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Proceedings

The VII International Workshop on

Management of the Diamondback Moth and other Crucifer Insect Pests

23-27 March 2015
University of Agricultural Sciences
Bengaluru, India

Issue Editors:

Srinivasan, R., Zalucki, M.P., Shelton, A.M., Kumar, A.R.V., Chandrashekara, K.

Organisers

AVRDC
The World Vegetable Center

University of Agricultural Sciences
Bengaluru, India

Cornell University
New York, USA

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Foreword

The diamondback moth, *Plutella xylostella* (L.) (Lepidoptera: Plutellidae) is an insect herbivore of plants in the Brassicaceae. The diamondback moth (DBM) is a consummate survivor in Darwinian terms and its ability to rapidly multiply under ideal conditions virtually makes it pre-adapted to exploit the increased resource availability in cropping systems. Not surprisingly, DBM is the single most important pest of several crops like cabbage (*Brassica oleracea* L. var. *capitata*), cauliflower (*B. oleracea* var. *botrytis*), broccoli (*B. oleracea* var. *italica*), kale (*B. oleracea* var. *alboglabra*), mustard (*Brassica juncea* (L.) Czern. and *Sinapis alba* L.) and canola (*Brassica napus* L. and *Brassica rapa* L.). DBM along with other pests has kept a legion of entomologists busy for over fifty years and continues to inflict heavy economic loss on crucifer based vegetable and oil cropping systems estimated at about four to five billion USD.

Current approaches to manage DBM and other insect pests of crucifers, worldwide, rely heavily on chemical pesticides in combination with cultural and biological methods. However, DBM has rapidly evolved resistance to insecticides and even *Bacillus thuringiensis* (Bt) toxin leading to an intensification of pesticide use in crucifers. The increased cost of production of crucifers coupled with unstable market prices has plunged cultivation of crucifers into a serious crisis. As a short-term measure, avocation and promotion of integrated approaches for managing DBM and other crucifer pests has been reasonably successful. In the long run however, the success of developing effective management strategies against DBM critically hinges on addressing the following important challenges: mechanism of insecticide resistance in DBM and its management; improving the efficacy of biological control; using genomics to identify targets for chemical control and host-plant resistance; meta-population dynamics, migration and dispersal, and genetic manipulation of DBM.

The progress achieved in addressing these challenges was discussed at the 7th International Workshop on Management of the Diamondback Moth and other Crucifer Insect Pests held from 23 to 27 March, 2015 by the University of Agricultural Sciences, Bengaluru in association with World Vegetable Center, Taiwan, Republic of China and Cornell University, New York, USA. The workshop was attended by entomologists and plant protection experts from across the world and comprised a total of 68 papers including lead talks and poster presentations. The workshop on DBM and other crucifer pests was divided into seven sections: (i), Global challenges, (ii) Biology, ecology and behavior of pests, (iii) host plant resistance and chemical ecology, (iv) insecticides and insecticide resistance, (v) biological control, (vi) biotechnological tools and other novel approaches and innovations and (vii) IPM at the farm level. The proceedings of the workshop have been brought out as a special issue of the Mysore Journal of Agricultural Sciences with full papers highlighting the major advances made in the management of DBM and other crucifer insect pests. The editors are thankful to all the authors for submitting the full version of their papers for compilation of the proceedings.

This volume would not have been possible but for the unstinted support from University of Agricultural Sciences, Bengaluru, AVRDC- World Vegetable Center, Taiwan, Cornell University, Ithaca, New York and our sponsors who facilitated the workshop.

– Editors

Mysore J. Agric. Sci., 51(A): 1-227, 2017

CONTENTS

Session 4 : Insecticides and insecticide resistance in diamondback moth and other crucifer pests

Session 5 : Biology and non-chemical methods of management of diamondback moth and other crucifer pests

Session 6 : Biotechnological tools and novel approaches to manage diamondback moth and other crucifer pests

Session 7 : At the farm level ; Barriers to and innovations for management of diamondback moth and other crucifer pests

Introduction

Mysore J. Agric. Sci., 51(A): 1-3, 2017

Quo vadis: Diamondback Moth Management– the Next Installment

Srinivasan, R., Zalucki, M.P.[1], Shelton, A. M.[2]
Entomology Unit, World Vegetable Center, Shanhua, Tainan, Taiwan, Republic of China
[1]School of Biological Sciences, The University of Queensland, Brisbane, Australia, 4072
[2]Department Of Entomology, Cornell University / NYSAES
630 W. North St., Geneva, NY 14456 USA

Abstract

Plutella xylostella (L.) (Lepidoptera : Plutellidae), the diamondback moth (DBM) is the most widely distributed insect pest of crucifers (Talekar and Shelton 1993; Zalucki and Furlong 2011). The pest status of DBM seems to have increased since the 1960s, when large-scale application of chemical insecticides began on vegetable crucifers (Talekar and Shelton 1993) and now costs the world economy USD $ 4-5 billion annually (Zalucki *et al.* 2012). Because of its pest status, there have been six international workshops devoted to the biology, ecology, and management of this pest (Talekar and Griggs 1985; Talekar1992; Sivapragasam *et al.* 1997; Endersby and Ridland 2004; Shelton 2008; Srinivasan *et al.* 2011). These proceedings represent papers presented at the seventh workshop, held in Bengaluru, India from 23-26 March, 2015.

Over the years the "DBM and other Crucifer Pests" workshops have changed. Here we summarize the changes in research and extension areas over the years and then ask: how is it that with all this time and effort DBM remains one of the key pests of Brassicas?

The focus of papers (first 6 proceedings) and presentations (in Bengaluru) has changed dramatically, although some things remain the same (Figure 1). In all of the workshops there has always been an emphasis on insecticides and insecticide resistance management (Figure 1a) (*e.g.*, Juil *et al.* 2017a & b; Kodandaram *et al.* 2017; Onkarnaik *et al.* 2017; Sridhar *et al*. 2017; Yin *et al*. 2017; this proceedings), with various emphases on "alternatives" to insecticides (excluding biological control). The alternatives have at various times included pheromones (*e.g.*, Topagi *et al*. 2017; this proceedings), botanical products (*e.g.*, Cerda *et al.* 2017; this proceedings) and more recently genetic techniques (*e.g.*, Good *et al.* 2017; Perry *et al.* 2017; Russell *et al.* 2017; this proceedings). Basic biology/ ecology accounted for a large proportion of papers in early workshops and biological control (the 4 P's: Parasites, Pathogens, Predators and Parasitoids) have always been focal areas (Figure 1b), and this workshop is no exception (*e.g.*, Amalina *et al.* 2017; Furlong *et al.* 2017; Ismail *et al.* 2017; Khatun *et al.* 2017; Nadia *et al*. 2017; this proceedings). But emphasis on biological control over the years has waxed and waned, in partperhaps because of the failure of insecticides and availability of "new" chemistries (Figure 1). IPM has been a central theme over all workshops although the percentage of presentations devoted to it has varied by workshop (Figure 1b). This variation in classifying the "broad theme of IPM" may be misleading since it probably reflects the variation in components of IPM: host plant resistance (see Srinivasan *et al*. 2017a; this proceedings); IPM tactics, decision tools and implementation (see Alam *et al.* 2017; Ketelaar *et al.* 2017; Patil *et al.* 2017; Srinivasan *et al.* 2017b; this proceedings). IPM should remain a central theme in future workshops, however, the success of IPM has been mixed judging by the evolution of insecticide resistance to many active ingredients (Li *et al.* 2012). The type of research under basic biology and ecology has changed from descriptive studies in the early years to more focused experimental and fundamental studies (see Sivapragasam 2017; Zalucki *et al.* 2017; this proceedings).

It has long been recognized that diamondback moth pest problems are exacerbated due to poor insecticide application and mismanagement (Talekar and Shelton 1993; Furlong *et al*. 2013; Li *et al.* 2016b), the disruption of biological control agents by the application of broad-spectrum insecticides (Furlong *et al.* 2004a & b), planting practices (year round production and poor post harvest hygiene) and the effects of climate (Li *et al.* 2016a; Zalucki *et al.* 2017; this proceedings).

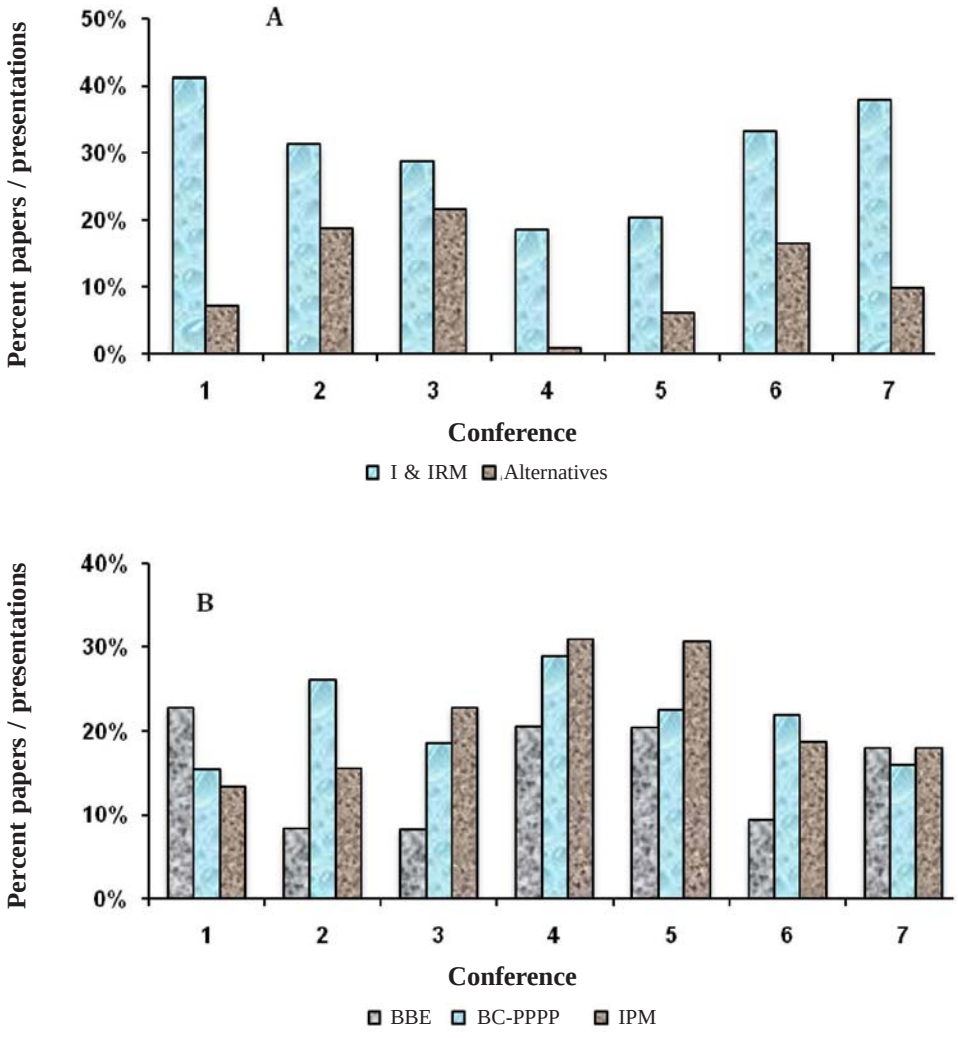

FIGURE 1

The percent of papers from previous DBM conference proceedings (1-6, see text for details) and presented at Bangaluru (7, see these proceedings) that address Insecticide application, resistance and management (I & IRM), soft alternatives to insecticides (BT, botanicals, pheromones, genetic methods) (A). Basic Biology and Ecology (BBE), biological control and the four agents (Parasites, pathogens, parasites and predators) (BC-PPPP) and IPM including host plant resistance, IPM tactics, decision support and implementation (B)

The difficult question that has to be posed is how is it that despite all that has been done to address the problem, the DBM problem persists and has even increased in some areas. How is it that with all the lessons supposedly learned, the research and IPM extension and implementation through farmer field schools and similar efforts that DBM persists as a major pest? Have researchers failed to address some of the key issues or is it a failure of implementation? Despite the need for IPM, knowledge about its needed components is often missing or only rudimentary. For example, few researchers are able to accurately partition mortality parameters under field conditions and estimate the natural enemy and predation component (Furlong and Zalucki 2010; Zalucki *et al.* 2015). Without such knowledge, it is easy to see why farmers may reach for an insecticide as the first tactic.

For such a major pest, surprisingly little research has been conducted on long-term dynamics and forecasting of populations (but see Zalucki and Furlong 2008, 2011; Li *et al.* 2016a). One of the great difculties in the management of this pest has been the occurrence of outbreaks, many due to insecticide resistance, that greatly strain management (Li *et al.* 2012). Would timely forecasts actually make a difference to management? Are these possible?

As preparations for the next workshop begin, the organizers might consider what are key failures in existing management programs and what can be done

to address them. Can a list of recommendations be formulated for the different areas in which DBM occurs? Can these recommendations be implemented at the grower level?

DBM has proven to be a fascinating insect worthy of the seven workshops because of its complex physiology, behavior, ecology and management, and there will be no shortage of new presentations for future workshops. But wouldn't it be satisfying if many presentations in the next workshop could be classified into the category "Successful IPM Programs?"

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Session 1

Diamondback moth and other crucifer pests : Global Challenges

Mysore J. Agric. Sci., 51(A): 5-8, 2017

The Talekar Challenge: What Have We Learned and Where are We Going with Practical DBM Research and Extension Since 1985?

Shelton, A. M.
Department of Entomology, Cornell University/NYSAES, 630 W. North St., Geneva, NY 14456 USA
E-mail: ams5@cornell.edu

Abstract

Dr. N.S. Talekar, an entomologist from AVRDC, was the workshop coordinator and editor of the proceedings for the first (1985) and second (1990) International Workshops on Diamondback Moth (DBM), both held in Tainan, Taiwan. For the occasion of the first and second conferences, Talekar also published an annotated bibliography that he made freely available to scientists worldwide. In 1993 he was the senior author of the first comprehensive review of DBM. Talekar has been an active researcher and promoter of applied entomology and biological control for insect pests on crucifers and other vegetables, especially in South- and Southeast Asia. His work and leadership continues to inspire us as we address the continuing challenges of managing DBM in the varied cropping systems globally. These challenges include: the continuing development of resistance to insecticides and applying strategies to delay the evolution of resistance; understanding the role biological control plays in reducing DBM populations and how biological control can be enhanced; utilizing the information from the DBM genome for creating useful management strategies; developing a better understanding of DBM ecology on a landscape level; researching genetic control strategies including modifying the insect and its host plants; and creating practical outreach programs that enable farmers to manage DBM in a more sustainable manner.

***Keywords*:** Diamondback moth, *Plutella xylostella*, integrated pest management

My presentation honors the man who started these workshops in 1985 and asks the question he asked me several months ago: what have we learned and where are we going with practical DBM research and extension? It is appropriate that Dr. N. S. Talekar ask this question since he did so much over several decades to advance sustainable control of DBM.

Dr. N.S. Talekar is a native of India so it is appropriate that this 7thworkshop be in his home country. Talekar was very proud to have received his PhD in 1973 from the University of Wisconsin, and in 1974 he began his career at the Asian Vegetable Research and Development Center (AVRDC), which was founded in 1971 in Taiwan. Dr. Robert Chandler from the International Rice Research Institute (IRRI) was appointed as the first Director General of AVRDC and appointed Talekar to a newly created program to deal with pesticide residues on vegetable crops.

Toward the end of the 1970s, DBM had developed resistance to practically all synthetic insecticides so growers increased their rates and spray frequencies. Officials in Singapore, the major market for vegetables grown in Malaysia and Indonesia, rejected cabbage because of high insecticide residues. These two events forced farmers to start using sprays of *Bacillus thuringiensis* which resulted in increased parasitoid populations and reduced DBM damage.

The story of how Talekar came to appreciate the importance of parasitoids for DBM can best be described in Talekar's own words. "My inspiration in going for biological control came from the success of Indonesian scientists in introducing *Diadegma semiclausum* from Java to Brastagi town's intensive vegetable growing area in North Sumatra Province. Brastagi is in the highlands. That parasitoid was introduced on Java (highlands near major city of Bandung) by the Dutch, who were a colonial power in Indonesia until the early 1950s. Brastagi was then a major supplier of vegetables to Singapore. When the Singapore Government found that those farmers were using all kinds of pesticides to combat DBM, they immediately banned import of vegetables from Indonesia. That drove Indonesian scientists into action. They imported the parasitoid from Java to Brastagi and made a pact with farmers, who were badly hit by the import ban imposed by Singapore,

that they would not use any pesticide on their vegetable (crucifer) farms. They agreed and kept their promise. *Diadegma semiclausum* was immediately established and farmers never reached for pesticides again".

Talekar continued his work on biological control of DBM but says that he just "copied the idea of biological control, tested it in farmers' fields in Taiwan, and spread it elsewhere." Talekar humbly claims that he did not have time for much research and published few papers on this topic, although we all now recognize the impact his work has had on biological control of DBM throughout Asia and SE Asia, and even in East Africa.

What was it that drove Talekar to work so hard on finding solutions for DBM control? In my recent correspondence with him, he described how AVRDC's Director General, Dr. Robert F. Chandler Jr. developed and enforced the preamble for AVRDC: "The farmer is our client, it is he whom we must serve, and any other objective is trivial compared to our aim to improve the well-being of the rural population and to strengthen agricultural production". Based on this strong philosophy of serving the grower, Talekar saw the need for collaborative efforts on a global scale.

Forming the DBM Community

As Talekar tells the story, "Sometime in early 1983, I was in Indonesia on the outskirts of a town named Bhogitingi (on Sumatra Island) and saw a farmer's field badly damaged with DBM. I stood in the farm and looked around and told myself - DBM is indeed a serious problem and I alone cannot solve it in my lifetime. I immediately decided to get all researchers together and see what they are doing and how we can learn from successes of others in combating this problem. As soon as I returned from the trip, I walked into Chandler's office and briefed him on what I found and my feeling about the problem and explained my idea of getting all scientists together to see how we can coordinate our research to fit their needs. My Director General approved the idea immediately, and thus started the first International Workshop on DBM in 1985". It is because of his vision that we are here today at the 7th International Workshop on Management of the Diamondback Moth and other Crucifer Insect Pests.

Besides arranging and hosting the First and Second International Workshops in 1985 and 1990, respectively, Talekar also spearheaded publication of the Proceedings for both workshops (Talekar & Griggs 1986; Talekar 1992) and the accompanying annotated bibliographies (Talekar *et al.* 1985; Talekar 1990). In 1989 he spent a year in my laboratory and we wrote the first comprehensive review of DBM (Talekar & Shelton 1993).

The Talekar Challenge

Over the last couple of years of correspondence with Talekar, I have heard him questioning whether the global DBM community is as focused on long-term solutions for the DBM problem as it should be. Many of us continue to introduce new insecticides into our integrated pest management (IPM) programs only to find out that they are harmful to the natural enemy population and the DBM quickly evolves resistance to them. It is an old story for DBM and one that is well documented in the DBM proceedings and reviews. Why don't we hear more stories about more sustainable approaches such as the introduction of *Diadegma semiclausum* into the highlands and *Oomyzus sokolowskii* into the lowland areas of Taiwan where they continue to provide control of DBM? Is it possible to enhance this biological control program even more by introducing pupal parasitoids into the lowlands? Why is not more of this work being done?

Talekar also cooperated with scientists in other countries to help establish parasitoids that provided successful control in the Philippines, Malaysia, Lao PDR, Indonesia, India, Kenya, China, Japan and Korea.

It has been 30 years since the first DBM workshop and now is an appropriate time to ask: what have we learned and where are we going with practical DBM research and extension? While our knowledge of DBM has certainly increased, has it led to better management of this devastating pest? Another way of asking this question is whether our increased knowledge of DBM over the last 30 years has been incorporated into extension programs that helped us move toward more sustainable IPM programs for DBM.

Talekar constructed a figure to illustrate this question. Fig.1 shows the relationship between the number of papers published on DBM over time and the control cost plus yield loss. Based on this figure, the data suggest that there is an unfortunate relationship between papers published and increasing control costs and damage. One could look at this relationship several ways. The first is that despite the increased number of papers, and presumably the increased knowledge about DBM, the losses to this pest continue to increase. However, one might argue that losses would be even greater if people didn't publish on this subject. Or perhaps what is being published has not yet made its way into useable management strategies. Both views are optimistic ways of viewing this seemingly unfortunate relationship.

But another way of viewing this relationship is more troubling and should cause us to examine why we choose to do certain research projects. Is the work that the DBM community is doing contributing to solving the DBM problem? Many of the papers being published now document cases of resistance (Fig. 2) and, while useful for justifying abandoning a particular insecticide in a specific area, we have heard that story too many times before. Shouldn't we be reading more publications about how resistance was managed properly so that judicious use of an insecticide led to its long-term place in an IPM program? Likewise, some of the published work over the last 30 years describes elegant biological phenomena but has it been incorporated into extension programs adopted by farmers? As we decide our research and extension agenda, we should ask what is the proper balance for basic and applied research that will lead to sustainable contributions for DBM management?

