June 2010.

Mating cage

Cuboid shaped cages (Height 90 × Width 60 × Length 60cm) were constructed of a steel flame and covered with nylon mesh (Fig. 5-1). For the construction of mating tables, I referred to the report by Zhu et al. (1997). Two mating cages and two equal-sized experimental rooms (Height 3 × Width 5 × Length 5m) were prepared. Each mating cage was placed in the center of each room under a natural photoperiod at 23 ± 5°C.

Dispenser

To determine the effect of Z11-16Al and Z9E11-14Ac to mating disruption of *A. nigrisigna*, two types of treatments were examined in the mating cage.

(A) Treatment with *A. nigrisigna* pheromone components only (1063.6μg).

(B) Treatment with Z11-16Al (3497.2μg), Z9E11-14Ac (3036.0μg) and *A. nigrisigna* pheromone components (1063.6μg).

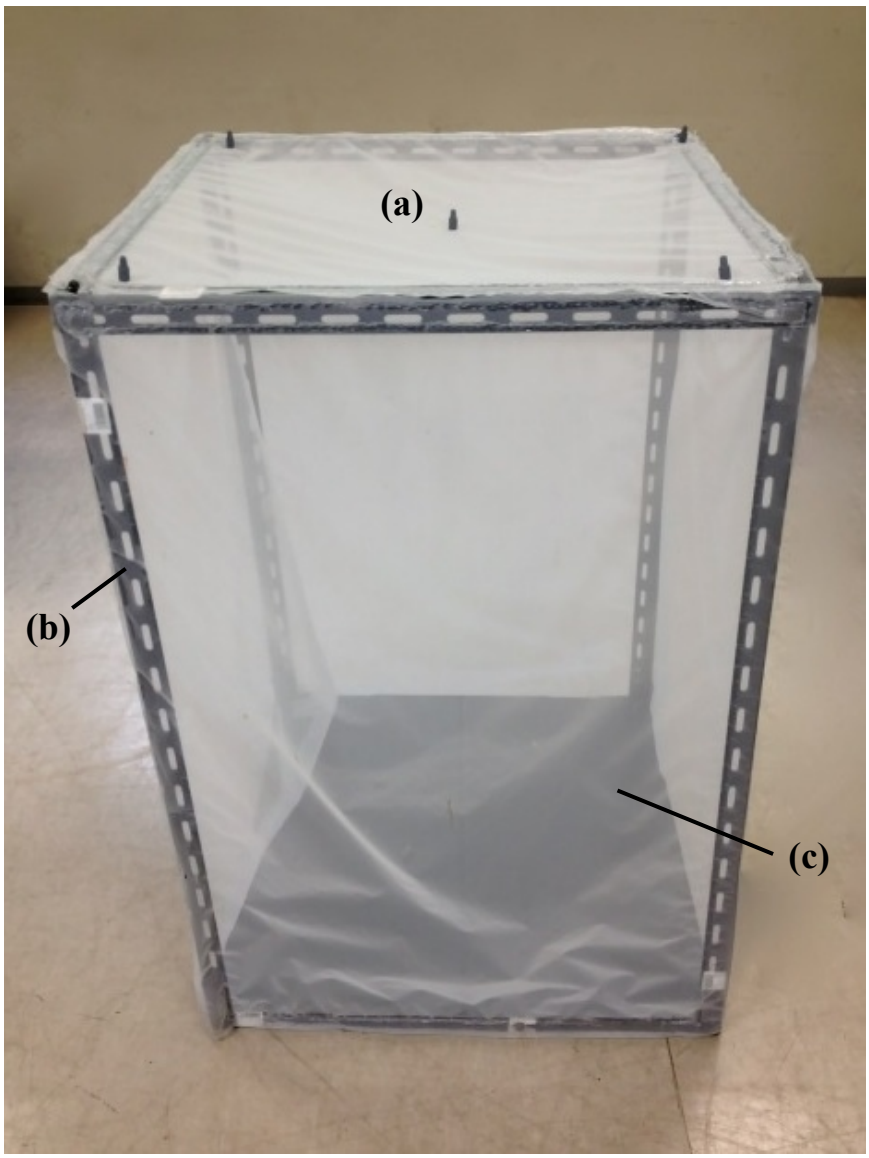

Fig. 5-1. Structure of the mating cage used in this study. (a) Dispenser. (b) Steel flame. (c) Nylon mesh.