Times have certainly changed since Talekar first began his career at AVRDC with the mandate of dramatically reducing the pesticide residues on vegetables shipped to Singapore. Such problem-oriented work may not be fully appreciated by administrators in universities or lead to articles in high-impact journals that help advance one's career. But nevertheless such work is needed and should be recognized as contributing to food security, environmental benefit and healthier food for a growing population.

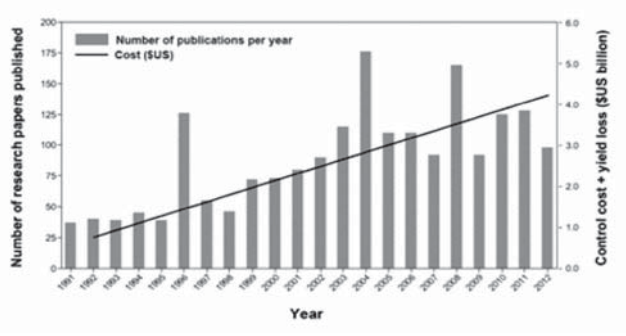

FIGURE 1

Relationship between number of research publications and cost of pest control and yield loss for DBM per year.

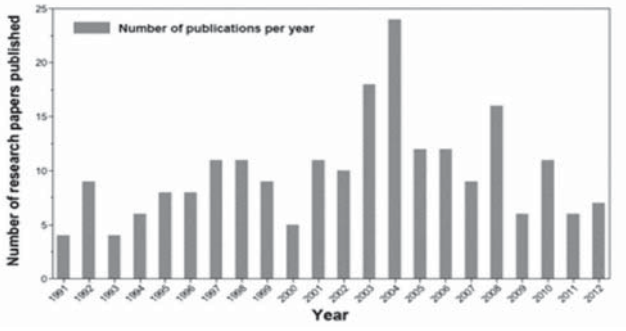

FIGURE 2

Number of research publications on insecticide resistance per year.

FINAL THOUGHTS

Talekar had a mandate from his Director General to solve a problem. The message given to him was clear: his client was the farmer who needed help in controlling DBM.

During his career at AVRDC, according to Talekar, he worked on "strictly applied research" that was initially confined to Southeast Asia. His contributions in enhancing biological control of DBM in Southeast Asia are legendary and inspirational. With new funding, he was able to expand his work subsequently into South Asia and East Africa where again he emphasized biological control as the most essential component in IPM.

Talekar retired from AVRDC in July 2005 and is a visiting professor at the National Chung Hsing University, Taichung, Taiwan where he teaches courses to undergraduate and graduate students.

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I am deeply grateful to Dr. N.S. Talekar for his stimulating insights and for the figures he provided.

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Mysore J. Agric. Sci., 51(A): 9-17, 2017

Modelling the Population Dynamics & Management of Diamondback Moth: the Role of Climate, Natural Enemies & Cropping Patterns

Zalucki M.P., Li, Z.Y.[1], Yonow T.[2], Kriticos D.[3], Furlong M.J.[4] and Feng X.[5]

School of Biological Sciences,
The University of Queensland, Brisbane, Australia, 4072
E-mail: M.Zalucki@Uq.Edu.Au
[1]Institute of Plant Protection, Guangdong Academy of Agricultural Sciences and
Guangdong Provincial Key Laboratory of High Technology for Plant Protection, Guangzhou 510640, P.R. China
E-mail: Shower99@163.Com
[2]CSIRO, Gpo Box 1700, Canberra, Australia, 2601
E-mail: Tania.Yonow@Csiro.Au
[3]CSIRO, Gpo Box 1700, Canberra, Australia, 2601
E-mail: Darren.Kriticos@Csiro.Au
[4]School of Biological Sciences, The University of Queensland, Brisbane, Australia, 4072
E-mail: M.Furlong@Uq.Edu.Au
[5]institute of Plant Protection, Guangdong Academy of Agricultural Sciences and
Guangdong Provincial Key Laboratory of
High Technology for Plant Protection, Guangzhou 510640, P.R. China
E-mail: Fengx@Gdppri.Com

Abstract

Diamondback moth, or DBM, is the major pest of *Brassica* vegetable production worldwide. Control has relied on insecticides and resistance to these has evolved widely and rapidly. We use the DYMEX modelling package to describe the effects of climate on the population dynamics of an age-structured DBM population. We show that there is a strong climate signal in DBM population changes and that natural enemies have a considerable effect on pest pressure and subsequent management. The scale of the pest problem that is experienced varies strongly with cropping practices; production breaks and strict post-harvest hygiene are associated with lower pest pressure in large-scale production units.

***Keywords*:** age structured population modelling, population outbreaks, *Plutella xylostella,* IPM

Plutella xylostella (L.) (Lepidoptera: Plutellidae), the diamondback moth (DBM), is one of the most destructive pests of crucifers; worldwide annual DBM management costs and lost production amount to US$ 4-5 billion (Zalucki *et al.* 2012). The pest status of DBM appears to have increased with the large-scale, widespread application of chemical insecticides in vegetable crops (Talekar and Shelton 1993; Furlong *et al*. 2013), coupled with the rapid evolution of resistance to every class of insecticide used so far (Furlong *et al.* 2013; Li *et al.* 2016b). Yet life-table studies (e.g. Harcourt 1963; Iga 1985;Wakisaka *et al.* 1991) and exclusion experiments (Furlong *et al*. 2004a, 2004b, Murtiningsih 2015) point to the strong effect exerted by parasitoids and predators. That successful classical biological control of DBM can be achieved if broad-spectrum insecticide use is reduced (Furlong *et al.* 2016; Ketelaar and Abubakar 2016), suggests DBM is an induced secondary pest, at least in areas that experience temperate climates. The pest status of DBM appears to be exacerbated due to poor insecticide application and management, the disruption of biological control agents by the application of broad-spectrum insecticides, planting practices and the effects of climate; however, the relative contribution of these factors is still the focus of considerable debate (Shelton 2004; Ridland and Endersby 2008; Li *et al.* 2012; Furlong *et al.* 2013; Arvanitakis *et al.* 2014).

For such a major pest, little research has been conducted on long-term population dynamics. DBM abundance can vary greatly among years (Zalucki and Furlong 2009, 2011; Li *et al.* 2012) and perhaps one of the great difficulties in the management of this pest has been the occurrence of outbreaks and insecticide resistance, which greatly strains management (e.g. Endersby and Ridland 1997; Shelton *et al*. 2000; Shelton 2004; Feng *et al.* 2011; Li *et al.* 2012).

Interpreting insect population dynamics is a difficult undertaking because many factors influence abundance (see e.g. Yonow *et al.* 2004; Zalucki and Furlong 2005; Schellhorn *et al.* 2008; Muthuthantri *et al.* 2010). Analysis of long-term pest population data based on, for example,light or pheromone trap catches of the adult stageisconfounded as the series represent the effects of local climate, cropping patterns, natural enemies and insecticide use. As DBM can migrate, the effects of these factors in source regions will influence such data sets and they are further complicated by the vagaries of migration (Zalucki and Furlong 2011)! Models that describe the effects of climate on population dynamics can be used as an aid in the interpretation of population changes and the impacts of management practices (e.g. Carriere *et al.* 2003).

Here we are interested in the contribution of climate, natural enemies and crop management practices on the population dynamics of DBM. We use an age structured population model written in DYMEX (Maywald *et al.*1999) to analyse and interpret patterns of DBM abundance data across a wide latitudinal gradient in eastern China; from the tropics (Haikou, 20.01°N) to the cold, temperate north (Beijing, 34.00°N). We use the model to simulate within and between year variations in DBM abundance using climate data from 2000-2012. For one location, Guangzhou, we run simulations with and without a parasitoid and with and without insecticide applications, for various immigration scenarios and cropping practices.

Methods

Modelling climate, cropping patterns, natural enemies and insecticide effects

Apart from the standard housekeeping modules in DYMEX models (e.g. Timer, Circadian, Met-manager etc., see Yonow *et al.* 2004) our model has four key "modules": a crop life-cycle, a DBM life-cycle, a *Diadegma* life-cycle and insecticide application. Each module and its parameterisation is described briefly below and more fully in Li *et al.* (2016a). Parameters were based on expert estimates or fitted to data in the literature. In nearly all cases we have kept equations that describe processes as linear functions above and below thresholds, bounded so as not to produce biologically meaningless values (e.g. negative mortalities or proportions greater than 1).

Crop Life-cycleModule

This module has three life-stages (Crop, Harvested and Stubble) and few processes. The user defines the planting dates and the number of plants in each crop. The number of plants is notional as we are modelling population size in the region as a whole and in this instance "Crop" simply means that hosts are available for oviposition and as food for larvae;"Crop" is also used to calculate a density so that sprays can be timed if required (see below). Crop duration is based on physiological age: the crop develops when temperatures exceed 0°C (with a development rate of 0.001) up to a maximum temperature threshold of 27°C, whereupon rate of development declines (-0.01). Crops are planted at a time specified by the user. A proportion (user specified) transfers to the "Harvested" life-stage when a physiological age of 1 is reached with the remainder transferring to the "Stubble" life-stage. For example a Stubble Proportion of 0.1would mean that 90% of the crop transfers to the Harvested life-stage, and 10% transfers to Stubble. Some time later (again user specified) the stubble is "cleaned up" or removed. We use this feature to simulate different crop management strategies, from a production break, to strict or lax crop hygiene.

DBM Life-cycle Module

This module contains seven life history stages; an egg stage, three larval stages; first (L1), second and third instars combined (L2-3), fourth instar (L4); pre-pupae, pupae and adults; and captures all of the developmental, mortality and reproductive functions that define the way in which these stages interact with their environment.

Processes such as development, mortality and transfer from one life stage to the next, act at the cohort level, not the life stage level. Cohorts are created in each daily time step in which one or more individuals enter a life stage. Each cohort is tracked independently. Cohorts have a number of state variables. These state variables track particular processes, within the life stage (e.g. number, age, stress and thermal accumulation) that drive other processes (e.g. development, mortality, reproduction) in the same or subsequent life stages. The concept of

cohort properties provides an elegant mechanism to account for the range of responses to the different conditions experienced by members of a population (Yonow *et al.* 2004).

Development rates for the immature DBM stages are taken directly from Liu *et al.* (2002) and are modelled as simple two segment linear functions with a lower development threshold and slope to an upper threshold, after which development declines (Li *et al.* 2016). The development rate is driven by the 12 steps per day Daily Temperature Cycle, which is generated in the Circadian Module provided by DYMEX. Using daily maximum and minimum temperatures from a location as input (read using met-manage module) and day-length (generated from a location's latitude by a DYMEX provided feature), the Circadian module generates a daily temperature cycle by applying a 12 segmented sine curve between the daily maximum and minimum temperatures. All of the temperature dependent development and mortality functions in the model are calculated using these temperatures.

When a cohort attains a physiological age of 1 all surviving individuals transfer to the next stage using a simple step function. Mortality functions for rainfall, temperature, harvest, and spray application and if relevant a parasitoid, are included for each of the immature stages (Li *et al.* 2016a). In general immature stages will die at a certain rate on a daily basis if it is too hot (heat stress accumulates for eggs and all larval stages at temperatures above 32^{o}C) or too cold (cold stress accumulates for these stages at temperatures below 8^{o}C), if there is too much rain (when rain exceeds certain threshold), if the crop is harvested, if there are fewer than 0.1 plants available, and/or if the density of L2 – L4 is too high, as determined by the user, and a spray event is triggered. Mortality due to insecticides will only occur if this module is initialised and a spray event triggered.

Larvae are particularly susceptible to rainfall immediately upon hatching and when they emerge from leaf mines at the second instar (e.g. Wakisaka *et al.* 1991; MPZ & MJF unpublished data). Consequently we have included an "establishment mortality" process that affects L1 and L2 on their first day of existence. Older larvae are subject to some rainfall effects (Harcourt 1963). Harvest mortality operates on all immature stages by removing the same proportion of each immature stage from the population as the proportion harvested. Crop residues (Stubble) that harbour immature stages can remain after harvesting. The proportion of immatures removed by harvesting can be adjusted but here we use 90% in all scenarios.

Potential egg production by females is a function of temperatures experienced by a cohort during immature development; both accumulated heat and cold stresses reduce potential fecundity from a notional maximum of 300 eggs per female. Fecundity records for DBM based on laboratory studies range from 100 to 400 eggs per female depending on a range of factors (Muhamad *et al.* 1994; Golizadeh *et al.* 2009; Zhang *et al.* 2012; Soufbaf *et al.* 2014). Potential egg production is a user defined cohort variable that was fitted to laboratory studies that reared DBM under various temperatures and recorded fecundity. Both temperature and rainfall affect egg laying on any particular day (Li *et al.* 2016a). Mortality for the adult stage is specified as a constant proportion per day (0.25). All individuals in a cohort are removed when adults reach aphysiological age of 1.

Diadegma *Life-cycle Module*

This module allows investigation of biological control using a larval parasitoid loosely modelled on *Diadegma semiclausum* Hellen (Hymenoptera: Ichneumonidae) that parasitises theL2-L3 stage larvae of DBM. The *Diadegma* life cycle module consists of 3 stages: *Diadegma* E-A, *Diadegma* Adults and *Diadegma* Eggs. The first stage, *Diadegma* E-A, represents the development and mortality processes acting on *Diadegma* eggs to when they emerge as adults (essentially the parasitised DBM larvae). This life-stage is subject to all factors that affect DBM larval mortality (above, including insecticides if used). Parameters for the parasitoid were taken from Dosdall *et al.* (2012), Wang *et al.* (2004), Soufbaf *et al.* (2014). Development of parasitised larvae is a linearabove threshold (2.8^{o}C) function with a slope of 0.0085. *Diadegma* E-A cohorts transfer to the *Diadegma* Adult life-stage when they reach a physiological age of 1. As with DBM, parasitoid fecundity is determined by rearing temperatures, exactly the same as for DBM (above) but with a maximum potential fecundity of 200. *Diadegma* adult mortality is a linear above threshold function of chronological age and progeny production (=eggs available to parasitise larvae) is a function of temperature; increasing from 5-18^{o}C and declining at temperatures above 18^{o}C and with chronological age; peaking at about 4-5 days of age;

and the number of L2-L3 larvae available to parasitise. Progeny production (number of eggs laid on any one day) is restricted either by how many eggs can be laid or by the number of small DBM larvae (L2 & L3 DBM instars) available to be parasitised. Parasitism increases with L2-L3 density (See Li *et al.* 2016a).

Insecticide application Module

If this module is used it is based on either a threshold density of L2-L3 plus L4 larvae per plant, L1 larvae per plant or on a time-scheduled basis. The spray residue remaining that kills larvae is based on three parameters that determine the number of days the insecticide effect lasts once a spray event is triggered: number of days the spray is maximally effective, an exponential decay rate and a scaling factor that determines the maximum effect. The user can choose different values that notionally represent how well sprays are applied (coverage, formulation, application, *etc.*) and hence the likely effect of the application. The effectiveness of the spray can be adjusted for the life stage to represent the susceptibility of different instar stages, insecticide persistence on the crop and notional resistance levels.

Initialization, climate input and model running

Initialisation of the model defines the scenario being simulated. One can initialise with adults immigrating into a field at the start once and/or at different times, and/ or with different numbers in all stages. The former represents a scenario wherein a field is planted and moths come in from elsewhere, the latter might represent a region with an established population age structure. Modules such as spraying and biological control by *D. semiclausum* can either be used or excluded. Comparisons of simulations with and without the particular module give an indication of how effective that process is in contributing to managing DBM populations.

All climate data entered into the model is from standard meteorological sources and uses daily minimum and maximum temperatures (°C) and rainfall (mm). We use climate data for locations to represent the range of conditions experienced by DBM from the South to the North of China for illustrative purposes: Haikou, Hangzhou and Beijing, with year round cropping.

DYMEX offers enormous flexibility to the user in scenarios that can be simulated. We run simulations with and without the parasitoid, with and without insecticide sprays. We use daily climate data from 2000-2012 for Guangzhou to represent the effect of a range of conditions. Using average conditions in Guangzhou, we investigate the effect of crop planting and crop hygiene.

RESULTS

DBM population dynamics: the effect of climate & spraying

The effect of climate on generation time and population build up is apparent as one moves from South (Haiku) to North (Beijing) in China (Figure 1). The effect of high summer temperatures can be seen in the decline of populations in Haikou and Hangzhou during this period and in limiting generation number – high temperatures extend generation time. Haikou has 11 generations and populations would increase if unmanaged. In Hangzhou there are 8 generations and populations decline with the onset of winter. In Beijing conditions only become suitable in late spring and they remain highly favourable for 5-6 generations, before conditions deteriorate and populations decline.

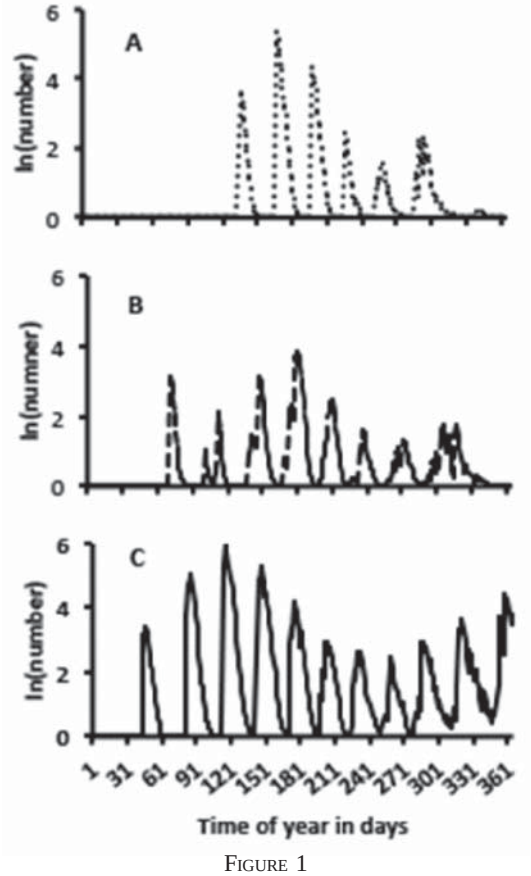

FIGURE 1

Simulated population dynamic of DBM adults based on average climate conditions (average of 2000-2012) over a year for: (A) Beijing, (B) Hangzhou and (C) Haikou. Simulations had year round cropping and were colonised by 100 adults on 1 January (not shown) for Hangzhou and Haikou and 1 April for Beijing

The effect of climate at a location across years (Fig. 2) indicates the wide variation amongst years and the consistent phenology. Populations are high in Guangzhou in spring, decline in summer and increase in autumn in simulations (Fig. 2A); a similar pattern is also observed in field data, e.g. for Shijing (Figure 2B) (data from Li *et al.* 2012). It is apparent that some years can be much worse than others.The population was sprayed on threshold, which effectively acts as a limitation on population size. The simulated and observed abundances are similar in magnitude (Fig. 2).

The effect of cropping pattern, migration and poor spray application

The effect of harvest and when the next crop is planted on pest pressure depends on how long stubble (crop residue) is retained and on the presence of natural enemies (Table 1, Figure 3). Immediate removal of stubble effectively removes an infesting population and DBM populations are greatly reduced (Figure 3A). With no migration the population goes extinct in early summer (data not shown). Leaving stubble for a week greatly increases pest pressure (Table 1), both in terms of mean abundance and the need to manage with sprays. Adding natural enemies reduces spray requirements (Table 1) as expected. The timing of migration input is critical. The pest problem is greater if moths colonise each new crop at its planting rather than by regular monthly input. This is because harvest and clean-up effectively remove the population in the crop, whereas colonisation at planting allows DBM to sometimes complete more than one generation and the population to build up. Not surprisingly weekly migration input leads to greater pest problems (Table 1, Figure 3B).

We simulated the effect of ineffective sprays due either to poor coverage/ application or resistance by simply reducing the level of mortality following a single spray event. Not surprisingly, the worst-case scenario of sequential cropping, poor hygiene and weekly migration input resulted in the number of sprays increasing to 24 and in Guangzhou the population was the highest in the sequential cropping scenario with poor crop hygiene (average population of 51/day). Spaying using farmer practice of once a week from February with an ineffective spray regime increased average population to 281/day at a cost of 48 sprays! This result highlights the need for well-timed sprays, which are part and parcel of an IPM approach.