The rubber septa dispenser (Shin-Etsu Chemical Co., Ltd.) received the treatment dissolved in hexane (1000$\mu\ell$) and left to dry for over 30min in a laboratory hood. The septa of each treatment were put in zip-lock aluminum foil bags and stored until used. Each treatment were conducted in each mating cage. Around the cage, ten treated rubber septa were attached. In the center of mating cage, the plastic cup (Diameter 7 × Height 3.5cm) filled with 3% honey solution were set. After setting dispensers and plastic cup, 10 males and 10 females were released into the cage. All adults were released at dusk and caught in the third morning after release. The females were dissected under a microscope to determine whether mating took place and calculate the mating rate. The control cage was similarly treated except that septa were loaded with 100$\mu\ell$ hexane alone. Five replicates were created for each treatment and new septa were used for each replicate. The influence of the different treatments on mating rates was analysed by two-way ANOVA and Tukey-Kramer's HSD test.

5.3 Results

5.3.1 GC-MS/EAD analysis

GC-MS analysis of diluted Confuser V® solution detected and eight components (Fig. 5-2). Based on the retention index and analyses of synthetic standars, components (a) ~ (h) were hypothesized to be Z7-12OH, Z7-12Ac, (Z)-9-Tetradecen-1-ol (Z9-14OH), (Z)-9-Hexadecenal (Z9-16Al), Z11-16Al,