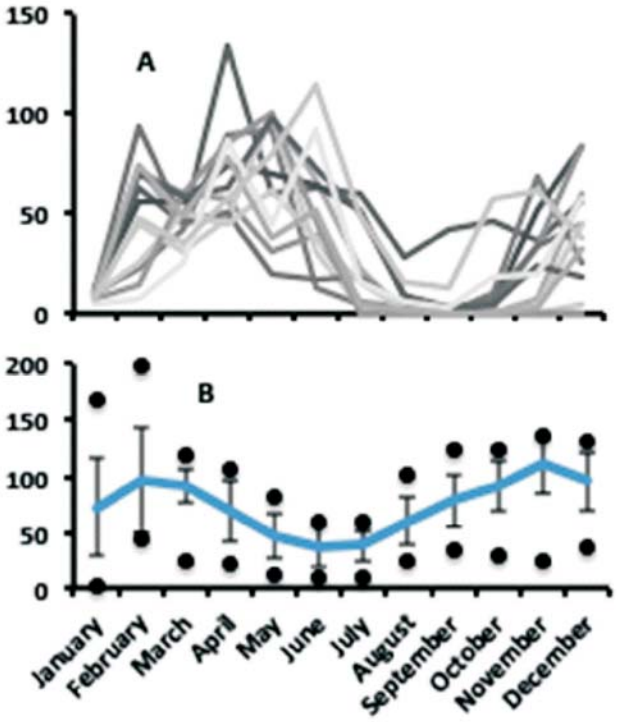

FIGURE 2

Simulated variation in the mean monthly abundance of DBM in Guangzhou based on climate data for each year from 2000-2012, with year round crop production, initial population of eggs (465), L1 (400), L2 & 3 (350), L4 (310), pupae (300), males (150) and females (150) and spraying on L2-L4 threshold of 10/plant (A) and observed variation in male trap catch at Shijing (mean±SD and min and max values) for a similar period, 2003-2013 (B)

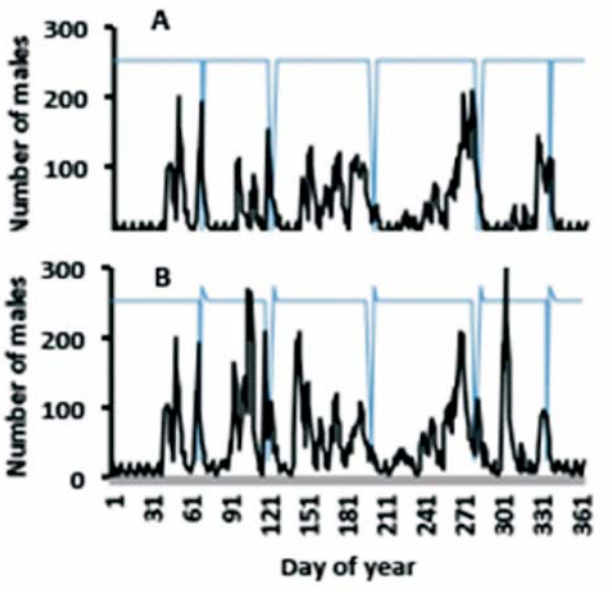

FIGURE 3

Simulation of DBM population dynamics in a cropping system with strict hygiene (A) and with poor crop stubble hygiene (B) with moths migrating into the crop weekly (light line is crop phenology)

TABLE 1

Pest status of DBM expressed as the average number of males/day over the year and number of sprays required based on an L2-L4 density of 10/ plant for an average climate year in Guangzhou for two cropping regimes; rapid clean-up of residues (1 day retention) and stubble remaining for 7 days for each of 2 migration scenarios: no migration except on day 1, migration input of 100 females every weekly (see Figure 3 for population traces and crop phenology). First value in brackets is the number of sprays required over the year, the second is sprays with biological control added

Migration scenario	**Stubble Retention time**	
	1 day	**7 days**
No migration	3.2 (2)(2)	10.9 (5)(3)
Weekly	38.3 (8)(6)	47.3 (14)(7)

DISCUSSION

The DYMEX approach to population modelling allows investigation of age structured population dynamics as influenced by climate for a wide range of scenarios. Insects have discrete generations with known temperature mediated development times and effects on survival and reproduction (e.g. Liu *et al.* 2002). Different stages have very different susceptibilities to extreme climate variables of temperature and rainfall (e.g. Yonow *et al.* 2004), and host plant effects (e.g. Soufbaf *et al.* 2010a, 2010b). These stages may or may not be present in the population when the events occur and the timing of events is critical to their subsequent population effects.

DBM shows wide variation in abundance and timing of population peaks depending on location and climate. From the tropical south to the extreme cold of the north, the patterns of variation suggest broad climate effects; summer in the south and central coastal areas are generally too hot, development slows (Figure 1) and populations decline (Figure 1). Further north winters are too cold and populations can only persist in the warmer months, but populations that migrate to Beijing type climates can do very well seasonally. These observations are consistent with our earlier analysis of climate effects using CLIMEX (Li *et al.* 2012).

Our DYMEX model captures temporal patterns (Figure 2) with some caveats that suggest management practices, particularly crop hygiene, are also crucial (Figure 3). However our simulation suggests that adding biological control can dramatically reduce insecticide inputs (Table 1), as has been suggested by research assessing natural enemies (Furlong *et al.* 2004a, 2004b) and classic biological control. DBM may well be an induced pest (Furlong *et al.* 2013; Li *et al.* 2016b).

Although there is a climate signal in both the observed and simulated population data, strong management and migration effects are indicated. The importance of crop hygiene is again highlighted. Our simulations capture the dynamics for the organic production farm at Zengcheng that exports to the Hong Kong market versus farms that produce for local consumption. With strict hygiene, as occurs on the organic farm, DBM only becomes problematic with high migration input (weekly) and even then it is manageable. Whereas leaving crop residues, as occurs on farms in Huadu and Shijing effectively doubles to quadruples the management spray requirements, depending on the migration pressure (Table 1). Timely spraying as required, based on appropriate thresholds, with good spray application (e.g. Guo *et al.*2004) is essential. Spraying once a week with an ineffective spray leads to the highest population pressure.

Management responses needed, if growers are prepared to implement them, are fairly obvious: better cultural control or crop hygiene (as in the organic farm at Zengcheng), sample crops for pests and then spray only if required, be sure to target the right stage (small larvae not large), use appropriate spray equipment (Guo *et al.* 2004) and preferably use insecticides that are biocontrol compatible (Furlong *et al.* 2008). Above all rotate insecticides (e.g. Heckel 2004) as part of an IRM strategy. This is the standard IPM mantra of the SSP (sample, spray and pray) kind (Zalucki *et al.* 2009) and it by and large works (e.g. Liu *et al.* 2004, 2014). The problem, as always, is implementation and adoption (Heisswolf *et al.*1997).

We believe at this stage that our model is too simple. Our intention is to reproduce the actual observed dynamics for any one site over a number of years. We have shown here that initialisation is important. To better capture crop dynamics and migration of both DBM and natural enemies we will incorporate “demes”- essentially sub-populations that interact. We will need to refine our parasitoid attack functions and the effect of rainfall on the DBM dynamics.

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Genome-Wide SNP Discovery in Field and Laboratory Colonies of Australian *Plutella* Species

K.D. Perry[1], S.M. Pederson[2] and S.W. Baxter

[1]School of Agriculture, Food & Wine, University of adelaide, South Australia 5005

[2]School of Biological SCiences, University of adelaide, South Australia 5005

Abstract

Understanding dispersal and gene flow is an important focus of evolutionary biology, conservation biology and pest management. The diamondback moth, *Plutella xylostella*, is a worldwide pest of *Brassica* vegetable and oilseed cropping systems. This insect has high dispersal ability, which has important consequences for population dynamics and the potential spread of insecticide resistance genes. Population genetic studies of the diamondback moth have found little evidence of population structure, suggesting that frequent intermixing occurs within regions, however the patterns of local and regional dispersal remain to be identified. For this and many other pest species, understanding dispersal is crucial for developing integrated management tactics such as forecasting systems and insecticide resistance management plans. In recent years, next generation sequencing (NGS) methods have provided previously unparalleled resolution for population genetic studies in a wide range of species. Here, we assessed the potential of NGS-derived molecular markers to provide new insights about population structure in the diamondback moth. We use restriction-site-associated DNA sequencing (RAD-Seq) to discover hundreds to thousands of single nucleotide polymorphism (SNP) markers in nine field and laboratory-reared populations collected from Australia. Genotypic data from RAD-Seq markers identified a cryptic species, *P. australiana*, among individuals collected from a wild host, *Diplotaxis* sp., indicating strong divergence in the nuclear genomes of two Australian *Plutella* lineages. Significant genetic differentiation was detected among populations of *P. xylostella* used in our study, however this could be explained by reduced heterozogosity and genetic drift in laboratory-reared populations founded by relatively few individuals. This study demonstrates that RAD-Seq is a powerful method for generating SNP markers for population genetic studies in this species.

Keywords: Gene flow, population structure, RAD-Seq, *Plutella australiana*, insecticide resistance

Dispersal is a fundamental life history trait with important consequences for the spatial and temporal dynamics of populations. 'Effective' dispersal (resulting in reproduction) also affects allele frequencies and the genetic structure of populations, and consequently, evolutionary processes (Broquet and Petit 2009). For example, adaptation and speciation depend on a balance between selection and gene flow (Turelli *et al.* 2001, Endersby *et al.* 2008). For pest species, quantifying dispersal and its effects on genetic structure is crucial to developing integrated management tactics, such as forecasting systems (Lushai and Loxdale 2004, Zalucki and Furlong 2005) and insecticide resistance management strategies. Molecular markers are a powerful tool for assessing the geographic structure of populations and inferring patterns of gene flow at large scales (Roderick 1996). Population genetic approaches have been successfully employed to infer patterns of long distance dispersal in a range of insect pests (Kim and Sappington 2013, Sun *et al.* 2015).

The diamondback moth, *Plutella xylostella*, is a worldwide pest of *Brassica* vegetable and oilseed crops. (Furlong *et al.* 2008, Zalucki *et al.* 2012, Furlong *et al.* 2013). The success of this insect is due in part to its remarkable genetic plasticity (Henniges-Janssen *et al.* 2011), large effective population sizes and high genetic diversity (You *et al.* 2013) that enable it to rapidly adapt to local environments and evolve insecticide resistance (Furlong *et al.* 2013). Furthermore, the diamondback moth is highly mobile and well-adapted to exploit its short-lived Brassicaceous hosts. It displays predominantly short-range dispersal within high quality host patches (Mo *et al.* 2003) but when habitat quality deteriorates, may utilize high altitude air currents to migrate long distances and colonise new habitats (Chu 1986, Chapman *et al.* 2002, Leskinen *et al.* 2011, Fu *et al.* 2014). In some years, large scale migration events instigate damaging outbreaks in crops (Dosdall *et al.* 2004, Wei *et al.* 2013). In most regions, the dispersal ecology of diamondback moth is poorly understood, yet this knowledge is critical for the development of forecasting systems and insecticide resistance

management plans (Furlong et. al. 2008). One reason for this is the challenging nature of studying long distance dispersal in small insects (Lushai and Loxdale 2004).

Previous population genetic studies in diamondback moth have employed a range of molecular markers, including allozymes (Caprio and Tabashnik 1992, Noran and Tang 1996, Kim *et al.* 1999, Pichon *et al.* 2006), ISSRs (Roux *et al.* 2007), microsatellites (Endersby *et al*. 2006) and mitochondrial genes (Chang *et al.* 1997, Kim *et al.* 2003, Li *et al.* 2006, Saw *et al.* 2006, Niu *et al.* 2014). Several authors have reported population differentiation at inter-continental scales (Endersby *et al*. 2006, Pichon *et al*. 2006, Roux *et al*. 2007). However, most studies from around the world have found little evidence of population structure within regions, including China (Kim *et al.* 1999, Li *et al.* 2006), Korea (Kim *et al.* 1999, Kim *et al.* 2000, Kim *et al.* 2003, Li *et al.* 2006), the USA (Caprio and Tabashnik 1992, Chang *et al.* 1997) and Australia (Endersby *et al*. 2006, Saw *et al*. 2006). These findings suggests that frequent intermixing occurs within regions, however the local and regional patterns of dispersal remain to be identified. More recently, studies using mitochondrial markers, or complementing these with microsatellite (Wei *et al.* 2013) or ISSR (Yang *et al.* 2015) nuclear markers, have provided new insights into seasonal migration routes from southern to northern regions of China, and identified potential geographic barriers to gene flow (Niu *et al.* 2014). Mitochondrial markers have also recently identified a novel *Plutella* lineage in Australia (Landry and Hebert 2013).

In recent years, next generation sequencing (NGS) has revolutionized the fields of molecular ecology and population genetics. Reduced representation sequencing methods (Narum *et al.* 2013) combined with the power of high-throughput NGS platforms (Glenn 2011) facilitate rapid and cost-effective marker discovery and genotyping in a wide range of organisms (Davey *et al.* 2011). Restriction-site-associated DNA sequencing (RAD-Seq) (Baird *et al.* 2008) is one of several reduced-representation methods for sequencing targeted regions across the genome at high sequencing depth, providing numerous advantages over traditional markers for population genetic studies (Davey and Blaxter 2010). RAD-Seq studies have provided new insights into previously undetected population genetic structure in a wide range of contexts (Narum *et al.* 2013, Reitzel *et al.* 2013) including terrestrial invertebrates (Nadeau *et al.* 2013, Lozier 2014).

Here, we assess the potential of NGS methods to provide new insights about population structure in the diamondback moth. We use RAD-Seq to discover single nucleotide polymorphism (SNP) markers in nine field and laboratory-reared populations collected from Australia. The RAD-Seq markers facilitate an initial assessment of genetic diversity within and among these populations.

Materials and Methods

Sample collection

Samples of diamondback moth were collected from *Brassica* vegetables, canola or wild *Brassica* hosts from nine locations in Australia between September 2012 and April 2014 (Table 1, Figure 1B). At each location, individuals were collected using a sweep net or by direct sampling. Seven populations were reared in laboratory cages on cultivated cabbage and 10% honey solution for between one and six generations. Individuals from two field populations and six of the laboratory-reared populations were preserved in 20% DMSO, 0.25M EDTA salt saturated solution (Yoder *et al.* 2006) and stored at -80°C. Individuals from the Nundroo population were stored in USP Grade propylene glycol and stored at -20°C.

RAD library preparation and sequencing

Libraries for RAD sequencing were prepared following a protocol modified from Baird *et al.* (2008). Genomic DNA was extracted from individual larvae or pupae by homogenizing tissue in DNA isolation buffer (Zraket *et al.* 1990) followed by two phenol and one chloroform extractions. DNA was treated with RNase A then precipitated and re-suspended in TE buffer. Genomic DNA was quantified using a Qubit 2.0 fluorometer (Invitrogen) and 200 ng digested with 10 units of *Sbf1* in Cutsmart Buffer (NEB) for 1 hour at 37ºC then heat inactivated at 80ºC for 20 minutes. P1 adapters with one of six molecular identifiers (MIDs) (AATTT, AGCTA, CCGGT, GGAAG, GTCAA or TTCCG) were annealed then ligated to digested DNA (top strand 5'-GTTCAGAGTTCTACAGTCCGACGATCxxxxxTGCA-3', bottom strand 5'-Phos-xxxxxGATCGTCGGACTG

TAGAAC-3', x represents sites for MIDs) using 1 μL T4 DNA ligase (Promega), 1 mM ATP, Cutsmart Buffer. Six individuals from different populations were pooled to form 12 library groups, each containing different P1 adapters to facilitate sample multiplexing. Library pools were then sheared using a Bioruptor sonicator (diagenode), ends blunted (NEB), adenine overhangs added then P2 adapters ligated (top strand 5'-Phos-TGGAATTCTCGGGT GCCAA-3', bottom strand 5'-CCTTGGCACCCGAG AATTCCAT-3'). DNA purification between each step was performed with magnetic beads (AMPure). PCR library amplification conditions were 16 cycles of 98ºC for 10 seconds, 65ºC for 30 seconds and 72ºC for 30 seconds using RP1 (forward) 5'-AATGATACGGCGACCACCGAGA TCTACACGTT CAGAGTTCTACAGTCCGA-3' and 12 unique RPI-indexed (reverse) 5'-CAAGCAGAAGACGGCATAC GAGATxxxxxx GTGACTGGAGTTCCTTGGCA CCCGAGAATTCCA-3' primers. Libraries were run on agarose gel to size select DNA fragments 300-700 base pairs in length. Paired end sequencing using 100 bp reads was performed over two lanes of Illumina HiSeq2500 at the Australian Cancer Research Foundation (ACRF) Cancer Genomics Facility.

Read filtering and variant calling

A total of 131.6 million raw sequence reads were de-multiplexed using RADTOOLS v1.2.4 (Baxter *et al.* 2011) then 50.3 million PCR duplicates were removed using the clone_filter tool in STACKS v1.19 (Catchen *et al.* 2013). Read trimming, adapter removal and quality filtering were performed in TRIMMOMATIC v0.32 (Bolger *et al.* 2014). First, a thymine base overhang added during P2 adapter ligation was trimmed from reverse reads, then paired end trimming was performed using the ILLUMINACLIP tool to remove adapter, trailing low quality bases (quality score<3), bases within a 4-base sliding window with average quality below 15 and trimmed reads shorter than 40 bp. Paired reads were aligned to the *Plutella xylostella* reference genome (version 1.1, modified to include the mitochondrial genome, accession number: JF911819) using STAMPY v1.0.21 (Lunter and Goodson 2011) with —baq and —gatkcigarworkaround options and expected substitution rate set to 0.005. Genotypes were called using the GENOME ANALYSIS TOOLKIT (GATK) v3.3-0 (McKenna *et al.* 2010, DePristo *et al.* 2011) HaplotypeCaller tool following the GATK Best Practices Workflow for GVCF-based Cohort Analysis (Van der Auwera *et al.* 2013). Sites with a genotype quality (GQ) ≥ 30 were retained. Filtering was performed using VCFTOOLS v0.1.12a (Danecek *et al.* 2011) to identify a set of variant sites for population genetic analysis. We removed indels and retained bi-allelic SNPs that passed the following quality filters: genotyped in at least 60 of 72 individuals, QUAL≥400, average read depth between 20 and 100 across individuals, minor allele frequency ≥ 0.2 and in Hardy-Weinberg equilibrium with p-value set to 0.05. To avoid closely linked markers, variants were separated by a minimum distance of 2 kb using the VCFTOOLS —thin function. A final set of 1285 SNP variants were retained after filtering. In addition, from the GATK HaplotypeCaller output, we generated a set of all confidently called variant and invariant sites (GQ≥30). Filtering was performed using VCFTOOLS v0.1.12a (Danecek *et al.* 2011) to remove indels and sites located within transposons, and retain sites genotyped in 60 of 72 individuals with mean depth between 20 and 100 across individuals. After filtering, we retained 491 831 confidently called variant and reference sites, including 623 sites from the mitochondrial genome.

Population genetic analysis

We used the 491 832 sites to generate a phylogeny for 72 individuals using a neighbor-joining clustering method implemented in the program GENEIOUS v7.1.9 (Kearse *et al.* 2012). An individual representing a cryptic species, *P. australiana*, 'Calca-6', was used as the out-group. We assumed the Tamura-Nei (1993) model and resampled with 1000 bootstraps to generate a consensus tree displaying nodes with at least 50% consensus support and visualized the resulting tree in the program FIGTREE v1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/).

Summary population statistics were calculated for variant SNPs (n=1285) and separately for all confident variant and invariant sites (n=491 832). The VCFTOOLS (Danecek *et al.* 2011) —depth function was used to calculate average site depth, and vcf-stats used to calculate the average number of genotyped sites and private alleles. Expected and observed heterozygosity and the inbreeding coefficient, F_{IS}, were calculated and significance determined by bootstraps (1000 bootstraps for the SNP variants, and 100 bootstraps for the 491 832 sites due to computational limits) using modified functions in the R package diveRsity

TABLE 1

Collection details of Plutella *populations from Australia genotyped using RAD-Seq*

Location	Latitude	Longitude	Host	Collection date	[a] Lifestage collected	[b] F_1 Individuals	Generation sequenced
Esperance, Western Australia	-33.8588	121.8931	Canola	26/9/2012	L, P	59	F6
Nundroo, South Australia	-31.7516	132.0565	Canola	19/8/2013	L	86	F2
Calca, South Australia	-33.0492	134.3729	Wild *Brassica* [c]	8/4/2014	L	-	Field
Picnic Beach, South Australia	-34.1696	135.2744	Wild *Brassica* [d]	7/4/2014	L	40	F1
Mallala, South Australia	-34.4383	138.5099	Canola	11/9/2013	L	173	F5
Virginia, South Australia	-34.715	138.557	Cauliflower	7/3/2013	L, P	-	Field
Glenore Grove, Queensland	-27.528	152.407	Cabbage	11/10/2012	L, P	25	F5
Mt Sylvia, Queensland	-27.717	152.220	Cabbage	3/10/2012	L, P	59	F5
Tenthill, Queensland	-27.566	152.235	Red cabbage	11/10/2012	L, P	40	F5

[a] Larvae (L), pupae (P), [b] Number of F_1 individuals used to establish laboratory populations, [c] *Diplotaxis* sp., [d] Sea rocket, *Cakile maritima*

(Keenan *et al.* 2013). To examine the effects of laboratory-rearing on genetic diversity, we plotted the distributions of average heterozygosity in R (R Core Team 2014).