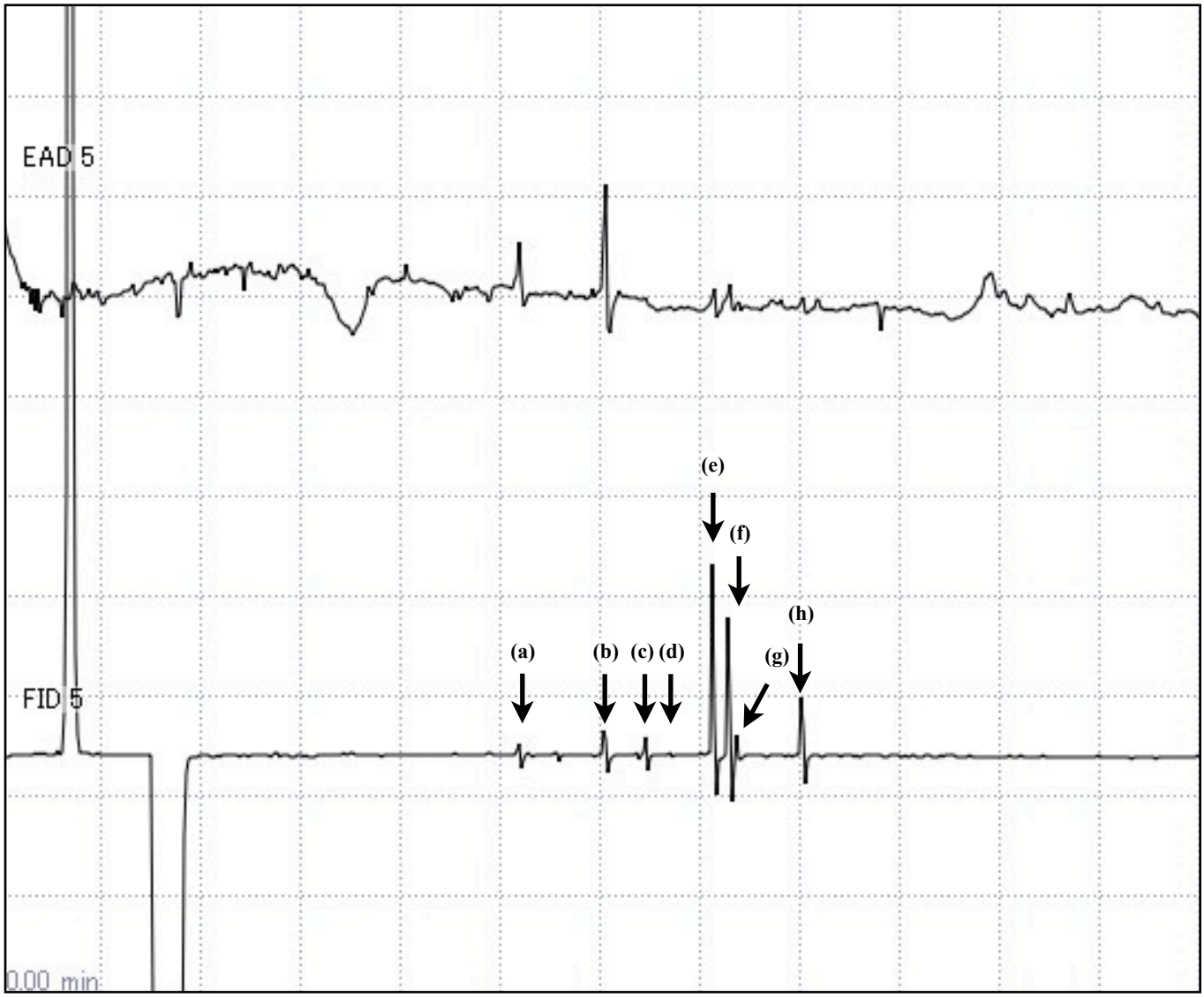

Fig. 5-2. Flame ionizationdetector (FID) and electroantennographic detector (EAD: male *A. nigrisigna* antenna) responses to diluted Confuser V® solution. (a) Z7-12OH. (b) Z7-12Ac. (c) Z9-14OH. (d) Z9-16Al. (e) Z11-16Al. (f) Z9E11-14Ac. (g) Z11-16OH. (h) Z11-16Ac.

Z9E11-14Ac, (Z)-11-Hexadecen-1-ol (Z11-16OH) and (Z)-11-Hexadecenil acetate (Z11-16Ac). As expected, *A. nigrisigna* pheromone components, Z7-12OH and Z7-12Ac, indicated remarkable EAD response. Other seven components (FID positive) are for other lepidopteran species. Of these components, Z11-16Al and Z9E11-14Ac indicated recognizable EAD response in all replicates. Z11-16Al is the lure for *Plutella xylostella* (Lepidoptera: Plutellidae). Z9E11-14Ac is for *S. litura*. Therefore, I assumed Z11-16Al and Z9E11-14Ac as excitatory substance that induced antennal stimulation of *A. nigrisigna* males.

5.3.2 Field trap test

As expected, *A. nigrisigna* males were attracted to lures loaded with only the *A. nigrisigna* pheromone (74.7 ± 11.1 males captured) (Fig. 5-3-A). The addition of Z11-16Al to the lures baited with *A. nigrisigna* pheromone reduced the catches of males of this species (1.0 ± 1.0 males captured) (Fig. 5-3-B). Same tendency was also seen in Z9E11-14Ac added trap (13.0 ± 5.1 males captured) (Fig. 5-3-C). The traps that were added both Z11-16Al and Z9E11-14Ac recorded the lowest male catches in four treatments (0.7 ± 0.7 males captured) (Fig. 5-3-D).

5.3.3 Mating disruption test

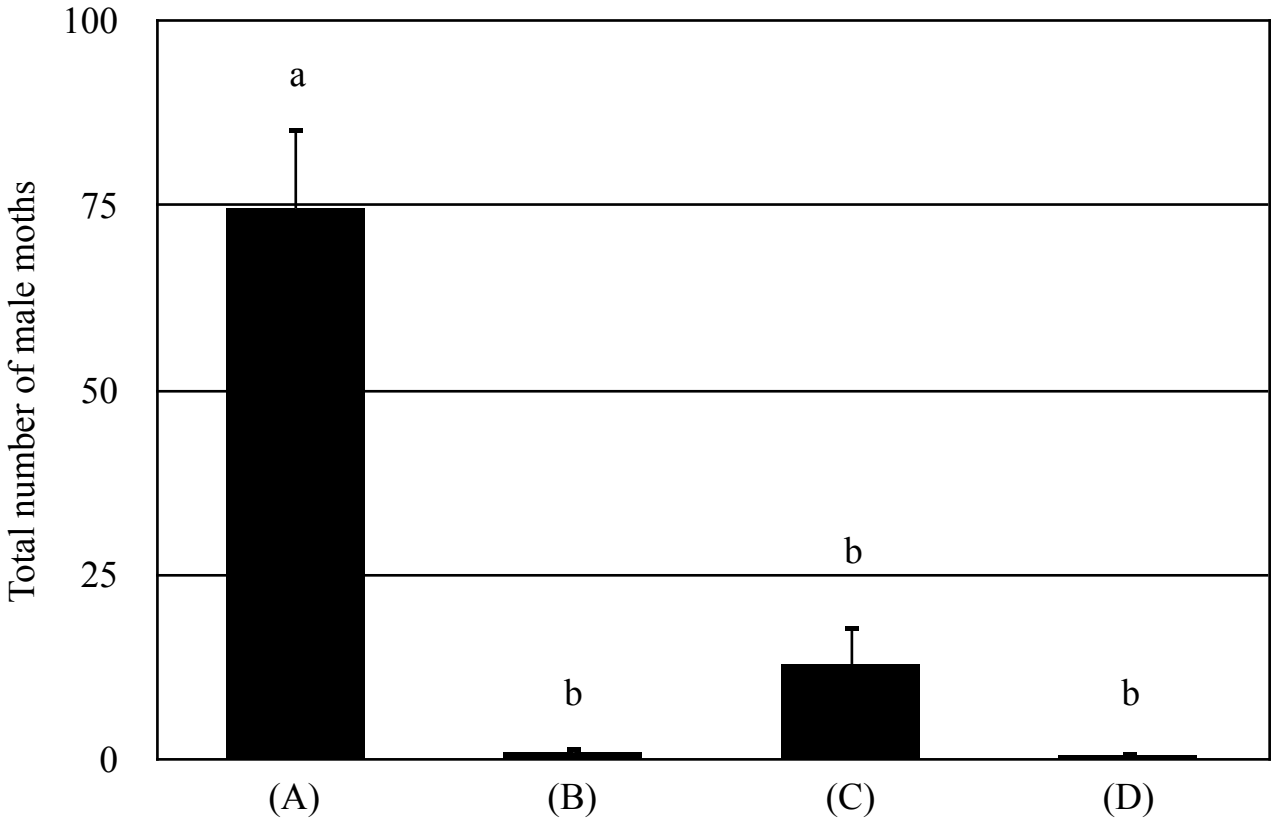

Fig. 5-3. Total trap catches of male *A. nigrisigna* moths with synthetic sex pheromones (July 4th- August 8th, 2011). Means followed same letter are not significantly different at the 5% level by Tukey-Kramer's HSD test. (A): *A. nigrisigna* pheromone. (B): Z11-16Al and *A. nigrisigna* pheromone. (C): Z9E11-14Ac and *A. nigrisigna* pheromone. (D): Z11-16Al, Z9E11-14Ac and *A. nigrisigna* pheromone.

In the control cage, the proportions of mated females were high (Table 5-1). The dispensers loaded with *A. nigrisigna* pheromone reduced the mating rate to 8 %. However, 24 % of females were mated in the cage treated with *A. nigrisigna* pheromone and other two components (Z11-16Al + Z9E11-14Ac).

5.4 Discussion

To date, three components have been identified from the pheromone glands of *P. xylostella* females (Suckling et al., 2002; Yang et al., 2007). All of three pheromone componets, Z11-16Al, Z11-16Ac and Z11-16OH, were contained in Confuser V®. In the case of *S. litura*, four components have been identified (Tamaki et al., 1976; Sun et al., 2002) and the two major components, Z9E11-14Ac and Z9E12-14Ac, were contained in Confuser V®. Although *A. nigrisigna, P. xylostella* and *S. litura* share their habitats and host plants with each other, any components were not shared by these species. However, Z11-16Al and Z9E11-14Ac induced antennal stimulation of *A. nigrisigna* males and its addition to *A. nigrisigna* pheromone decrease the number of males.

Results of GC-EAD and trap test suggested that *A. nigrisigna* males perceived some of other lepidopteran pheromones in Confuser V® and if these components were added to their natural sex pheromones, males avoided the pheromone source. In the Confuser V® treated environment, *A. nigrisigna* males might be able to distinguish their natural sex pheromone from fake pheromone of Confuser V® and copulate with true females. This prediction also conforms to

Table 5-1. Mean mating rates of *A. nigrisigana* females in the sex pheromone treated cages.