To investigate population differentiation, a global estimate of Weir and Cockerham's (1984) F_{ST} with 99% bootstrap confidence intervals (10 000 bootstraps) was calculated in the R package diveRsity (Keenan *et al.* 2013). Pairwise F_{ST} values (Weir and Cockerham 1984) were calculated and significance determined using exact G tests implemented in GENEPOP v4.3 (Rousset 2008) after Bonferroni correction for multiple comparisons (Dunn 1961). To test for genetic isolation by distance (Wright 1943), we performed a Mantel test (Mantel 1967) using 10 000 permutations on the regression of Slatkin's (1995) linearized F_{ST} transformation ($F_{ST}/(1- F_{ST})$ onto the natural log of geographic distance (Rousset 1997) using the R package ade4 (Dray and Dufour 2007). Geographic distances were calculated using the GEOGRAPHIC DISTANCE MATRIX GENERATOR (Ersts 2007).

We used the 1285 SNP variants to analyse population structure using a Bayesian clustering method in the program STRUCTURE v2.3.4 (Pritchard *et al.* 2000). Variant data were converted from VCF to STRUCTURE file format using PGDSPIDER v2.0.8.2 (Lischer and Excoffier 2012). STRUCTURE analysis was used to infer the number of genotypic clusters and assign individuals to clusters. Analyses were performed for all individuals (n=72) and separately for *P. xylostella* individuals only (n=69). For each analysis, we assumed the admixture model with correlated allele frequencies and the locprior model, specifying nine geographic populations. For each analysis, we performed ten independent STRUCTURE runs for each value of *K*=1-10, where *K* is the number of genotypic clusters. For all runs, we used 500 000 burn-ins and 500 000 MCMC replicates. The optimal *K* was determined using the delta *K* method of Evanno *et al.* (2005) visualized in the program STRUCTURE HARVESTER (Earl and vonHoldt 2012). Individual and population Q-matrices (containing posterior probability of assignment to genotypic clusters) across replicate structure runs were aligned in the program CLUMPP v1.1.2 (Jakobsson and Rosenberg 2007) and visualized in DISTRUCT v1.1 (Rosenberg 2004).

PCR genotyping assays

To examine the frequency of mutations associated with pyrethroid resistance, we performed PCR based genotyping assays for three point-

mutations in the voltage gated sodium channel, T929I (*skdrl*), L1014F (*kdr*) and F1020S (*cdr*) according to Endersby *et al.* (2011). MyTaq polymerase (Bioline) was used for amplification in a Verity thermocycler (ABI).

To distinguish between *P. xylostella* and *P. australiana* lineages, we developed a PCR-RFLP genotyping assay using COI sequence published by Landry and Hebert (2013). Genomic DNA was amplified using a modified LCO1492_Px primer (5'-TCAACAAATCA TAAA GATATTGG-3') and HCO2198 (5'-TAAACTTCA GGGTGACCAAAAA ATCA-3') (Folmer *et al*. 1994). Ten microliter reactions were run with 2 µL of MyTaq 10x buffer, 0.4 µL of each primer (10 µM stocks), 1 µL of DNA (approx. 5 ng) and 0.05 µL of MyTaq polymerase (Bioline). Samples were amplified at 95 ºC for 2 minutes, then 35 cycles at 95ºC for 10 seconds, 52ºC for 20 seconds, 72ºC for 30 seconds) followed by a 5 minute final extension at 72ºC. PCR products were then digested at 37ºC for 1 hour with *AccI* restriction enzyme with 2 µL Cutsmart Buffer and 1 unit of *AccI* (NEB) to a final volume of 20 µL. Following digestion, products were separated using agarose gel electrophoresis (1.5%). *P. xylostella* products are approximately 517 bp and 191 bp and *P. australiana* products are 348 bp and 360 bp (Figure 1C).

Results

Population genetic analysis

Using RAD sequencing we identified a set of 491 832 variant and invariant sites, representing 0.146% of the *P. xylostella* reference genome, and a subset of 1285 SNP variants, for population genetic analysis. Individuals from nine field and laboratory-reared populations from Australia were genotyped at an average read depth of 45 (Table 1).

The 491 832 confident sites were used to generate a neighbour-joining phylogeny for all 72 individuals (Figure 1A,B). Seven individuals collected from South Australian locations, three from Calca, three from Nundroo and one from Picnic Beach, were confidently resolved with 100% consensus support. Three individuals, Calca-4,-6 and -7, showed particularly long branch lengths, although the branch length estimate for Calca-4 is inflated due to missing data (48% sites genotyped). We performed PCR mitochondrial genotyping assays to assess whether the dataset contained multiple *Plutella* lineages. The assays identified the three divergent individuals, Calca-4,-6 and -7, as a cryptic species, *P. australiana*, and confirmed that all other individuals (n=69) were *P. xylostella* (Figure 1C). These results based on RAD-Seq markers indicate strong divergence in the nuclear genome between the two Australian *Plutella* lineages, consistent with high mitochondrial sequence divergence already reported (Landry and Hebert 2013). Four basal *P. xylostella* individuals, Picnic Beach-5, -6, -9 and Nundroo-8, were also confidently resolved (100% consensus support), and relative branch length estimates suggest strong divergence from other *P. xylostella*. All remaining *P. xylostella* individuals could not be confidently resolved (<50% consensus support), despite some evidence of individuals grouping according to geographic location.

The levels of heterozygosity were used to assess genetic diversity within and among populations. For all genotyped sites, the average observed heterozygosity ranged from 0.0085 to 0.0150 for *P. xylostella* populations but was notably higher at 0.0220 for the three *P. australiana* individuals (Table 2). The average number of private alleles among *P. xylostella* populations ranged from 20-91, while three *P. australiana* individuals had a much higher average of 1476 private alleles (range 606-2063), consistent with strong divergence. For the variant SNP dataset, the average observed heterozygosity ranged from 0.3052 to 0.5115 for *P. xylostella* populations, and again was higher at 0.5619 for the three *P. australiana* individuals (Table 2). Private alleles were filtered out of the SNP variant dataset. Average levels of heterozygosity were variable within and among populations (Figure 2). Laboratory-reared populations, Esperance, Glenore Grove and Mt Sylvia, show the lowest levels of genetic diversity (Figure 2), and were founded with relatively low numbers of individuals, ranging from 25-59 (Table 1). The values for the inbreeding coefficient, $F_{IS,}$ were significantly different from zero for these three populations (Table 2).

The global estimate of F_{ST} across all *P. xylostella* populations and loci was significantly different from zero (F_{ST} = 0.0487, 99% CL 0.0187-0.0905), indicating significant genetic differentiation among the nine populations. The pairwise F_{ST} values indicate that the differentiation is associated with populations from the three regions, Western Australia, South Australia and Queensland (Table 3). However, patterns of differentiation did not clearly relate to geographic proximity and may reflect inbreeding in laboratory populations. Glenore Grove, for example, was highly differentiated from all other populations including those from Tenthill and Mt Sylvia in close proximity (18-28 km). Lower F_{ST} values among some South.

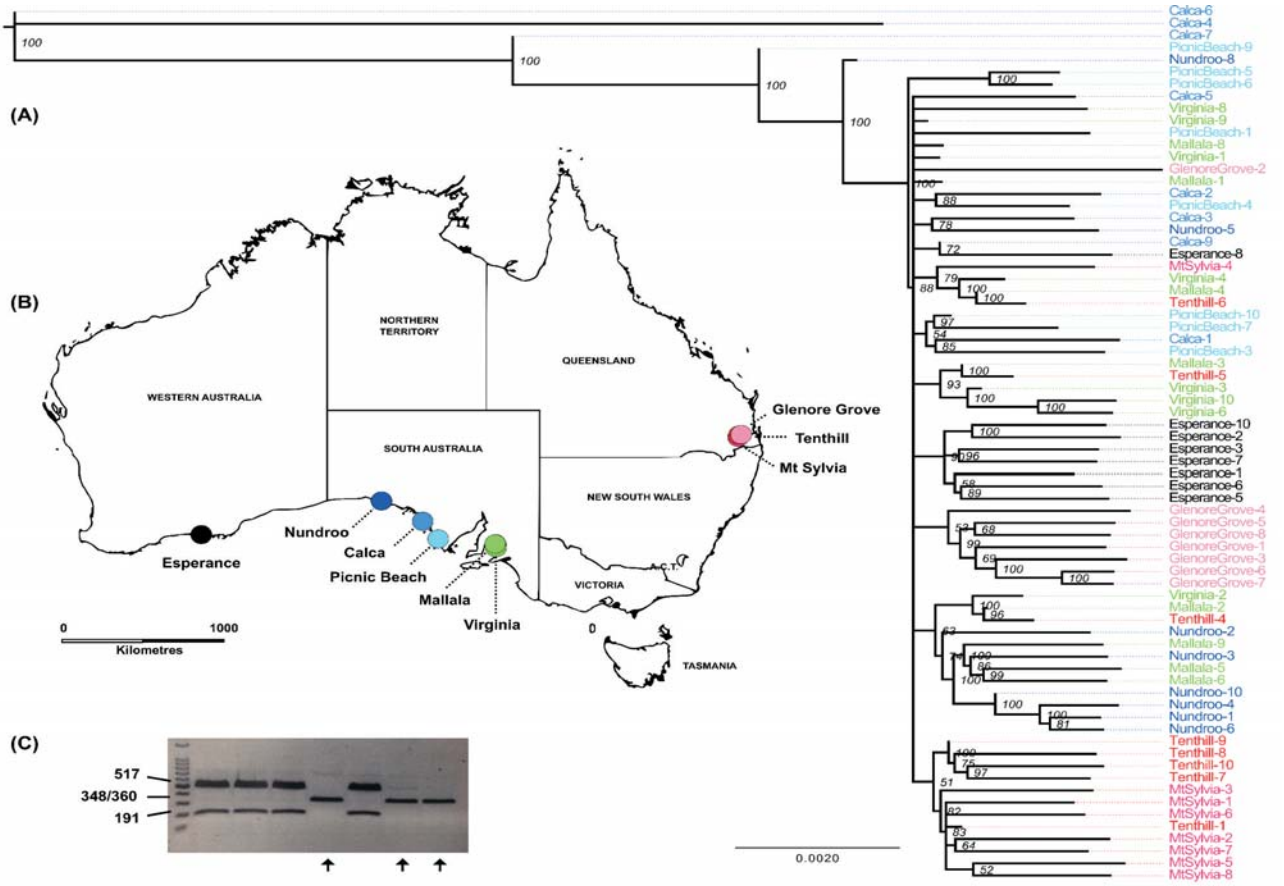

FIGURE 1

(A) Neighbour-joining consensus tree generated from 491 832 variant and invariant sites for 72 Plutella individuals, displaying nodes with at least 50% consensus support. Node labels are the percentage consensus support for 1000 bootstrap replicates. (B) Map of collection sites for nine Plutella populations from Western Australia (n=1), South Australia (n=5) and Queensland (n=3). (C) Gel electrophoresis image showing the results of a mitochondrial CO1 gene assay to distinguish the two Plutella lineages. P. australiana is genotyped as a single band, shown by black arrows and P. xylostella as two bands

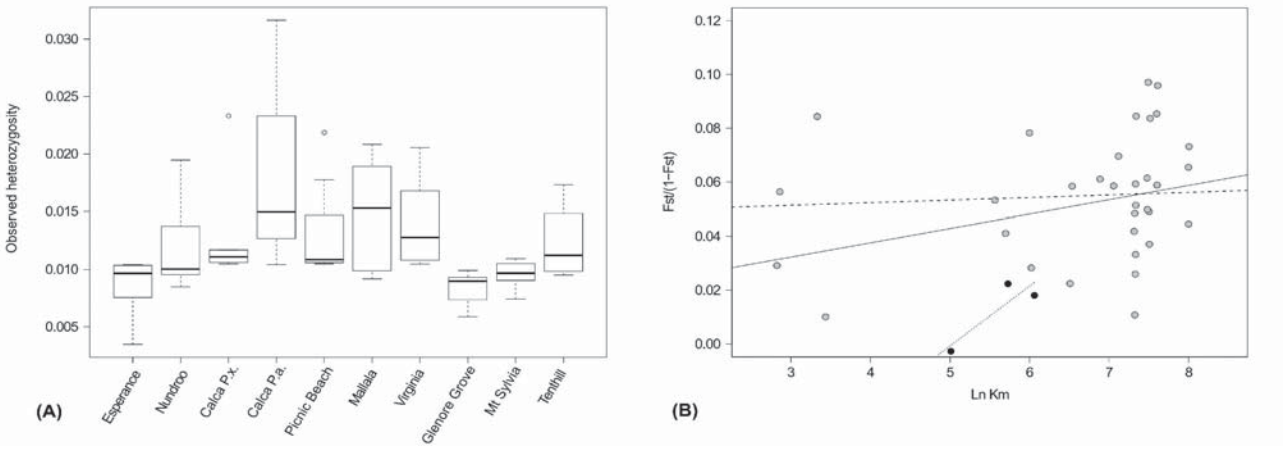

FIGURE 2

(A) Distributions of observed heterozygosity for Plutella populations. Each population contains eight individuals. Calca is split into two lineages, Calca P.x. (n=5) and Calca P.a. (n=3). (B) Regressions of Slatkin's linearized genetic distance (F_{ST}/1- F_{ST}) against the natural log of geographic distance (Ln km) for all pairwise population comparisons of P. xylostella (n=69 individuals). Lines represent the fitted linear regression model for pairwise comparisons for 'all populations' (grey and black circles, solid line, y= 0.00529x+0.01646, Mantel's r=0.3048, p=0.0215), 'cage' populations (grey circles, dashed line, y=0.00096x + 0.04854, Mantel's r=0.07388, p=0.36226) and 'field' populations (black circles, dotted line, y=0.02224x-0.11197, Mantel's r=0.88862, p=0.33307)

TABLE 2

Population statistics calculated for the SNP variant sites (top) and for all confidently called (GQ≥30) variant and invariant sites (bottom) for populations of Plutella *species collected from Australia. Populations each contain eight sequenced individuals with Calca split into* P. xylostella *(n=5) and* P. australiana *(n=3) individuals. Statistics include the population means for the number of individuals genotyped per locus (N), number of sites genotyped, site depth, number of sites unique to each population (private alleles), the proportion of observed (H_O) and expected (H_E) heterozygosity, and Wright's inbreeding coefficient (F_{IS})*

Population	N	Sites genotyped	Site depth	Private alleles	H_O	H_E	F_{IS}
SNP variants (n=1 285)							
Esperance	6.7	1073	32	0	0.3485	0.3650	0.0451
Nundroo	7.6	1215	54	0	0.4008	0.3763	-0.0653
Calca_Px	4.8	1246	65	0	0.4327	0.3844	-0.1256
Calca_Pa	2.0	855	34	0	0.5249	0.3233	-0.6173
Picnic Beach	7.7	1238	73	0	0.3977	0.3821	-0.0408
Mallala	7.7	1229	42	0	0.5619	0.4263	-0.3181
Virginia	7.4	1194	30	0	0.5115	0.4032	-0.2685
Glenore Grove	7.2	1158	34	0	0.3052	0.3469	0.1202
Mt Sylvia	7.3	1169	33	0	0.3490	0.3667	0.0482
Tenthill	7.3	1175	32	0	0.4827	0.4026	-0.1991
All variant and invariant sites (n=491 832)							
Esperance	7.5	462076	33	41	0.0090	0.0108	0.1715*
Nundroo	7.8	479764	54	32	0.0120	0.0120	0.0041
Calca_Px	4.9	482269	65	91	0.0135	0.0129	-0.0492
Calca_Pa	2.4	398538	39	1476	0.0220	0.0185	-0.1923
Picnic Beach	7.9	482882	72	77	0.0132	0.0133	0.0113
Mallala	7.9	484254	43	20	0.0150	0.0129	-0.1651
Virginia	7.8	480662	31	53	0.0142	0.0129	-0.1017
Glenore Grove	7.6	464262	35	28	0.0085	0.0103	0.1800*
Mt Sylvia	7.7	473837	34	30	0.0098	0.0112	0.1313*
Tenthill	7.8	479215	33	20	0.0126	0.0118	-0.0658

* F_{IS} values in bold are significantly different from zero according to bootstrapped 95% CL.

TABLE 3

Pairwise comparisons of genetic distance measured by Weir and Cockerham's (1984) F_{ST} (lower diagonal) and geographic distance in km (upper diagonal) for all population pairs of P. xylostella

	Esperance (F_6)	Nundroo (F_2)	Calca_Px (F_0)	Picnic Beach (F_1)	Mallala (F_4)	Virginia (F_0)	Glenore Grove (F_5)	Mt Sylvia (F_5)	Tenthill (F_5)
Esperance, WA		979	1162	1234	1530	1534	2993	2968	2975
Nundroo, SA	0.0576***		261	403	672	689	2021	1997	2004
Calca_Px, SA	0.0554	0.0506		150	413	429	1836	1811	1819
Picnic Beach, SA	0.0651***	0.0726***	-0.0027		299	307	1793	1767	1776
Mallala, SA	0.0322	0.0220	0.0275	0.0395		31	1531	1504	1515
Virginia, SA	0.0489	0.0553	0.0178	0.0219	0.0100		1541	1514	1525
Glenore Grove, Qld	0.0682***	0.0875***	0.0772***	0.0885***	0.0560**	0.0779***		28	18
Mt Sylvia, Qld	0.0615***	0.0787***	0.0467	0.0579***	0.0402	0.0462	0.0778***		17
Tenthill, Qld	0.0427	0.0556	0.0358	0.0474	0.0108	0.0253	0.0534**	0.0284	

Significance of exact G tests after Bonferonni correction: P<0.05 *, P<0.001**, P<0.0001***

Australian populations suggest high levels of gene flow within this region, however these populations were also not differentiated from Tenthill in Queensland despite large geographic separation (1515-1819 km). The Mantel test for all pairwise population comparisons indicated a weak but significant effect of isolation by distance (n=69 individuals, r = 0.3048, p=0.0215) (Figure 2B). When analysed separately however, there was no relationship between genetic and geographic distance for pairwise comparisons of either 'cage' populations (n=33, F_2-F_6; Mantel's r= 0.07388, p=0.36226) or 'field' populations (n=3, F_0-F_1; Mantel's r= 0.88862, p=0.33307).

We performed an analysis of population structure using a Bayesian clustering approach in the program STRUCTURE. For the analysis of all population samples (n=72), the data most likely formed three genotypic clusters (*K*=3) (Figure 3). Inspection of STRUCTURE barplots shows that individuals from Calca and Picnic Beach populations were assigned to similar genotype clusters, however STRUCTURE did not identify the three *P. australiana* individuals from Calca at that value of *K*. Although statistically less likely, *K* values of

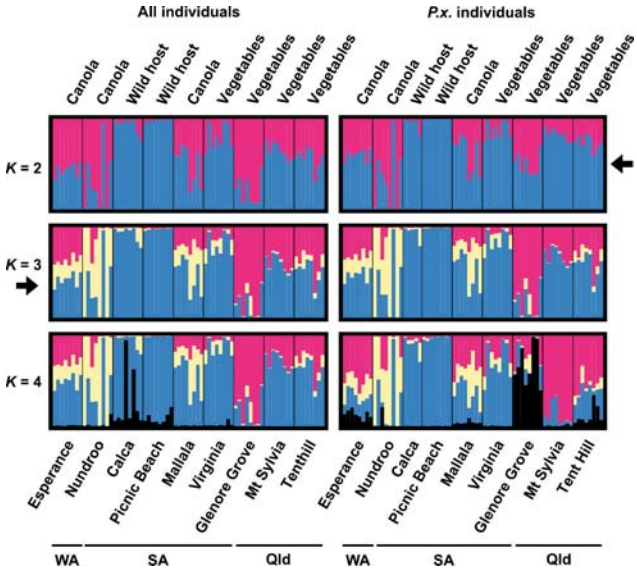

FIGURE 3

Posterior probability of assignment to inferred genotypic clusters, K, generated in the program STRUCTURE for 'all individuals' (n=72), and separately for *'P. xylostella individuals'* (n=69) where three *P. australiana* individuals from the Calca population were excluded. The most likely K for 'all individuals' and '*P.x. only*' is indicated by black arrows. Each vertical bar represents a single individual, populations are separated by black vertical lines, and different genotypic clusters for K=2-4 are represented by different colours

2 and 4 are also presented. At *K*=4, the *P. australiana* individuals are more clearly resolved, as seen by tall black bars for individuals Calca-4 and -6 (Figure 3A). Moderate sharing of the black-coloured genotype cluster occurs across all individuals from Calca and Picnic Beach, and rarely elsewhere. For the analysis excluding the three *P. australiana* individuals, the most likely number of clusters was reduced to two (*K*=2). At this value of *K*, a high degree of admixture is evidence among most populations, as seen by sharing of pink and blue-coloured genotypic clusters. The two populations collected from wild hosts in a similar region, Calca and Picnic Beach, share similar genotypic clusters. At *K*=3, the assignment of individuals to genotypic clusters is consistent with patterns of population differentiation inferred from pairwise F_{ST} values (Table 3).

Frequency of insecticide resistance alleles

We examined the frequency of mutations associated with pyrethroid resistance among nine populations collected from canola crops, *Brassica* vegetable crops and wild hosts. The average frequencies for *skdrl*, *kdr* and *cdr* were 0.29, 0.51 and 0.27 respectively (Appendix). The frequencies were comparable among populations for *skdrl* (range 0.2-0.38) and *kdr* (range 0.25-0.83), but more variable for *cdr* (0.05-0.6). Interestingly, the populations collected from wild hosts, Calca and Picnic Beach, had a moderately high frequency of the *cdr* mutation relative to most other populations. In contrast, the three *P. australiana* samples were all found to be susceptible for *skdrl* and *cdr* however the *kdr* assay failed, possibly due to variation in primer binding sites.