Treatment	No. of moths per cage (♀×♂)	Replication	Mean mating rates (%)
Hexane (Control)	10 × 10	5	88 a
(A)	10 × 10	5	8 b
(B)	10 × 10	5	24 c

Means followed same letter are not significantly different at the 5% level by Tukey-Kramer's HSD test. (A): *A. nigrisigna* pheromone. (B): Z11-16Al, Z9E11-14Ac and *A. nigrisigna* pheromone.

the result of mating disruption rate. Mating rate was significantly reduced in the pheromone treated cages as compared to the control cage. And more, mating rate in the cage added two components to *A. nigrisigna* pheromones was significantly higher than the cage added *A. nigrisigna* pheromones only. The addition of Z11-16Al and Z9E11-14Ac to *A. nigrisigna* pheromones resulted in an increased mating rate. When the other pheromone components were added to natural sex pheromones, *A. nigrisigna* males might recognize that it was not their pheromone source and be able to mate with real females. To demonstrate this prediction, further research using wind tunnel should be carried out. Additionally, electrophysiological approach along with GC-EAD will be also needed. Hansson et al. (1987) used single-sensillum recording method and revealed that there were three types of pheromone receptor neurons in the antenna of *Ostrinia nubilalis* (Lepidoptera: Crambidae) males. Of these receptor neurons, two types respond to conspecific pheromone components and a third type responds to the behavioral inhibitory components. And more, Campanacci et al. (2001) discovered that the pheromone-binding proteins (PBPs) from *Mamestra brassicae* (Lepidoptera: Noctuidae) and *Antheraea polyphemus* (Lepidoptera: Saturniidae) respond to pheromone components from other species. Same phenomena might be occured in the receptor neurons and the PBPs of *A. nigrisigna* males and females. By perceiving other species pheromones, males might enhance the sensitivity to their own phermones and females might produce their own pheromones more actively.

In some cases, the addition of heterospecific components to the conspecific blend causes inhibition of male upwind flight. These so-called pheromone antagonists play a role in maintaining reproductive isolation of closely related species (belonging to same genus) sharing common pheromone components, and, therefore, susceptible of mating mistakes (Leal, 1996; Borden, 1997; Mustaparta, 1997; Cardé and Haynes, 2004). And more, pheromone antagonism also occurs between species that are not closely related (belonging to different genera) and do not share pheromone components (e.g. Eigzaguirre et al., 2002). However, the effects of releasing a high amount of pheromone antagonists as pest control programs have rarely been studied. Some studies have investigated the effects of releasing pheromones in the environment for mating disruption purposes on insects taxonomically close to the target species (Johnson et al., 1991; Ferrao et al., 1998) and also on its parasitoid complex (Niwa and Daterman, 1989). More research should be done to consider whether target species are sensitive to other target species pheromone and whether the females may also respond to other species pheromones.

In this chapter, I considered the possibility that false response of *A. nigrisigna* males to other lepidopteran pheromones. It could be one of the reasons for the low effectiveness of Confuser V®. From the results reported here, it was revealed that other lepidopteran pheromone components in Confuser V® induced antennal stimulation of *A. nigrisigna* males, and altered the trap catch number and mating disruption rate.

General Discussion

6.1 Constraints on mating disruption

In this study, I described four possibilities contributing to the low effectiveness of the mating disruption for *A. nigrisigna* moths. These possibilities could be roughly divided into two types according to male side or female side problem. The possibility that migration into the pheromone treated area (discussed in Chapter 2) was the problem which belongs to female side. Host plant selection (discussed in Chapter 3) was also the problem on female side. Weak response caused by the absence of minor components in Confuser V® (discussed in Chapter 4) was the problem on the part of male. False response to other lepidopteran pheromones (discussed in Chapter 5) was also the problem on male side. From the results reported in this study, the female motility (Chapter 2) and the male responsiveness (Chapter 4 and 5) were considered as the factors of the low effectiveness of Confuser V®.

Female motility was suggested as the one of the key points for the successful mating disruption. Many researchers mentioned the relationship between success rate of mating disruption and migration of mated females (Wakamura et al., 1989; Fadamiro et al., 1999; Kerns et al., 2000; Trimble et al., 2001; Vassiliou, 2009). However, flight ability of mated females varies greatly according to species. In some of moth species, mated females hardly moved from their eclosion site until they finished to oviposit (Elzinga et al., 2011). In

other species including *A. nigrisigna*, mated females maintain the capability of dispersing to more suitable host plants. To control these movable moths by mating disruption, areawide treatment is required. By treating mating disruptant outside the farm, females which stay the outside will become difficult to mate. As a result, the probability of migration by mated females from the outside will be reduced. Thus, mating disruption will likely work best as an areawide management tool (Ogawa, 1990; Staten et al., 1997). The efficacy of mating in large areas is not only because large areas reduce the impact of mated females, but also because homogenous air permeation is facilitated. Incomplete permeation with pheromone, especially along crop borders, is an obstacle. This has been confirmed by field measurements of aerial pheromone concentrations (Milli et al., 1997). Border effects become negligible when large surfaces are treated. Indeed, dispenser spacing and overall pheromone application rate can be reduced as the treated area increases, resulting in considerable cost savings to farmers.

Besides the female motility and border effects, there are various factors that constrain the effect of mating disruption. A high population density of the target insect can also thwart control efforts (Cardé and Minks, 1995), especially if disruption is based on competition between synthetic and female-produced pheromone plumes. The number of generations per year is another important consideration (Cardé and Minks, 1995). The effectiveness of the disruption technique for a pest in a given region is sometimes correlate with the number of

generations per year. The attributes of the mating system also can be modified by environmental conditions. The nature of the canopy and shifts in wind direction and wind speed determine the pheromone plume's turbulent structure (Schmitz et al., 1997; Sauer and Karg, 1998). Accessibility to females may also be changed by whether the males are active during the day or night. At night, wind speed are on average lower, and obviously there is less light. Thus, males become difficult to follow a pheromone plume and use visual information. Finally, continuous application of synthetic pheromone could lead to the development of resistance against mating disruption through an alteration in the chemical communication system of the target pest (Haynes and Hunt, 1990; Evenden et al., 2002; Mochizuki et al., 2002). For example, one of the plusiine species, *T. ni*, has genetically-based variations in signale and response (Evenden et al., 2002). Females of two pheromone strains, normal and mutant type, produce the same six components but in different ratio. Spohn et al. (2003) revealed that the effects of a mating disruptant differed according to pheromone strains. In the case of *A. nigrisigna*, any genetical or geographical variation has not found until now. However, selection pressure always exists under pheromone treated environment and it might provide resistance for pest species. More research will be needed to examine whether such variation exist in *A. nigrisigna* agricultural population that are controlled with Confuser V®.

As described above, there are populational, environmental and genetical constraints on mating disruption. We need to keep in mind that the effect of

mating disruption can differ according to behavioral attributes of target insects and geographical conditions of treated field. However, limitations of mating disruption as a control tactic may be best overcome with an increased knowledge of target insects and continual observation of ecological circumstances.

6.2 Efficacy of newly-developed disruptant

In this study, it was revealed that the disruptant specialized for *A. nigrisigna* was more effective for *A. nigrisigna* control than the conventional disruptant, Confuser V®. In the mating cage experiment (Chapter 5), I saw a number of males gathering around the dispensers loaded with complete *A. nigrisigna* pheromones. On the other hand, the pheromone traps baited with Confuser V® as a lure did not attract *A. nigrisigna* males. From these findings, the attractiveness of newly-developed disruptant might be built upon the different mechanism with Confuser V®. If we understood the underlying mechanisms that cause disruption of mating, we will be better placed to understand why some applications are successful and others not (Cardé and Minks, 1995; Sanders, 1997; Miller et al., 2010). In order to understand the mechanisms, the investigation should be conducted from two perspectives, thus, behavior of moths and molecules. Male moth behavior depends on various factors, including pheromone blend, release rate, and aerial concentration. Measurement of these factors and their contribution to efficacy will help to predict the outcome of mating disruption

(Bengtsson et al., 1994). For measuring male moth behavior, the wind-tunnel will become a useful tool. The wind-tunnel assay allowed assessment of the effects of a wide range of component combinations and ratios on sequential stages of the male moths' flight responses, from activation of quiescent moths, to initiation of flight, flight upwind along the pheromone plume, landing on the pheromone source, and time spent in contact with the odor source (Kanno et al., 2010). Many past work adopted this assay and measured the proportion of males reaching and contacting a point source of odor (e.g., Linn et al. 1988; Zhu et al. 1999; Trimble and Marshall, 2008). To reveal the superior attractiveness of complete pheromone blends, additional studies should be undertaken by using these methods.