Discussion

RAD sequencing was used to identify thousands of SNP markers and hundreds of thousands of invariant loci from across the genome of two *Plutella* species. These markers facilitated an initial assessment of genetic diversity within and among nine *Plutella* populations collected from different locations and host plants across Australia.

Analysis of RAD-Seq markers and mitochondrial genotyping identified three individuals of a cryptic *Plutella* lineage, *P. australiana*, among our 72 *Plutella* individuals. The relative branch length estimates in the neighbouring-joining tree, differences in heterozygosity and high numbers of private alleles within the *P. australiana* populations (albeit only three individuals) provide the first evidence that the *P. australiana* and *P. xylostella* lineages are strongly divergent in their nuclear genomes. Among the three *P. australiana* individuals, it was interesting to note that individual Calca-7 had the fewest number of private alleles (606, compared to 1760 and 2062) despite having the highest heterozygosity (3.2%, compared to 1.5% and 1.04%). The phylogeny also shows this individual most closely related to *P. xylostella* individuals Picnic Beach-9 and Nundroo-8, which grouped separately from all other *P. xylostella* individuals. As yet, the potential for hybridization between these lineages remains to be tested.

The original discovery of *P. australiana* in Australia was made through sequencing the mitochondrial COI gene from moths collected in light traps, rather than from known host plants (Landry and Hebert 2013). Hence, the fundamental biology of this species and its potential pest status remain to be understood. In our study, individuals of *P. australiana* and *P. xylostella* were collected from a wild Brassicaceous host, *Diplotaxis* sp., at the same location and date, indicating that these lineages can co-exist in similar environments and exploit at least one common host. Considering that there have been several previous genetic studies of *P. xylostella* in Australia (Endersby *et al.* 2006, Pichon *et al.* 2006, Roux *et al.* 2007, Endersby *et al.* 2008), including mitochondrial markers (Saw *et al.* 2006), the discovery of this novel lineage only recently is intriguing. It is possible that differences in sampling strategies (direct sampling from plants vs trapping), times or locations between studies, or differences in the biology of *Plutella* lineages (e.g. host range), meant that *P. australiana* was not collected in previous studies. Alternatively, some molecular markers designed for *P. xylostella* may not amplify efficiently in *P. australiana*. These questions require further investigation.

We examined genetic diversity in field and laboratory-reared populations of *P. xylostella*. Significantly reduced heterozygosity was observed in the laboratory populations, Glenore Grove, Mt Sylvia and Esperance, as measured by the inbreeding co-efficient, F_{IS} (Table 2). These populations were founded by 25-59 individuals at the F_1 generation and then reared for five to six generations. The population from Mallala was also reared for five generations but established from a higher number of individuals

(n=173), and maintained higher levels of heterozygosity in culture, comparable with the field (F_0) populations.

We assessed genetic structure among our population samples using a range of approaches. The global estimate of F_{ST} (0.0487, 99% CL 0.0187-0.0905) indicated significant genetic differentiation among our populations. Pairwise F_{ST} comparisons showed that most differentiation was associated with the three most inbred laboratory populations, Glenore Grove, Mt Sylvia, Esperance, but also Nundroo (F_2). There was no evidence for isolation by distance in pairwise population comparisons among laboratory populations only. Hence, the estimates of genetic isolation are inflated by inbreeding in population cages. The STRUCTURE analysis for all population samples (n=72) inferred that individuals most likely form three genotypic clusters, however failed to resolve the two lineages at that value of *K*. This result could reflect that alleles unique to *P. australiana* individuals were filtered out of the variant SNP dataset used for this analysis. Removing *P. australiana* reduced the optimal value of *K* to two. At this *K* value, a large degree of admixture was observed among populations, supporting the neighbor-joining phylogeny which failed to clearly resolve clusters for the majority of *P. xylostella* individuals (<50% consensus support). Overall, these findings are consistent with high levels of gene flow previously reported for Australian populations of diamondback moth (Endersby *et al.* 2006).

The frequency of pyrethroid resistance alleles has previously been documented for Australian *P. xylostella* populations collected from 2003-2005 (Endersby *et al.* 2011). Endersby *et al.* (2011) reported average resistance allele frequencies for *skdrl* (0.139), *kdr* (0.609) and *cdr* (0.305), however considerable spatial variation was observed. To assess the potential change in frequencies over time, we re-examined these frequencies from populations collected between 2012 and 2014 (Table 1,4). The average frequency for *skdrl*, *kdr* and *cdr* were 0.29, 0.51 and 0.27 respectively, which is comparable between studies for *kdr* and *cdr*, however somewhat higher for *skdrl*. The stability of these frequencies may reflect that synthetic pyrethroid insecticides continue to be widely used to control a range of invertebrate pests in Australian crops.

Conclusion

RAD-Seq is a powerful method for generating SNP markers for population genetic studies in the diamondback moth. We recommend that future studies focus on field sampling design and wherever possible strive to use field-collected (F_0) populations to adequately represent genetic diversity.

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Appendix

TABLE 4

Frequency of resistance alleles for three known point mutations in the voltage-gated sodium channel (Endersby et al. 2011) in nine populations of P. xylostella*: super-kdr-like (skdrl), knockdown resistance (kdr), and crashdown (cdr)*

Locality	Host type	Individuals genotyped (skdrl/kdr/cdr)	Resistance allele frequency		
			skdrl	Kdr	cdr
Esperance, Western Australia	Canola	7/6/8	0.36	0.58	0.06
Nundroo, South Australia	Canola	10/10/10	0.20	0.25	0.1
Calca, South Australia	Wild host	9/9/9	0.38	0.63	0.31
Picnic Beach, South Australia	Wild host	12/9/12	0.33	0.72	0.29
Mallala, South Australia	Canola	10/6/10	0.35	0.67	0.05
Virginia, South Australia	Vegetables	10/10/10	0.30	0.40	0.60
Glenore Grove, Queensland	Vegetables	8/5/8	0.25	0.60	0.19
Mt Sylvia, Queensland	Vegetables	8/6/9	0.31	0.83	0.06
Tenthill, Queensland	Vegetables	10/10/10	0.30	0.40	0.20
By Host type:					
Canola (n. pops = 3)	C	27/22/28	0.30	0.45	0.07
Wild hosts (n. pops = 2)	W	21/18/21	0.35	0.65	0.30
Vegetables (n. pops = 4)	V	36/31/37	0.29	0.51	0.27

Session 2

Biology, ecology and behavior of diamondback moth and other crucifer pests

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Comparison of Biological and Chemical Control Methods in Suppressing *Plutella xylostella* (L.) (Lepidoptera: Plutellidae) on Cabbage in South Africa

Bopape, M.J., Nofemela, R.S.[1], Mosiane, M.S.[2] and Modise, D.M.[3]
Baviaanspoort Correctional Centre, Department of Correctional Services,
Private Bag X01, Lynn East 0039, South Africa
Department of Agriculture And Animal Health, College of Agriculture and Environmental Sciences,
University of South Africa, P.O. Box 392, Unisa 0003, South Africa
[1]insect Ecology Division, Arc–Plant Protection Research Institute, Private Bag X134, Queenswood 0121, South Africa
E-Mail: Nofemelar@Arc.Agric.Za
[2]insect Ecology Division, Arc–Plant Protection Research Institute,
Private Bag X134, Queenswood 0121, South Africa
[3]department of Agriculture and Animal Health, College of Agriculture and Environmental Sciences,
University of South Africa, P.O. Box 392, Unisa 0003, South Africa

Abstract

The diamondback moth, *Plutella xylostella* (L.) (Lepidoptera: Plutellidae), is a serious cosmopolitan pest of *Brassica* crops. In South Africa, there are no action thresholds for its chemical control leading growers to apply insecticides indiscriminately. To promote judicious use of insecticides and to quantify impact of parasitoids on *P. xylostella* populations, we compared the efficacy of weekly and bi-weekly application regimes of Dipel and Dichlorvos against parasitoids in suppressing pest densities and optimising cabbage yield in replicated field trials over two cropping seasons. Since cabbage is attacked by other insect pests, the use of selective (Dipel) and broad-spectrum (Dichlorvos) insecticides enabled us to determine if crop yield depends largely on suppression of *P. xylostella*. During October–December 2011, *P. xylostella* infestations were lowest on plants that received weekly Dipel application followed by bi-weekly Dipel, then weekly Dichlorvos application and were higher on bi-weekly Dichlorvos and control plants. There was a negative relationship between cabbage head weights, % marketable heads and *P. xylostella* infestations. During March–May 2012, *P. xylostella* infestations were higher on control plants followed by Dichlorvos treatments, and lowest on Dipel treatments. However, average infestation per plant during this time was <1 *P. xylostella* in all treatments, which led to similar cabbage head weights and % marketable heads across treatments. Parasitism of immature *P. xylostella* was high (>50 %) in early stages of crop development during this season, which prevented pest density build-up. This result is in contrast to the first half of October–December cropping season. *Cotesia vestalis* (Haliday) (Hymenoptera: Braconidae) was the most abundant parasitoid in all treatments and in both cropping seasons. Based on our findings, weekly application of Dipel suppressed *P. xylostella* better than Dichlorvos and parasitoids, and are necessary to protect crop yield during October–December, a season that coincides with high *P. xylostella* density. However, weekly applications of Dipel are not economically justifiable when parasitoid activity is high as observed during the March–May season, as crop yield and quality in the control treatment was similar to all insecticide treatments. Occasionally, pests such as cabbage aphid, Bagrada bug and cabbage webworm were recorded at very low densities that did not cause significant crop damage. While maintenance of *P. xylostella* infestations below 1 individual per plant had a positive influence on cabbage yield, successful cultivation of *Brassica* crops will also depend on monitoring and effective suppression of the pest complex.

***Keywords*:** Infestation level, Dipel, Dichlorvos, parasitoids, cabbage yield

Effective suppression of diamondback moth, *Plutella xylostella* (L.) (Lepidoptera: Plutellidae), population density is considered key to successful production of *Brassica* crops in many parts of the world (Talekar and Shelton 1993; Furlong *et al.* 2013). This pest is estimated to cost growers worldwide about US$ 4-5 billion annually in direct crop losses and management costs (Zalucki *et al.* 2012). Its high pest status stems mainly from the ability to develop resistance to all insecticide classes (Zhao *et al.* 2006; Furlong *et al.* 2013). Largely in response to insecticide resistance, biological control programmes were initiated in several countries, mainly relying on parasitoid introductions (Lim 1986; Talekar and Shelton 1993;

Sarfraz *et al.* 2005). As successful suppression of the pest by parasitoids led to significant reduction of insecticide applications (Kfir and Thomas 2001; Furlong *et al.* 2004a,b; Löhr *et al.* 2007), biological control can be used effectively for insecticide resistance management (Roush 1997; Justum *et al.* 1998). However, indiscriminate insecticide applications, particularly of broad-spectrum insecticides, still remain a nemesis to sustainable management of *P. xylostella* in many parts of the world (Heckel 2004; Sarfraz and Keddie 2005; Furlong *et al.* 2013). As these insecticide applications are often made without prior evaluation of the impact of natural enemies (Nofemela 2013), it is believed that over-reliance on chemical control has not only led *P. xylostella* to develop high insecticide resistance, but also widespread decimation of its natural enemies (Grzywacz *et al.* 2010).Evidence for this is readily seen in Asia where all the gains accrued with parasitoid introductions in the 1970s and 1980s (Poelking 1986; Sastrosiswojo and Sastrodihardjo 1986; Talekar *et al.* 1986; Ooi 1992) have been reversed by indiscriminate insecticide applications (Waage 1996; Williamson 1998; Upanisakorn *et al.*2011). The situation is so bad in some places that growers apply insecticides up to three times a week in an attempt to overcome the high level of insecticide resistance, which typically develops within two to three years of introduction of a new product (Wang and Wu 2012; Furlong *et al.* 2013). To give growers the benefit of doubt, *P. xylostella* coexists with other insect pests and the damage they cause can be substantial (McCully and Salas Araiza 1986; Biever 1997; Furlong *et al.* 2014), but are generally sporadic in many areas (Annecke and Moran 1982; Lim *et al.* 1997). In many instances, there are no appropriate monitoring or biological and cultural control methods for these pests, and as a consequence, their chemical control has a negative impact on biological control of *P. xylostella* (Shepard and Schellhorn 1997; Sivapragasam 2004; Nofemela and Mosiane 2011).

Plutella xylostella is a persistent and key pest of *Brassica* crops in South Africa (Ullyett 1947; Kfir 1997; Nofemela 2010). For decades now, studies have shown that indigenous parasitoids are an important natural mortality factor of *P. xylostella* in South Africa (Ullyett 1947; Kfir 1997; Nofemela 2010). During February 1992–January 2008, detailed studies were conducted on population dynamics of *P. xylostella* and its parasitoids on unsprayed cabbage fields, and the patterns are well-established. Parasitoid-inflicted mortality is very high (e”50 %) during November–May maintaining infestation levels mostly below 1 *P. xylostella* per plant. Due to low parasitism rates (<50 %) in winter (June–August) and early spring (September–October), infestation levels become very high, mostly >10 *P. xylostella* per plant in spring (Kfir 1997; Waladde *et al.* 2001; Nofemela and Kfir 2005; Nofemela 2010). To convince growers that parasitoids, when effective, can eliminate the need to apply insecticides, we need to demonstrate that high parasitism levels observed during November–May have an impact to *P. xylostella* infestations and crop yield that is comparable to chemical control. Quantifying these not only helps in conserving efficacy of insecticides and parasitoid populations (Sarfraz and Keddie 2005; Zalucki *et al.* 2015), but it also paves a way for integrating biological and chemical control methods where efficacy of parasitoids varies between seasons (Nofemela 2013).Although it is necessary to apply insecticides against *P. xylostella* to suppress the high infestation levels during spring (Nofemela and Kfir 2005; Nofemela 2010), it remained undetermined whether insecticides need to be applied weekly and throughout the season to optimise yield? This study compared the efficacy of weekly and bi-weekly applications of a) Dipel® (*Bacillus thuringiensis* Berliner var. *kurstaki*) and b) Dichlorvos, an organophosphate, against c) parasitoids in suppressing pest densities and optimising yield in replicated field trials during two cabbage cropping seasons. Dipel selectively kills Lepidoptera larvae whereas Dichlorvos is a broad-spectrum insecticide. Since cabbage is attacked by other insect pests (Annecke and Moran 1982; Nofemela and Mosiane 2008), the use of selective and broad-spectrum insecticides enables determination of whether or not crop yield depends largely on effective suppression of *P. xylostella*.

Materials and Methods

The study site and experimental plots

The experiments were conducted at Baviaanspoort Correctional Services Centre (25º38'S 28º30'E, altitude 1164m) in Pretoria, South Africa, during October–December 2011 and March–May2012. In both seasons, cabbage (*B. oleracea*L. var.*capitata*) seedlings, cultivar Green Star (Starke Ayres, Centurion, South Africa), were transplanted on 28 plots of 10 m × 16.6 m each with a spacing of 2 m between plots. In each plot, 10 raised-bed ridges of approximately 1.2 m wide and 10 m in length each were made. The spacing between ridge beds was 60 cm, and three rows were made on each ridge bed with plant spacing of 40 cm. Thus, a total of 750 cabbage seedlings were transplanted in each plot. At the landscape level, there were other independently managed cabbage plots at any given time, which may have served as reservoirs for *P. xylostella* and other pests and natural enemies. Since experimental plots were not contained in insect exclusion cages, the pests and their natural enemies were free to move between plots and this movement is assumed to be similar for all plots.

Treatments and experimental design

Seven treatments were used in this study: a) untreated Control; b) Dipel[1] (Dipel® DF, a *Bacillus thuringiensis* Berliner var. *kurstaki*, Valent Biosciences, Somerset West, South Africa) applied at supplier's recommended low dose of 250g/ha at weekly intervals; c) Dipel[2]applied at bi-weekly intervals (i.e., every second week); d) Dipel[3] applied weekly only for the first eight weeks after seedling transplants; e) Dichlorvos[1] (Dichlorvos EC, an organophosphate, Villa Crop, Kempton Park, South Africa) applied at supplier's recommended dose of 1ml/l at weekly intervals; f) Dichlorvos[2] applied at bi-weekly intervals; and g) Dichlorvos[3] applied weekly only for the first eight weeks after seedling transplants. Both insecticide classes had not been used at the study site prior to this study. Complement® Super (a non-ionic wetter/ spreader/ penetrant surfactant, Syngenta SA, Halfway House, South Africa) was mixed with each insecticide treatment at supplier's recommended dose of 100 ml/ha. All chemical solutions were applied using 16-litre hand-operated knapsack sprayers with flat fan nozzles at 4.8–5.5 bar, and the quantities used were adjusted accordingly. We reduced spray drift by restricting the width of the spray swath and holding the nozzle closer to the plant being sprayed.

The seven treatments were allocated among the 28 plots in a complete randomized block design making four replicates for each treatment. All chemical applications commenced three weeks after seedling transplants, and were stopped one week before harvest for weekly and bi-weekly treatments. Standard cultivation practices that included fertilizer application, irrigation and weeding were followed. The plots were irrigated using overhead sprinklers twice a week, but irrigation was withheld for two days after each insecticide application.

Plutella xylostella *density and determination of parasitism levels*

Plutella xylostella larvae and pupae were monitored on each treatment plot at weekly intervals from three weeks after transplanting cabbage seedlings until one week before harvest of cabbage heads. In each treatment plot, 10 randomly selected plants (excluding outer two rows in each plot) were thoroughly inspected every week and the numbers of *P. xylostella* larvae and pupae (i.e., infestation levels) found on each plant were recorded. All insecticide applications were on the day following crop monitoring events.

In order to determine parasitism levels, samples of *P. xylostella* larvae (third and fourth instars) and pupae and pupae of its parasitoids were collected during scouting at weekly intervals. Sample sizes ranged from 1 to 206 individuals depending on infestation levels. The samples were taken to the insectary (ARC-Plant Protection Research Institute, Rietondale campus (25°442S 28°132E) in Pretoria) where they were maintained at 25 ± 1°C, 65 ± 5 % RH, and 16L:8D photoperiod. The larvae were provided with sections of fresh cabbage leaves and held individually in Petri dishes. The leaves were replaced every second day until host larvae pupated

or parasitoid pupae formed. *Plutella xylostella* pupae and parasitoid pupae were individually placed in glass vials. All emergent parasitoids were identified and their incidence determined. Parasitism rates (pooled per treatment) were calculated as the percentage of emergent parasitoids out of the total samples of *P. xylostella* and parasitoid pupae for each week. However, samples that died of unknown causes were excluded from calculations of parasitism.

Crop quality and yield

On the day before crop harvest, the total plant population, numbers of multiple heads and marketable heads were counted and recorded on each plot. Multiple heads are defined here as cabbage plants whose apical meristem was damaged by herbivory in the early stages of plant development such that multiple cabbage heads formed from the same stem. We regarded cabbages to be marketable when the head size exceeded 30 cm in maximum diameter using a flexible measuring tape. At harvest, 100 marketable cabbage heads were randomly selected from each treatment (i.e., 25 cabbage heads per plot), and each was cut at the interface between the head and the stem. The 4 outer leaves were removed from each cabbage head, and they were weighted individually on a calibrated electronic scale (Digital Weighing Indicator, model DI-20, Teraoka Weigh Systems PTE LTD, Singapore, Singapore).

Statistical analysis

As only 10 plants were scouted and sampled every week out of 750 plants in each plot, it is reasoned that *P. xylostella* infestations at the landscape level were not influenced by sampling 1.33% of the plants per plot every week. Therefore, Analysis of Variance (ANOVA) was chosen as an appropriate method of analysis. Prior to ANOVA, the data were checked for normality using Shapiro-Wilks' test and for homogeneity of variance using Levene's test. As data for % of multiple heads and % marketable plants were not normally distributed, the data were square-root transformed prior to analysis. One-way (ANOVA) were performed to compare 1) *P. xylostella* infestation levels between treatments, and 2) across the season; 3) % multiple heads; and 4) cabbage weights between treatments. Where significant differences were detected, the means were compared using Fisher's protected least significant difference (LSD) test. Linear regression was used to determine the relationship between infestation levels and the proportion of plants infested. Prior to analysis, the data were square-root transformed. The statistical analyses were performed at 5 % level of significance (Statistica 2013).