6.3 Future prospects of pheromone research

In 1959, a german team led by Adolf Butenandt achieved the first chemical identification of pheromones (Hecker and Butenandt, 1984). About 50 years after this discovery, a large number of insect pheromones were identified and pheromone-based pest control reached nearly every corner of the world. Nowadays, we embarked on a new stage of pheromone research. It is now within our reach to facilitate the discovery of relevant chemical signals with emerging molecular tools (Witzgall et al., 2010). An odorant binding protein recently has been used to select oviposition attractant candidate compounds in a mosquito (Leal et al., 2008; Pickett et al., 2010). And more, structural chemistry

software combined with statistical analyses was established to calculate a physicochemical odor metric that predicts neuronal responses (Haddad et al., 2008). In ecological aspect, impacts of a long-term mating disruption on non-target insects were studied (Martinez and Mgocheki, 2012). As for the the formulations of mating disruptant, microcapsules and hollow fibers were developed. Hand-applied type formulations enabled fewer point application of highly-concentrated synthetic pheromone. Season-long field life and reduced application cost in combination with fungicides are main advantages of hand-applied type (Leonhardt et al., 1990; Weatherston, 1990; Trimble et al., 2003; Tcheslavskaia et al., 2005; Il'ichev et al., 2006). These new knowledge of pheromone in the IPM programs suggests that there is still a vast potential for intervention with pheromone research.

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要約

本研究では野菜用交信かく乱剤コンフューザーV®を施用した環境でタマナギンウワバ *Autographa nigrisigna* (チョウ目: ヤガ科) が発生する要因を解明するとともに、キンウワバ類に対する分子同定法を構築することを目的として実験を行なった。交信かく乱剤は、害虫数種の合成性フェロモンを吸着させた農業資材であり、これを大量に配置することで、圃場全体に性フェロモンが充満し、交尾行動が阻害され、結果的にメス成虫の交尾率が低下する。タマナギンウワバは野菜用交信かく乱剤コンフューザーV®のターゲット害虫でありながら、本剤では圃場での発生が抑えられず、防除効果が現れにくいことを博士前期課程での研究で明らかにした。

交信かく乱剤施用環境下においてタマナギンウワバをはじめとするキンウワバ類の発生が報告されるようになると、「発生したウワバの幼虫が一体どの種か特定できない」という問題が生じてきた。キンウワバ類は、日本において 59 種が存在する。種同士は各ステージで外部形態が酷似しており、一見して識別できない種が多いということは以前から指摘されていた。しかし、実際に圃場での発生が多くなったことにより、この問題が表面化してきたようだ。キンウワバ類の同定は主に成虫の前翅斑紋によって行われる。幼虫の場合は腹部にある刺毛配列でキンウワバ類を A から F までの 6 グループに分類することが可能であるが、各グループには複数種が含まれるため、刺毛配列のみでは確実に種を特定するまでに至らない。また、寄生蜂が脱出した後の幼虫や罹病した幼虫では体表の損傷・変色により、刺毛配列を確認

することは困難になるし、孵化以前の卵の状態では識別はほとんど不可能である。しかし、寄生や病気によって種が不明な個体ばかりになってしまった場合、各圃場における種構成の比較はできない。IPMの観点からも圃場の害虫相を正確に把握することは重要だと考えられる。

そこで、本研究では確実に卵と幼虫期での同定が行なえるように分子生物学的な手法を構築することを目指した。レタスでの発生が特に顕著であったキンウワバ類 3 種を対象に、ダイレクトシークエンス法を用いて、ミトコンドリア DNA の COI（Cytochrome Oxidase I）領域の塩基配列約 900 bp を決定した。この塩基配列を基に、それぞれの種に特異的なプライマーを設計し、マルチプレックス PCR を行なった。その結果、アガロースゲル上のバンドパターンから 3 種を識別することができた。さらに、本手法を日本国内の様々な地点で採集したキンウワバの生体や病気や寄生によって死亡した個体に応用したところバンドパターンから 3 種を見分けることができた。このように、外部形態による同定に加え、分子同定を行なうことで、圃場におけるキンウワバ類種構成をより明確に示すことが可能となった。