Results and Discussion

Plutella xylostella *density and parasitism rates*

October–December 2011

Crop infestations were low (about 1 *P. xylostella* per plant) during the first week of the experiment and were similar ($F_{6, 273}$ = 1.1617; P = 0.3271)across treatments (Figure 1). Except for weekly applications of Dipel (Dipel^1 and Dipel^3), infestations increased substantially in the other treatments until about week 4. The sharp decline in infestation levels in these treatments from week 5 coincided with high parasitism levels (>50%) of *P. xylostella* larvae and pupae (Figure 1). Parasitism levels were not significantly different ($F_{6,57}$ = 1.59; P = 0.1666) among the treatments. Four species of primary parasitic Hymenoptera were recorded from *P. xylostella* larvae and pupae during this season, namely *Cotesia vestalis* (Haliday) (Braconidae), *Apanteles halfordi* (Ullyett) (Braconidae), *Oomyzus sokolowskii* (Kurdjumov) (Eulophidae) and *Diadromus collaris* (Gravenhorst) (Ichneumonidae). *Cotesia vestalis* accounted for more than 80% of total parasitism levels. As infestation levels were as low as 0.5 *P. xylostella* per plant in all treatments by the end of the season ($F_{6,273}$ = 1.8740; P = 0.0854), the additional mortality that weekly application of Dipel (Dipel^1) provided in maintaining infestations low throughout the season was not significant by the end of the season (Figure 1).These results are in agreement with recent studies that *P. xylostella* infestations are low from November in South Africa (Nofemela and Kfir 2005; Nofemela 2010, 2013).

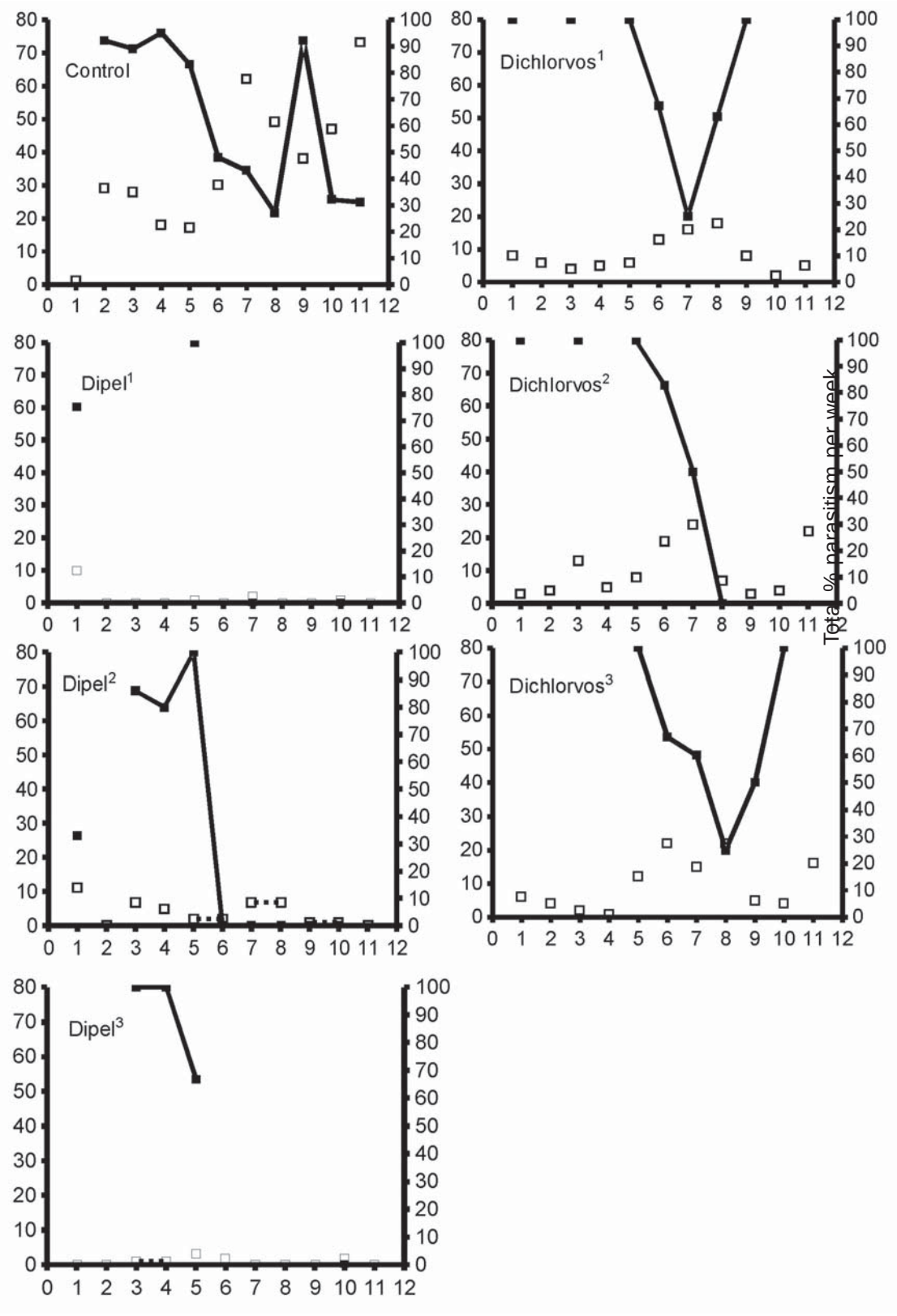

FIGURE 1

The influence of different insecticides and spray regimes on incidence of Plutella xylostella immature stages (dotted line) and parasitism levels (solid line) during October–December 2011.

March–May 2012

Infestations were very low (below 0.3 *P. xylostella* per plant) at the start of the season in all treatments, and remained at such low levels throughout the season, except for the control (Figure 2). While infestation levels only exceeded 1 *P. xylostella* per plant on four occasions during the second half of the season in the control treatment, the average infestations in the control were far lower (Figure 2) during this season than the October–December season (Figure 1). The starting parasitism levels of *P. xylostella* larvae and pupae during the March–May season were very high (>80%) in all treatments. While parasitism levels fell below 50% in several treatments in the second half of the season, parasitism levels bounced back up in the control and Dichlorvos treatments. Despite the fluctuation of parasitism levels in the different treatments, infestations remained fairly low in all treatments (Figure 2). The parasitoids recorded on *P. xylostella* larvae and pupae during this season were *C. vestalis*, *O. sokolowskii* and *D. collaris*. *Cotesia vestalis* was again the dominant parasitoid in all treatments, and parasitism levels were not significantly different ($F_{6,40}$ = 1.71; P = 0.1448) between treatments. These result imply that *P. xylostella* population density was low at landscape level during the March–May season. Again the findings of this study confirm those of previous studies that *P. xylostella* population density is very low during November–May in South Africa (Nofemela and Kfir 2005; Nofemela 2010, 2013).

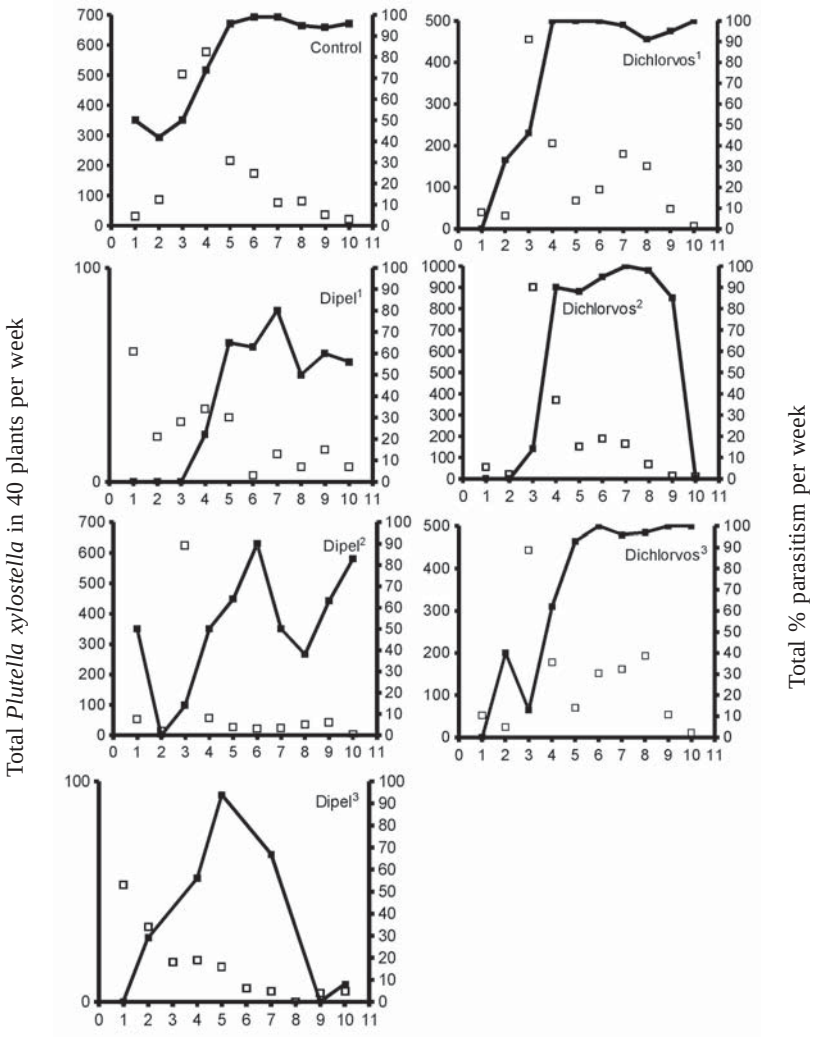

FIGURE 2

The influence of different insecticides and spray regimes on incidence of Plutella xylostella *immature stages (dotted line) and parasitism levels (solid line) during March–May 2012.*

TABLE 1

The influence of Dipel and Dichlorvos insecticides applied at different regimes compared to the untreated cabbage plants (Control) on mean (± S.E.) number of *Plutella xylostella* larvae and pupae and cabbage quality (i.e., % multiple heads, % marketable heads and cabbage weight (kg) during October–December 2011 and March–May 2012.

	October–December 2011				**March–May 2012**			
Treatments	Infestation per plant	% multiple heads	% marketable heads	Cabbage weight (kg)	Infestation per plant	% multiple heads	% marketable heads	Cabbage weight (kg)
Control	4.53 ± 0.36a	16.19 ± 2.12a	78.29 ± 1.87a	2.00 ± 0.06d	0.86 ± 0.08a	13.2 ± 0.92a	76.82 ± 2.7a	3.51 ± 0.06a
Dipel[1]	0.54 ± 0.06d	9.21 ± 1.23b	86.69 ± 1.64b	3.24 ± 0.08a	0.03 ± 0.01c	9.82 ± 1.46a	79.97 ± 3.9a	3.67 ± 0.04a
Dipel[2]	2.22 ± 0.25c	10.97 ± 1.71b	86.09 ± 1.91b	2.49 ± 0.07b	0.09 ± 0.02c	11.15 ± 1.39a	75.05 ± 3.17a	3.53 ± 0.07a
Dichlorovos[1]	3.21 ± 0.24b	11.4 ± 0.98b	85.38 ± 1.13b	2.46 ± 0.10c	0.21 ± 0.03b	13.23 ± 2.18a	67.57 ± 4.04a	3.59 ± 0.06a
Dichlorovos[2]	4.86 ± 0.41a	10.55 ± 1.14b	87.09 ± 0.91b	2.18 ± 0.05d	0.25 ± 0.03b	11.61 ± 0.95a	71.88 ± 3.67a	3.57 ± 0.06a
Dichlorovos[3]	3.2 ± 0.20b	8.96 ± 1.29b	86.84 ± 1.64b	2.85 ± 0.07c	0.25 ± 0.03b	13.73 ± 1.87a	72.73 ± 4.39a	3.6 ± 0.04a
Dipel[3]	0.4 ± 0.06d	10.03 ± 1.3b	86.57 ± 2.12b	3.45 ± 0.10a	0.01 ± 0.01c	10.1 ± 1.17a	73.71 ± 3.4a	3.64 ± 0.07a

Means in a column with the same letter are not significantly different at P = 0.05 Fisher's LSD.

[1]Weekly insecticide applications until one week before harvest.

[2]Bi-weekly insecticide applications until one week before harvest.

[3]Weekly insecticide applications only during the first 8 weeks of experiment.

Effects of infestations on cabbage quality

October–December 2011

Plutella xylostella infestations were significantly higher ($F_{6, 2793}$ = 47.296; P < 0.001) on control and Dichlorvos[2] treatments followed by Dichlorvos[1] and Dichlorvos[3], then Dipel[2] and least on Dipel[1] and Dipel[3] during October–December 2011 (Table 1). However, multiple heads were significantly higher ($F_{6,21}$ = 2.8512; P = 0.0343) on the control treatment than the insecticide treatments. Similarly, the percentage of marketable cabbage heads was significantly lower ($F_{6,21}$ = 3.5929; P = 0.0131) on control compared to the insecticide treatments. Cabbage head weights were significantly different ($F_{6, 723}$ = 44.545; P < 0.001) among treatments being higher on Dipel[1] and Dipel[3] followed by Dipel[2], then Dichlorvos[1] and Dichlorvos[3] and least on Dichlorvos[2] and control.

As the proportion of plants infested with *P. xylostella* was positively influenced (r^2 = 0.7859; $F_{1,54}$ = 198.2241; P < 0.001; y = 0.192 + 0.2289x) by infestation levels in the control plots, all plants were infested at an infestation level of just 4 *P xylostella* per plant. This implies that the faster infestations increase and are maintained at high levels, the higher the damage to the crop. Hence, the higher proportion of multiple heads and low marketable heads were observed on the control plots, as there was no significant top-down suppression of the pest density during the initial stages of crop development (Fig.1).

March–May 2012

Albeit *P. xylostella* infestation levels were again significantly high ($F_{6, 3073}$ = 59.086; P < 0.001) on control followed by Dichlorvos treatments and least on Dipel treatments during March–May 2012 (Table 1), average infestations were much lower in all treatments throughout the season compared to the October–December season (Table 1).The proportion of plants infested across treatments was also significantly lower during this season. As a consequence, there were no significant differences in the percentage of multiple heads ($F_{6, 21}$ = 1.1395; P = 0.3761), percentage of marketable heads ($F_{6, 21}$ = 1.1579; P = 0.3668) and cabbage weights ($F_{6, 717}$ = 0.8328; P = 0.5447) among the treatments.

Conclusion

Suppression of *P. xylostella* is key to successful cultivation of cabbage in South Africa. Although this is widely emphasised for many other parts of the world (Talekar and Shelton 1993; Furlong *et al.* 2013), control of *P. xylostella* remains dependent on insecticide applications. However, as *P. xylostella* develops resistance and insecticides lose their efficacy against it, growers are under pressure to reduce application rates of insecticides to prevent widespread insecticide resistance. Previous studies have shown that *P. xylostella* enters a population outbreak phase every spring if insecticides are not applied, which results in severe crop damage (Nofemela and Kfir 2005; Nofemela 2010). We found that weekly application of Dipel is necessary in the first half of October–December season to effectively suppress *P. xylostella* infestations and to maximise yield. As infestation levels, % marketable heads and cabbage head weights were similar when Dipel was applied every week until crop harvest (Dipel[1]) and when applied weekly only for the first eight weeks of crop development (Dipel[3]), these results suggest that there is no need to apply Dipel up to crop harvest, which can save growers a lot of money in insecticide and application costs estimated at US$ 63.13 per 500 g and US$ 8.05 per ha, respectively (Ayalew 2006). In contrast to early spring, the period November–May has been shown to correspond with low incidence of *P. xylostella* (Nofemela 2010), which explains why infestations were very low during the March–May season in all treatments, and as a consequence yield was similar among the treatments. The major mortality factor of *P. xylostella* during this period was the strong top down effect of parasitoids, which exceeded 50 % in all treatments from the beginning of the season. Thus, there is no economic benefit to growers to maintain infestations far below 1 *P. xylostella* per plant by application of insecticides during this period. Similar argument was made in previous studies elsewhere (Furlong *et al.* 2004a; Ayalew 2011).

To ensure that the pest population densities remain below damaging levels, growers need to monitor the crop for infestations on a regular basis. Previous studies demonstrated that *P. xylostella* infestation levels corresponded with synthetic sex pheromone trap catches of male moths (Nofemela and Kfir 2005; Nofemela 2010). An action threshold of 8.52 moths/trap/week was established which

corresponds with 1 *P. xylostella* per plant (Nofemela 2010). While aphids [*Myzus persicae* (Sulzer), and *Brevicoryne brassicae* (L.)] and the cabbage webworm [*Hellula undalis* Fabricius] were recorded on very few occasions at low densities during this study, they have a potential to devastate *Brassica* crops (Munthali 2009; Nofemela and Mosiane 2011). Thus, successful cultivation of *Brassica* crops will also depend on monitoring and effective suppression of the pest complex.

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Effect of Host and Temperature on Reproduction of *Diadegma semiclausum* Hellen

Chen, F.S.H., Wang, Y.[1], Zhang, H.M.[2], Yang, Y.X.[3] and Chen, Z.Q.[4]

Institute of Agricultural Environment and Resource,
Yunnan Academy of Agricultural Science, Kunming 650205, P.R. China
E-mail: chenfsh36@163.com

[1]Institute of Agricultural Environment and Resource,
Yunnan Academy of Agricultural Science, Kunming 650205, P.R. China
E-mail: piglucky@126.com

[2]Institute of Agricultural Environment and Resource,
Yunnan Academy of Agricultural Science, Kunming 650205, P.R. China
E-mail: bshrjs999@163.com

[3]Institute of Agricultural Environment and Resource,
Yunnan Academy of Agricultural Science, Kunming 650205, P.R. China
E-mail: yyyyx99@163.com

[4]Institute of Agricultural Environment and Resource,
Yunnan Academy of Agricultural Science, Kunming 650205, P.R. China
E-mail: zongqichen55@163.com

Abstract

The effect of temperature and host, *Plutella xylostella* (L.), density and instar on the reproduction and development of *Diadegma semiclausum* Hellen was studied at six constant temperatures (15, 20, 22, 25, 27, 30°C) in the laboratory. The parasitism rate of *D. semiclausum* decreased with an increase in larval host numbers from 30-90, but this decrease was not statistically different at 110 larvae/ plant. *D. semiclausum* could parasitize the 2nd, 3rd and, 4th instars of DBM larvae, however, parasitism of the 2nd and 3rd instars was significantly higher than the 4th instar. Developmental duration, eclosion success and sex ratio of *D. semiclausum* were observed. Developmental duration of different stages of *D. semiclausum* decreased significantly with increasing temperature from 15-27°C; at 30°C pupae failed to develop. The eclosion success and sex ratio increased with increasing temperature from 15-22°C, and decreased with increasing temperature from 22-27°C. The optimal larvae number, larvae instars of *P. xylostella* and temperature for the reproduction of *D. semiclausum* was 30-90 larvae, 3rd instar, and 22°C, respectively.

***Keywords*:** *Plutella xylostella*, natural enemy, mass rearing, developmental duration, eclosion success, sex ratio

Diadegma semiclausum (Hellén) (Hymenoptera: Ichneumonidae) is one of the important parasitoids of larvae of the diamondback moth (DBM), *Plutella xylostella* (L.), (Lepidoptera: Plutellidae) a serious pest of cruciferous vegetable crops. There is a high practical potential of this parasitoid in to the management of DBM (Zalucki *et al.* 2016). *D. semiclausum* originated from Europe (Hardy 1938; Chua and Ooi 1986), and was first introduced from England to New Zealand in 1936 where it successfully controlled DBM.

From there *D. semiclausum* was introduced to Australia, Malaysia, Japan, and Taiwan (Ooi 1992; Talekar and Shelton 1993; Amend and Basedow 1997; Noda *et al.* 2000; Saucke *et al.* 2000; Waterhouse and Sands 2001; Furlong *et al.* 2016). The species was introduced to Yunnan Province of P.R. China by Chen ZongQi from Vietnam and Taiwan in 1997, achieving field parasitism rates of up to 87% (Chen ZQ *et al.* 2001a, 2001b, 2003; Chen FSH *et al.* 2010). Now *D. semiclausum* is widely utilized in Yunnan, including the districts of Kunming, Yuxi, Dali, Baoshan, Lincang, Qujing, Chuxiong and so forth, and has become a key natural enemy in the control of DBM in these areas.

In order to more fully utilize *D. semiclausum* we plan to mass rear the species so as to establish control earlier in the field. Here we report the effect of various host factors (number and instar) and temperature on the rearing of *D. semiclausum* in the laboratory; see also Dosdall *et al.* (2012).

Materials and Methods

Insects

Rearing of *P. xylostella:* Eggs of *P. xylostella* laid on foil were put into 15% formalin for 15m, then rinsed twice in distilled water. Eggs and larvae, fed on cabbage of JingFeng variety, were kept under insectaria conditions (22±2°C, RH: 60%-75%, L: D=14:10). Larvae were used to rear parasitoids when they had developed to the 3rd instar.

Rearing of *D. semiclausum:* 3rd instars larvae were exposed to adult *D. semiclausum* in cages (50cm*50cm*50cm) for 48h. Parasitised larvae were reared on cabbage under insectaria condition (22±2°C, RH: 60%-75%, L: D=14: 10). Newly emerged mated adult females were used in experiments.

Experiments

The effect of larval number on D. semiclausum parasitism

Every cabbage plants were infested with 30, 50, 70, 90 and 110 3rd instar larvae. There were 4 repeats of each density treatment and each plant was exposed to a pair of *D. semiclausum* in cages (50cm*50cm*50cm) for 24h. Larvae continued to develop and the numbers of *D. semiclausum* pupae recorded, when the pupae formed.