また、本研究では交信かく乱剤を施用したレタス圃場でタマナギンウワバが発生する要因として以下 4 つの可能性を掲げ、それぞれの可能性について検証を行なった。

・既交尾メスの圃場外から圃場内への飛び込み

・寄主植物間での産卵選好性と摂食選好性の違い

・コンフューザーV®における微量成分の欠如

・ コンフューザーV® における他種害虫用成分の影響

1 点目の可能性を検証するため、交尾の前後におけるメス成虫の飛翔能力を調べた。飛翔能力の推定にはフライトミルおよびアクトグラフ装置を用いた。フライトミルを用いた実験では、交尾の有無によって飛翔時間や飛翔距離に違いがみられなかったが、交尾を終えたメスでも 1 晩に、最高 5 時間 6.3 kmの飛翔が可能であることがわかった。アクトグラフを用いた実験では、既交尾メスは未交尾メスに比べ、夜間活発に動き産卵していることがわかった。これらの結果より、交尾前後でメスの体力に大きな差はないが、交尾を終えたメスは寄主植物の探索と産卵のために活発に動き回ることがわかった。その結果、メスが交信かく乱剤を施用した圃場外で交尾を行ない、圃場内に侵入して産卵を行なう可能性もあると考えられた。

2 点目の可能性は農家や試験場関係者からの指摘に由来する。本研究の調査地である長野県軽井沢町は高原野菜の産地であり、レタスの生産が盛んに行われているが、その他にもキャベツがほぼ同時期・同所で栽培される。レタスとキャベツの圃場が密接しているような場所で交信かく乱を行なうと、タマナギンウワバの発生が顕著にあらわれるのは、キャベツ圃場ではなく、レタス圃場で圧倒的に多いという指摘が現地試験場の研究員や地元農家から寄せられた。このように被害が特定作物の圃場に片寄る現象は、タマナギンウワバの食性や産卵特性に起因しているのではないかと考えた。タマナギンウワバは本来の寄主植物とされているアブラナ科植物の他に、キク科・マメ科・バラ科など様々な植物に寄生する広食性昆虫である。しかし、これら寄主植物間でも、より好まれるもの・好まれないものがあると考

え、キャベツとレタスを用いた産卵選好性・摂食選好性の実験を行なった。その結果、寄主植物の種類によって幼虫の成長速度は変わらないものの、メス成虫はレタスよりもキャベツを産卵基質として好むことがわかった。

3 点目の可能性では、タマナギンウワバに特化した新規交信かく乱剤を作成し、その防除効果をコンフューザーV®と比較した。コンフューザーV®にはタマナギンウワバの性フェロモンのうち主要 2 成分のみが含まれているが、新規交信かく乱剤には 4 成分すべてが含まれており、メス成虫が放つ性フェロモンに近くなっている。野外試験の結果、新規交信かく乱剤処理区内に設置した性フェロモントラップにはオス成虫がほとんど誘殺されなかった。また、新規交信かく乱剤を用いると一晩当たりのメス交尾率が 0 %を示し、圃場での幼虫発生数も低下したことから、本剤はコンフューザーV®に比べタマナギンウワバに対する防除効果が高いと考えられた。この結果より、コンフューザーV®にはタマナギンウワバの性フェロモンのうち微量 2 成分が欠如していることが防除効果の低下に起因していると示唆された。

4 点目の可能性について、本研究では GC-EAD を用いてコンフューザーV®の各成分に対する触角の反応性を調べた。その結果、オス成虫は自身の性フェロモン成分以外にコナガ用およびハスモンヨトウ用の性フェロモン成分に強く反応することがわかった。さらに、これらの成分が本来のタマナギンウワバの性フェロモン成分に加わると、オス成虫の誘引性は低下し、交尾率は上昇することがわかった。これらの結果より、コンフューザーV®を処理した環境において、オス成虫はメス成虫が放つナチュラルの性フェロモンとコンフューザーV®が放つ疑似の性フェロモンを

区別できるため、本物のメスを見つけ出し交尾に至ることができるのではないかと考えられた。以上の検証結果をまとめると、既交尾メス成虫の活動性の向上とコンフューザーV®の成分に対するオス成虫の反応性の低下が、コンフューザーV®を施用した環境でタマナギンウワバが発生する要因に大きく関与するものと考えられた。