The effect of host instar on the rearing of D. semiclausum

Every Cabbage plants were infested with 100 DBM larvae at the 2nd, 3rd or 4th instar stage. There were four repeat for each instar treatment. Each plant was exposed to a pair of *D. semiclausum*, for 24h. Larvae continued to develop and the numbers of *D. semiclausum* pupae recorded, when pupae formed.

Temperature effects on the developmental of D. semiclausum

Newly parasitized 3rd instar larvae of *P. xylostella* (n=200) were reared on cabbage at 15, 20, 22, 25, 27, 30!, RH: 65%-75%, L: D=14: 10h. Time to parasitoid pupal formation was recorded and at least 100 pupae were collected for each temperature treatment. The time to adult parasitoid emergence, the exclusion success rate and sex ratio were recorded.

Results and Discussion

The effect of larval number on D. semiclausum *parasitism*

The number of *D. semiclausum* emerging increased with the larval numbers from 30-90, however the rate of parasitism was constant around 50%; but both the number emerging and rate decreased significantly at 110 larvae per plant (Table 1).

Table 1

Effect of number of P. xylostella *larvae on parasitism rate (%) of* D. semiclausum

Number of *P. xylostella*	Number of parasitoid emerging	Mean parasitism rate
30	16.00	53.33±2.17 a
50	25.75	51.50±2.24 a
70	35.25	50.36±1.64 a
90	42.00	46.67±1.97 a
110	37.27	33.88±1.88 b

In a column, means followed by the same letter(s) do not differ significantly

The effect of host instar on the rearing of D. semiclausum

The per cent parasitism of 2nd, 3rd and 4th instar DBM larvae by *D. semiclausum* was 51%, 51% and 42%, respectively: significantly higher for 2nd and 3rd instars than the 4th instars (Table 2).

Table 2

Effect of P. xylostella *larval instar on parasitism rate of* D. semiclausum

Instar of larvae	Number of pupae	Number of the parasitoid	Parasitism rate
2nd instar	81.75	41.75 aA	51.1±1.16 aA
3th instar	81.00	41.00 aA	50.6± 0.43 aA
4th instar	80.50	34.00 bB	42.3±1.76 bB

Means followed by the same alphabet do not differ significantly

Temperature effects on the developmental of D. semiclausum

The developmental durations of an entire generation at each rearing temperature were 36, 21.8,

18.6, 15.4, and 14.9 d, respectively; decreasing significantly with increasing temperature from 15-27°C. Pupae failed to develop at 30 °C (Table 3).

TABLE 3

Effect of temperature on the developmental duration of D. semiclausum

Temperature (°C)	Developmental duration (days)		
	Egg-pupae	Pupae-adult	Egg-adult
15	18.32±0.09a	17.73±0.13a	36.0±0.20a
20	10.21±0.08b	11.54±0.07b	21.8±0.11b
22	8.35±0.09c	10.20±0.07c	18.6±0.11c
25	7.92±0.05d	7.01±0.07d	14.9±0.10d
27	7.13±0.06 e	7.05±0.06d	14.3±0.07e
30	7.08±0.15e	—	—

In a column, means followed by the same letter(s) do not differ significantly

The rate of parasitoid eclosion was 70%, 83%, 92%, 88% and 75% at 15, 20, 22, 25, 27°C, respectively (Table 4). There was no effect of temperature on the sex ratio (@&/(@&+B&)) at 15 and 25°C, but at temperatures higher than 25 °C, the sex ratio decreased significantly to 19% at 27 °C (Table 5).

TABLE 4

Effect of temperature on the eclosion success of D. semiclausum

Temperature (°C)	Number of pupae	Number of adults	Mean eclosion rate
15	25	17.50	70.00±0.05c
20	25	20.75	83.00±0.03b
22	25	23.00	92.00±0.02a
25	25	22.00	88.00±0.02a
27	25	18.75	75.00±0.02bc
30	25	—	—

In a column, means followed by the same letter(s) do not differ significantly

TABLE 5

Effect of temperature on the sex ratio of D. semiclausum

Temperature (°C)	Number of pupae	Number of eclosing		Sex ratio
		Female	Male	
15	25	7.00	10.50	42.97±2.72a
20	25	9.75	11.00	47.07±2.35a
22	25	11.25	11.75	48.96±1.37a
25	25	8.25	13.75	37.29±5.31a
27	25	3.50	15.25	18.95±5.01b
30	25	—	— —	

In a column, means followed by the same letter(s) do not differ significantly

CONCLUSION

The number of larvae and instar of *P. xylostella* affect rearing of *D. semiclausum*. The rate of parasitism significantly decreased when the number of larvae reached 110/plant.

Temperature has a major effect on rearing *D. semiclausum* impacting on developmental duration, eclosion rate and sex ratio. The pupae of *D. semiclausum* did not develop at 30°C.

Sex ratio is an important factor in mass rearing *D. semiclausum*. The sex ratio declines significantly when temperature exceeded 25°C. The sex ratio in parastoids is a complex problem and we will continue to undertake further research in this area.

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Longevity and Release Rate of Sex Pheromone Component in Field Exposed Diamondback Moth Lures Across Different Seasons in Cabbage

Topagi S.C., Bhanu K.R.M.[1] and Ashok Kumar C.T.
Department of Agricultural Entomology, UAS, GKVK, Bangalore
E-mail: sanjay.topagi@gmail.com
1Bio-control Research Laboratories, Pci, Sreeramanahalli, Doddaballpur Road, Bengaluru
E-mail: bhanu.krm@pcil.in

Abstract

The diamondback moth (DBM), *Plutella xylostella* (L.) (Plutellidae: Lepidoptera) is the most destructive insect pest of cruciferous crops throughout the world, infesting particularly cabbage, broccoli and cauliflower. The use of pheromones for pest control promises to be an important component in the development of alternatives that may help to solve major environmental and human health problems associated with chemical pesticide use in agriculture. To understand the release/dissipation rate of the pheromone, or for how long the commercial lures would be able to attract the moths in Cabbage fields, a study was conducted at Bangalore Rural District across three different seasons viz., rabi-2012, kharif-2013 and summer-2014. The field installed pheromone lures were regularly brought to the laboratory at 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 days after installation and the quantity of pheromone remaining in the black rubber septa was assessed using standard procedures. In present study pheromone lures loaded with female sex pheromone (1 mg) was found to emit the active ingredient at an average of < 0.075 mg/day, <0.074 mg/day and <0.064 mg/day in rabi, kharif and summer seasons respectively. Almost 95, 94 and 86 per cent of the total chemical was released in 100 days during rabi, kharif and summer seasons respectively.The number of male moths trapped during the 100 day field exposure varied throughout cropping period. Since, the crop duration in the field varies between 90-100 days, one lure per trap is enough to attract DBM adults and no lure change during the season is necessary in mass trapping plans.

Keywords: Lure residue analysis, release rate,seasonalinfluence

The diamondback moth (DBM), *Plutella xylostella* (L.) (Lepidoptera :Plutellidae) is distributed worldwide and considered to be the most destructive insect pest of cruciferous crops, especially cabbage, broccoli and cauliflower (Furlong *et al.* 2013). The extent of damage on these crops by this pest alone has been estimated to be 52 % (Sachan and Gangwar 1990). The larvae damage the crop by feeding on the foliage. Severe attack hinders the health, growth and development of the plant resulting in considerable loss of yields and decline in market value due to the damage and even death of young plants. The difficulty in controlling this pest has forced some growers to abandon the production of cruciferous crops (Li *et al.* 2012).The use of pheromones for pest control promises to be an important component of the ongoing challenge to develop alternatives that may help to solve major environmental and human health problems associated with chemical pesticide use in agriculture (Kirsch 1988).

Pheromones can be mainly used in three ways in pest management programmes: (1) for surveillance or monitoring the pest population so that chemical control measures can be undertaken at the appropriate time, (2) for mass trapping and (3) to disrupt mating behaviour (Minks 1977). Sex pheromones in insects contain mixtures of several compounds, of which the primary component attracts the insects upwind from a distance and the secondary components in combination with the primary component stimulate aspects of mating behavior (Piccardi 1979). Males detect and mate with females cued by the reception of the female sex pheromone. When lured with sex pheromone traps, if sufficient males are trapped, females may remain unmated, thus leading to a reduction in population growth.

Work on release/dissipation rate of the pheromone, or field longevity of the lure in attracting adult males has not reported on DBM in cabbage fields. Here we examine the viability of the sex pheromone component in field exposed diamondback moth lures in cabbage ecosystem at Bangalore Rural District across three different seasons.

Materials and Methods

Release/dissipation rate of the pheromone was conducted at Byatha(13°11'18.80'' N 77°28'58.52''E elev-288 ft) during rabi-2012, Kadathanamale (13°12'59.77''N 77°33'01.92''E elev-2890 ft) during kharif-2013 and Sri Ramanahalli (13°12'01.29'' N 77°33'33.76''E elev-2953 ft) during summer-2014. 100 lures containing the pheromone component were kept in cabbage fields at each season. Then the field installed pheromone lures were returned (10 in numbers at each point of time) to the laboratory at 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 days after installation and the residual quantity of pheromone in black rubber septa was estimated using standard Gas Chromatography (GC) procedures (Romano*et al.* 2000; Jyothi *et al.* 2008).

Pheromone Sample/lures

Black rubber septa impregnatedwith 1 mg of a formulation of three components, Z-11-hexadecenal, Z-11-hexadecenyl acetate and Z-11-hexadecenol, in the ratio10: 10: 0.1, manufactured by Bio-control Research Laboratories, PCI were used for the study.

Extraction of pheromone from the lures

The field collected lures (10 numberseach time at ten days interval) were extracted with a solution containing 2 mg of tetradecanyl acetate (14:Ac) in 10mL hexane contained in a 20mL sampling vial and decanted after soaking overnight. The extracts were then subjected to Gas chromatographic analysis.

Analysis of lure extracts

The lure extracts were subjected to GC-MS analysis on Agilent 7820A GC system interfaced to a 5977E mass selective detector (MSD) fitted with a HP-5 column (both 30 m× 0.25 mm i.d., 0.25 ìm film; J&W Scientific, Folsom, CA, USA) and compared with the standard PCI commercial DBM pheromone blend to identify the peaks of different components of the blends as Z-11-hexadecenal, Z-11-hexadecenyl acetate and Z-11-hexadecenol. The contents of lure extracts were quantified using Shimadzu GC-2014 equipped with RestekStabilwax (Cross bond Carbowax Polyethylene Glycol) (30 m x 0.25 mm i.d 0.5µmdF), N_2 at the flow rate of 5 ml min^{-1} served as the carrier gas. The injector was kept at 225ºC and the detector at 225ºC, the column oven was kept in a temperature programme of 60ºC with a hold time of 2 min, then up to 220ºC at a rate of 60ºC min^{-1} with a hold time of 2 min. The GC was fitted with the Flame Ionisation Detector (FID). The split injector with the ratio of 10:1 was used.The residual quantity was calculated by comparing per cent area of pheromone with per cent area of internal standard and expressed as per cent released over time and regression analysis was performed to best fit the relationship between the amounts released over time. The data on progressive quantity of released chemical and mean weather variables over the sampling period were subjected to correlation analysis.

Field experiment

In order to determine the trapping capacity of the field exposed lures over a period of 100 days, the experiments were carried out in different cabbage (Unnati variety) fields across the same areas (see above). The monitoring traps with a density of 6 traps randomly positioned and covering an acre area were set up in the farmer's field on the same day of transplantation to cover the maximum time period of the crop. The main purpose of the experiment was to see the performance of the lures for catching DBM adults over a period of 100 days. The water traps baited with pheromone in black rubber septa fastened to wooden pegs were maintaining at a height of 0.3 m above the canopy level. The water trap consisting of a plastic basin (25×10 cm diameter); adapter, basin to hold water mixed with a spoon of detergent powder and a lure holder with a canopy. About three fourth of the container was filled with water to hold trapped moths attracted by the pheromones. The pheromone septa were suspended from the lure holder from the centre of the basin. Moths trapped were drowned and killed when fallen into the water containing detergent. Observation of total moth catches from 6 traps was made at every 10 days interval. Insecticidal sprays were continued as per the farmer's practice.

Results

Since the weather parameters of different seasons cannot be clubbed, the results of all the three seasons are presented separately as below.

Rabi-2012

Residue analysis of field exposed lures indicated that the pheromone release rate varied across 100 days; the initial quantity of release (after 10 days) was

47%, declining to 12, 9, 3 and 5% at 20, 30, 40 and 50 days, respectively. Up to 60 days 79% of the chemical was released after which the rate of release was reduced to 2, 5 and 6% for days 70, 80 and 90; at the 100th day 5% of the chemical remained in the septa. The rubber septa loaded with female sex pheromone (1 mg) emitted the active ingredient at an average of <0.075 mg/day (Figures 1 and 2).

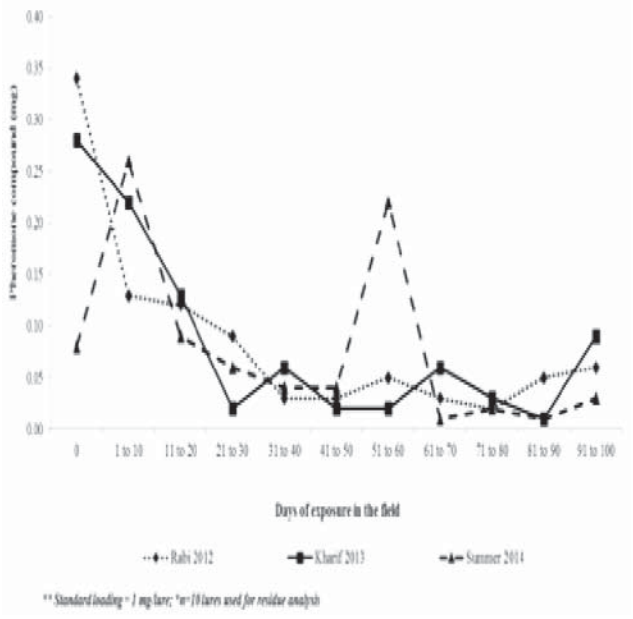

FIGURE 1

Mean release rate of pheromone component in the field exposed lures across different seasons

The data on release rate of DBM pheromone compound and mean weather parameters were subjected to simple correlation analysis. The progressive quantity released was positively correlated with morning relative humidity (r=0.038) and rainfall (0.228), whereas, negatively influenced by maximum temperature (r=-0.416), minimum temperature (r=-0.811), afternoon RH (r=-0.469) and wind speed (r=-0.011). However, theinfluence of these factors was found to be non-significant. It may be due to the combined effect of the chosen weather parameters together influenced the release rate of pheromone. When the data was subjected to linear regression analysis it revealed that, 93 per cent (R^2=0.937) lure release was influenced by factors other than those chosen or it might be a combination of all the chosen weather parameters and equation as follows.

$$Y = -2.672 + 0.146X_1 - 0.137X_2 + 0.007X_3 + 0.020X_4 - 0.004X_5 + 0.002X_6$$

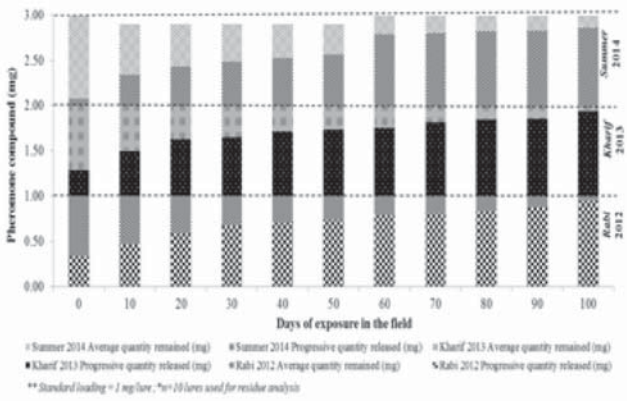

FIGURE 2

Progressive quantity of pheromone component released in the field exposed lures across different seasons

Kharif-2013

Residue analysis of pheromone compounds in field exposed lures, used in water traps, during kharifindicated that the pheromone release rate varied across 100 days, the initial quantity of release (after 10 days) was 50 per cent, later the rate of release was reduced to 13, 2, 2 and 2 per cent for 20, 30, 40 and 50 days, respectively. At 60 days 75 per cent of the chemical was released then the rate of release was reduced to 3, 1 and 9 per cent for 70, 80, and 90 days respectively and at the 100th day still 6 per cent of the chemical was remained on the lures (Figure 1 and 2). Pheromone lures of rubber septa loaded with female sex pheromone (1 mg) emitted the active ingredient at an average of <0.074 mg/day. Almost 94 per cent of the total chemical was released in 100 days.

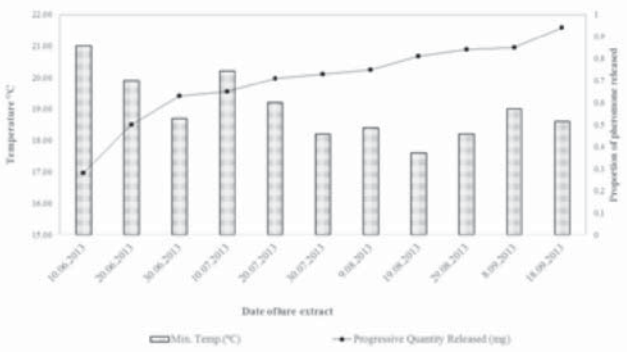

FIGURE 3

Influence of Minimum temperature on DBM Pheromone released rate during kharif-2013

The progressive quantity released exerted a negative association with maximum temperature (r=-0.191), afternoon relative humidity (r=-0.262) and wind speed (r=-0.267); while, positive relation with

morning relative humidity (r=0.577) and rainfall (r=0.570). There was a highly significant negative association with minimum temperature (Figure3). When the data was subjected to linear regression analysis it revealed that, pheromone release rate was influenced by weather parameters an extent of 82 per cent (R^2=0.821) with following linear regression equation

$$Y = 3.304 - 0.014X_1 - 0.123X_2 + 0.006X_3 - 0.006X_4 - 0.008X_5 - 0.001X_6.$$

Summer-2014

Residue analysis of pheromone compounds in field exposed lures, used in water traps, indicated that the pheromone release rate varied across 100 days, the initial quantity of release (after 10 days) was 34 per cent; later the rate of release was reduced to 9, 6, 4 and 22 per cent for 20, 30, 40 and 50 days, respectively. Up to 60 days 79 per cent of the chemical was released; later, the rate of release was reduced to 2, 1 and 3 per cent for 70, 80, and 90 days respectively (Figure 1 and 2) and at 100th day still 14 per cent of the chemical was remained on the lures. Pheromone lures of rubber septa loaded with female sex pheromone (1 mg) emitting the active ingredient at an average of < 0.064 mg/day. Almost 86 per cent of the total chemical was released in 100 days.

The release rate of pheromone during summerseason was found to be positively correlated with wind speed (r=0.310) and rain fall (r=0.562); while negative correlation was found with morning RH (r=-0.146) and afternoon RH (r=-0.288). The progressive quantity released pheromone was positively significant with maximum temperature (r= 0.690) and highly positive relation with minimum temperature (r= 0.957) (Figure4).

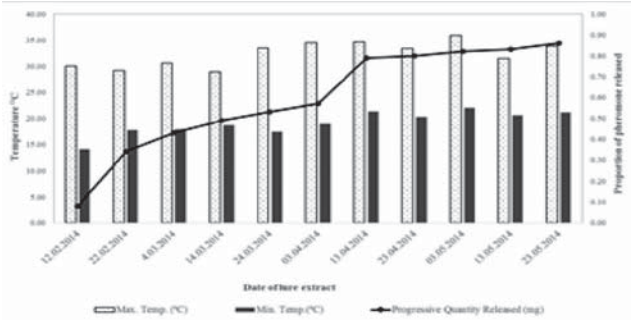

FIGURE 4

Influence of weather factors on DBM pheromone released rate during summer-2014

The data on release rate of pheromone and weather parameters were subjected to linear regression analysis; which revealed that, an extent of 98 per cent (R^2=0.985) of pheromone release rate was influenced by weather parameters with the following linear regression equation

$$Y = -2.583 + 0.053X_1 + 0.076X_2 - 0.009X_3 + 0.025X_4 - 0.040X_5 + 0.004X_6.$$

Trapping of DBM adults from the field exposed lures

The appearance of DBM infestation on cabbage occurred (4.50 moths/trap/10 days (rabi-2012); 7.17 moths/trap/10 days (Kharif-2013) and 5.17 moths/ trap/10 days (summer-2014) up to the first 10 days after the trap installation (DAT) (Figure 5). During rabi-2012, the incidence showed an increasing trend up to 30 days, as the plants put forth new tender leaves resulted in buildup of DBM population. The maximum number (116.33 moths/trap/10 days) of moth trapping was observed during 30 DAT followed by 40 DAT (91.50 moths/trap/10 days) as plants attained growth. Whereas during kharif-2013 and summer-2014 the trap catches showed an increasing trend up to 40 days with the maximum of 116.17 moths/trap/10 days and 269.83 moths/trap/10 days respectively, then there was a sudden fall in the mean number of moths (45.33 moths/trap/10 days (kharif-2013) and 76.50 moths/trap/10 days (summer-2014)) trapped at 50 DAT; again traps regained the maximum number (118.83 moths/trap/10 days (kharif-2013) and 221.67 moths/trap/10 days (summer-2014) at 60 DAT. There was a lot of fluctuation in the mean number of moths trapped up to 100 days in all the seasons.The moth trapping decreased and ceased as the crop attained senescence showing minimum number (13.33 moths/trap/10 days) at 90 DATduringrabi-2012 and between 91 to 100 DAT, 27.67 moths/trap/10 days were trapped. Whereas, the moth trapping decreased with total catches of 32.00 moths/trap/10 days and 23.83 moths/trap/10 days between 71 to 80 DAT in both kharif-2013 and summer-2014 seasons respectively. At 100 DAT the crop attained senescence showing minimum number of 9.50 moths/ traps/10 days and 6.33 moths/trap/10 days during kharif-2013 andsummer-2014 seasons respectively (Figure 5). Our

results clearly indicated the trapping capacity of male moths from the field exposed lures had lasted for 100 days during all the seasons.

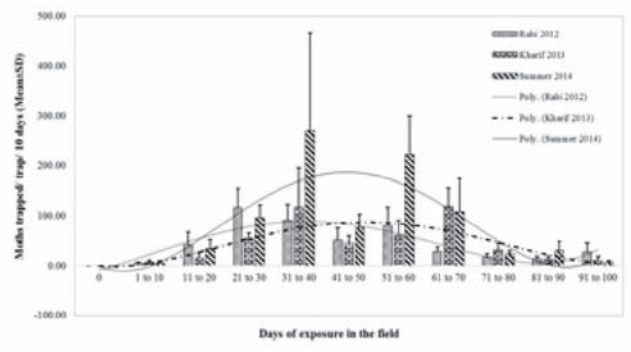

FIGURE 5

Number of DBM adults trapped across different seasons (vertical bar indicates SD for moth catches

DISCUSSION

As cabbage is grown in all the seasons the occurrence of DBM populations is expected throughout the year. Since the sex pheromone mediated mating is common among lepidopteron insects, the possibilities of this mechanism can be harnessed to check their multiplication through pheromone trapping resulting reduction in population.

Effective field application of sex pheromone depends on the type of lure used. Synthetic lures have to be designed to surpass calling females by releasing a higher pheromone signal. Mottus*et al.* (1997) evaluated the effects of pheromone release rate in cabbage fields in Estonia. Rubber mini dispensers (K-50) had an active exposure time of at least two months and delta traps were capable of monitoring a population of *P. xylostella* throughout the summer. Pheromone release rates between 8 and 17 ng per ha were recommended for maximum trap catches.

Here we examined only the performance of lures in attracting and trapping the DBM adults and the release rate of pheromone component for a period of 100 days across the different seasons but not the comparison between trap catches and larval infestation. We found pheromone lures loaded with synthetic female sex pheromone (1 mg) emitted the active ingredient at an average of <0.064 mg/day to <0.75 mg/day depending on the season. Almost 95% of the total chemical was released in a 100 days and the influence of weather conditions was found to be non-significant in rabi and kharifseasons.

Studies on monitoring field populations of diamondback moth using sex pheromone traps at Tsubaka, Japan, indicated a high population level throughout the year regardless of changes in climatic conditions (Kuwahara*et al.*1996). In our study male moths were trapped throughout the year but did vary with cropping period. The incidence of male moths showed an increasing trend as the plants put forth new tender leaves, resulted in buildup of DBM population in the middle of the cropping season across all the seasons. The reason for this has to be verified by conducting systematic field experiments by recording the population count or infestation levels periodically and comparing with monitoring trap catches.

There was a lot of fluctuation in the mean number of moths trapped over the 100 days. The collection of moths trapped started declining and ceased as the crop senesced. Kuwahara*et al.* (1996) too reported a sharp decline in population coincided with harvest of the vegetables in the furrows and a lag to recover the population density was detected for several weeks after harvest, indicating a low flight activity of the moths in the near proximity. Our results clearly indicate that the monitoring traps in trapping of male moths from the field exposed lures have lasted for 100 days during rabi-2012. Walker *et al.*(2001) showed that the pheromone traps were useful tools for predicting larval infestations in most crops in spring and early summer.

The progressive quantity releasedexerted a negative association with maximum temperature, afternoon relative humidity and wind speed; while positive relation with morning relative humidity and rainfall in rabi-2012. There was a highly significant negative association with minimum temperature in kharif-2013. Chandramohan (1995) studied the catches of males of*P. xylostella*using sex pheromone traps in the Nilgiris, Tamil Nadu, India. Weather elements cumulatively contributed to 16.34 % of the variation in trap catch. Minimum temperature, relative humidity and total rainfall had a negative influence on trap catch. There was a positive correlation existed between field pupal populations and pheromone catches.

In summer-2014, about 86 per cent of the total chemical was released in 100 days. The progressive

quantity released pheromone was found to be positive in significance with maximum temperature and highly positive relation with minimum temperature. Zhang *et al*. (2013) estimated that the polyethylene vial dispensers released pheromone at a higher rate and caught more male dogwood borer in the first two months of the trial, later catches in the traps declined, even though the release rate of pheromone did not change might be due to decrease in adult population.

Overall, 5 to 14% of the pheromone component was existed in the field exposed lures at 100th day after trap installation during all the three seasons. Therefore, the weather parameters would not have affected much on the release rate of pheromone component. The quantity released or the residual quantity remained in DBM pheromone lure during the study period would be sufficient to trap male moths for a period of 100 days after installation of the monitoring traps. Since the crop duration in the field varies between 90-100 days, the data suggests that one lure per trap is enough to attract male DBM in mass trapping plans and no change of lure during the crop period is required.

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Ecological and Foraging Aspects of the Cabbage Webworm, *Hellula undalis* Fabricius

SIVAPRAGASAM, A.
Cabi Southeast Asia Regional Center, Building A19, Mardi, Serdang, Selangor, Malaysia
E-mail: a.siva@cabi.org

ABSTRACT

The paper addresses the influence of temperature, ascertains the weed hosts and elucidates the foraging aspects of the larva of the cabbage webworm (CWW), *Hellula undalis* (Fabr.), a major pest of head or English cabbage (*Brassica oleracea* var. *capitata*) grown in the Malaysian lowlands. The optimal temperature for the development of CWW was 30ºC, whereby the intrinsic rate of increase r_m , was the highest. The ubiquitous weed, *Cleome rutidosperma* (Family: Capparidaceae) was found to be its natural host. The level of infestation of CWW on *C. rutidosperma* in the field was low, ranging from 0.1 % to 3.45%. Nevertheless, it provided a source population for the monocrop cabbage causing significant damage. The intra and inter plant foraging studies revealed that the CWW larva preferred the cabbage shoot to the leaf and this preference enhances its survival rate in the field.

***Keywords*:** Cabbage webworm, *Hellula undalis*, temperature, weed host, foraging behavior

INSECT pests and diseases are major biotic factors affecting head or English cabbage production both in the highlands and lowlands of Malaysia (Sivapragasam and Loke 1992). Among the pests which inflict serious damage, particularly in the lowlands, is the cabbage webworm (CWW), *Hellula undalis* (Fabr.) (Lepidoptera: Pyralidae). Although this insect has also been recorded in the Malaysian highlands (Ooi 1979), it has reached serious pest status only on cabbage grown in the lowlands. Stage specific crop life tables on cabbage (Sivapragasam, 1994) suggested that *H. undalis* is the most important pest contributing to about 41 percent of total mortality of the plants during the pre-heading stage.

Although CWW infests other cruciferous plants, such as chinese mustard, *Brassica chinensis* (L.), chinese kale, *B. oleracea var alboglabra* Bailey and radish, *Raphanus sativus* (L.), it is more serious on head cabbage because damage by a single larva (Figure 1) boring into the growing shoot of the cabbage plant (Figure 2) before its pre-heading stage could result either in the death of the plant or in producing small-sized multiple cabbage heads which are unmarketable. As such, there is no economic threshold level to initiate insecticide treatments but to advocate for preventive applications.

FIGURE 1. *Larva of* Hellula undalis

FIGURE 2. Hellula damage on head cabbage

There is also potential for indirect damage due to soft rot, *Erwinia carotovora*. In any case, complete control of CWW solely with insecticides is fraught with challenges due to the webbing behaviour of the larva and to some extend resistance development (Sivapragasam 1994). Thus, to develop pragmatic management strategies of CWW, an understanding of its biology and ecology in the Malaysian context is pertinent. Sivapragasam (2005) described some aspects of its biology and development. To complement the current repository of knowledge, in this paper some aspects of the ecology and foraging behavior are reported. The aspects are: (i) Effect of temperature on the development of CWW; (ii) Evaluating weeds as host of CWW and (iii) Dispersal and foraging behaviour of the CWW larva.

Materials and Methods

Here, general details of the materials and methods are described. For specific details, refer to Sivapragasam (1994).

Effect of temperature on development

The CWW used in this study was obtained from cultures maintained in the laboratory on cabbage leaves. All experiments were conducted in controlled temperature incubators (Rumed^{R}) maintained at different constant temperatures of 10, 15, 20, 25, 30, 35 and 40 ºC. Temperatures were maintained within ±1ºC and the R.H. between 80±5 percent at each temperature studied. The development and survival rates of the immatures at each temperature were determined using a cohort of at least 100 eggs laid within 24 h. At each temperature regimen, newly emerged larvae were isolated individually in Petri dishes (9.0 x 1.5cm^2) and fed with excised young cabbage leaves until pupation. The longevity and oviposition of the adult that emerged from each temperature regimen was determined using a rearing jar provided with leaves of *Brassica juncea* as the oviposition substrate. The number of eggs laid and the adults surviving were recorded for at least 20 pairs throughout their life span. The intrinsic rate of increase (r_m) was computed using tables of age specific oviposition (m_x) and survival rates (L_x) for the age interval (x) of one day using the analytical method described by Birch (1948).

Evaluating weed as hosts of CWW

To ascertain which of the weeds found in or near the lowland cabbage ecosystem were serving as hosts to CWW, common broad-leaf weed species were examined *in situ* and also collected and brought to the laboratory to examine for eggs and other stages of CWW. Feeding tests were also done under laboratory conditions with temperature ranging from 24ºC to 28ºC and R.H. 80±5%. Fresh young leaf samples of each weed were offered to five first and third instars of CWW confined in a Petri dish (10.0 x 2.0 cm) lined with moistened filter paper. First and third instars fed on cabbage leaves served as the 'control'. Further details of the experimental set-up are described in Sivapragasam (1994).For each weed species, the tests were repeated 3xand in all a total of 19 weed species belonging to 12 families were used in the tests.

Dispersal and foraging behaviour of the CWW larva

The intra- and inter-plant movement of CWW larva on cabbage were studied in a glasshouse usingcaged 1.5 month old cabbage plants with about 10 leaves. Details of the experimental procedures are given in Sivapragasam (1994). Briefly, for the intra-plant movement study, a cohort of eggs in an aluminium egg card (< 24h old) was clipped to the 3rd leaf of each cabbage plant (the 'egg leaf'). Leaf number 1 is the first open leaf from the top. The number of larva on each of the ten leaves was observed daily for 10 days. The experiment was replicated 10x and data from all ten replicates (or plants) were pooled for analysis.

The inter-plant movement studies were conducted using a larger cage (Sivapragasam, 1994). The design of the experiment was as follows: In the centre of the cage was a cabbage plant (hereafter called the source plant) with a cohort of 10 eggs (< 24h old). The 'source' plant was surrounded by four similar-aged cabbage plants placed equidistant from each other and the 'source' plant. However, one leaf from the 'source' plant and that of each of the neighbouring plants was stapled together to form a bridge between them. A corrugated cardboard paper sprayed with sticker was used as a barrier to prevent larval movement between plants except via the 'bridge'. Daily observations were made on the source and neighbouring plants for CWW larva. The experiment was terminated when all the larvae has

pupated in the pupal tray filled with vermiculite placed at the base of each cabbage plant. The experiment was repeated 10 x and the data from all the replicates were pooled for analysis.

Results and Discussion

Effect of temperature on development

Table 1 shows the development from egg to adult emergence (TDP), percent survival (egg to adult emergence), adult longevity for both the female and male, oviposition rate per day and intrinsic rate of increase (r_m) of CWW under various temperature regimens. Egg eclosion occurred from 15ºC until 35º C; none of the eggs hatched at 10ºC and 40ºC. TDP ranged from 14.64 days at 35º C to about 108.25 days at 15 ºC and the survival rate from 47percent at 25ºC to 3 percent at 15ºC. The number of eggs laid generally increased with temperature until 30ºC but declined at 35ºC. The longevity of the male and female differed significantly between temperatures (Table 1). The r_m values increased with temperature to a maximum at 30º C and then decreased.

Temperature affected the survival, development and other life table parameters including number of eggs laid and intrinsic rate of increase. This study suggested that the temperature most favorable for CWW appeared to be around 30ºC. At this temperature r_m was the highest. This finding agreed with that of Awai (1958) who reported that the optimal temperature for CWW development was 31º C. Messenger (1976)illustrated that for a given environmental condition, r_m could be used as a measure of favorability and that the higher the value r_m, the more favorable were the environmental conditions within which the population resides. Based on this, it can be inferred that the temperature conditions in the Malaysian lowlands with the mean monthly temperatures of ca 29º C (range: ca 24ºC to 35ºC) is optimal for CWW development. On the other hand, in the cooler highlands(e.g. Cameron Highlands, a major cabbage growing area at1,400 – 1,500 meters above sea level with a mean temperature of 18.7ºC (13.4ºC to 24.3ºC) development is impeded. Sachan and Gangawar (1980) reported the concomitant decrease in the importance of CWW with increase in altitude (with the concomitant decrease in temperature) at which the cabbage is planted. The effect of temperature on the distribution of CWW is explicitly shown from its data in temperate countries whereby CWW is reported to be major problem only in summer to late autumn (AVRDC 1978; Shirai and Kawamoto 1991).

Table 1

Development from egg deposition to adult emergence (TDP), percent survival, adult longevity oviposition rate and intrinsic rate of increase (r_m) of Helulla undalis under various temperature regimes

Parameters	Temperature ºC				
	15	**20**	**25**	**30**	**35**
TDP (mean ±day)*	108.25±2.10a	49.31±0.52 b	27.08±0.39 c	19.23±0.36 d	14.64±0.18 e
Survival (%, egg to adult)	3.00	18.00	47.00	35.00	28.00
Adult Longevity (days)(mean±day)					
· Female	11.75±1.91 a	8.77±0.76 b	8.00±0.74 b	3.79±0.48 c	3.08±0.78 c
· Male	12.25±2.42 a	6.70±1.11 b	6.79±0.55 b	4.28±0.71 b	4.15±0.66 b
Oviposition rate (eggs laid per female/day)	1.77	3.96	11.12	22.50	9.01
Intrinsic rate of increase (r_m)	0.01	1.02	1.11	1.15	1.08

***** Means with the same letter within a row are not significantly different at P< 0.05 using the Duncan's multiple range test.

Evaluating weeds as hosts of CWW

Amongst the weeds examined, CWW was found feeding only on *Cleome rutidosperma* DC (Family: Capparidaceae) (synonym: *C. ciliata* Schum. & Thonn.). Specimens of adults of both sexes emerging from *C. rutidosperma* were confirmed based on voucher specimens held at Malaysian Agricultural Research and Development Institute (MARDI), Malaysia. However, the level of infestation of CWW on *C. rutidosperma* in the field was low, ranging from 0.1 % to 3.45%.

C. rutidosperma is a ubiquitous herbaceous weed found in the waste grounds and cultivated lands of Malaysia (Barnes and Chandapillai 1972). The

damage by CWW larva on cleome is evident from the closely-webbed leaves in the apical region of the plant. In this study, only one larva of CWW was found per plant. In other studies, CWW was found on other species of *Cleome*, *viz*., *C. viscose* (synonym: *C. icosandra*) (Sivapragasam 1994).For example, Alam *et al*. (1961-62) recorded CWW on *C. viscosa* in Pakistan. In the Nearctic countries, the host plants reported for a congeneric species of *Hellula*, *viz*., *H. phidilealis*, seemed to be largely confined to plants in the families Cruciferae and Capparidaceae (Alam 1989). The infestation of cabbage and cleome by CWW is correlated to the fact that both these families share a similar phytochemical constituency characterized by the occurrence of isothiocyanates (mustard oils) (van Steenis 1972). The occurrence of other crucifer 'specialists' such as the flea beetles, *Phyllotreta* spp., the cabbage leaf webworm, *Crocidolomia pavonana*, and the diamondback moth, *Plutella xylsotella*, on *Cleome* (Sivapragasam 1994) supports this contention. In addition, *Cleome* and cabbage also share quite a similar suite of natural enemies (*e.g*., parasitoids) such as *Trathala flavoorbitalis* (Ichneumonidae), *Bassus* sp. and *Chelonus* sp. (both Braconidae) on CWW (Sivapragasam and Chua 1997).As for the low infestation level of CWW on *Cleomevis a vis* cabbage, from a foraging perspective, this could be attributed to the 'plantapparent' factor (Feeny 1977). By this it means that cleome grows in the field amongst other weeds and thus has low visibility to CWW, compared to cabbage which is grown as a monoculture in relatively larger patches. This can also be explained from a food resource perspective when insects forage for food. Root (1973) suggested the term 'resource concentration' for this phenomenon, *i.e*., low resource concentration in the case of cleome and a high one for cabbage.

Dispersal and foraging behaviour of the CWW larva

The within (or intra) plant distribution revealed that during the first 2 days after eclosion, the CWW larva remained on the egg leaf (*i.e*., the third leaf). On the third day after eclosion, one of the cabbage plants (out of 10 used in the experiment) had a larva on the shoot. From the fourth day onwards, most of the leaves had the larvae on them and from the sixth day until the ninth, most of the larvae had moved from the leaves to the shoot of the plants with about 43 % of the plants having two larvae per shoot on the sixth day. However, this number decreased to 10 % by the eighth day and by the ninth day, all the plants had only one larva per shoot.

In the inter-plant movement study, larval movement from the 'source' plant to the neighbouring plants was observed in 8 out of the 10 replicates. By the ninth day, 45 % of the neighbouring plants were found to have CWW larvae. Most of the larvae (84.6 %) found earlier on the 'source' plants, were found on the shoot. On the neighbouring plants, 40 % of the larvae were also found on the shoot and the rest on the leaves. Interestingly, the larvae on the neighbouring plants were first seen on the sixth day after the eggs were introduced, *i.e*., about 3 days after eclosion.

Both the intra-plant and inter-plant studies indicated that CWW larvae moved to the shoot of the plants from their initial location. Talekar *et al.* (1981) also indicated that the young CWW larvae preferred to burrow into the shoot rather than the leaves of Chinese cabbage, *Brassica campestris*. The initial increase in the number of larvae per shoot and the eventual decrease could be due to the limited space in the shoot to accommodate more than one CWW larva, particularly that of the later stages. Sivapragasam (1994) suggested that the preference of CWW larva for the cabbage shoot to the leaves (which were found to be nutritionally better) was largely due to seeking a natural refuge and protection against natural enemies, *viz*., predators, of the CWW larva under field conditions(Sivapragasam and Chua 1997).Although CWW does mine (e.g. in the first instar), web and fold the cabbage leaf in the later stages to protect itself, the cabbage shoot affords a better natural concealment for the larva to protect itself without much investment of energy for leaf folding or web making. The trade-off for the larva residing in the shoot was the higher survival rate (about 2.7x), and the higher intrinsic rate of increase r_m (about 1.5 x) than on the leaves (Sivapragasam and Chua 1997).It is not specifically known what attracts the larva to the shoot. William and Daxenbicher (1981) reported that younger and faster growing tissues of Chinese cabbage have higher quantities of glucosinolates than the older ones. It is not known whether there are any specific chemical cues attracting the larva to the shoot. In corn, the larva of *Pyrausta nubilalis* (Hueb) settles near those parts of the plant which are rich in sugar content (Beck 1956).

Conclusion

The study suggested two important factors that contributed to the distribution and pest status of CWW in lowland cabbages as opposed to those in the highlands. The first was the effect of ambient temperature, and the other was the presence of the weed, *C. rutidosperma*. The optimal temperature for the development of CWW was found to be 30ºC which is close to the mean ambient temperature in the lowlands.

The study on cleome underscores the fact that if a crop (*e.g*., cabbage) is introduced into an area where there is a closely related endemic weed (*e.g*., *Cleome*), one is likely to see a rapid adaptation by CWW to the newly introduced host. This is because of the common chemical cues (*e.g*., mustard oils) found in both crops that are conducive for feeding and oviposition. This was the case with CWW.

Based on the within and between plant dispersal studies, CWW prefers the shoot to the leaf. Although this provides greater survival for CWW larvae against natural enemies, from a management standpoint it is easier to control on the shoot. Thus, the farmer needs to deliver the insecticide as a shoot application to effectively manage CWW. In field trials, effective control of CWW on cabbage was achieved using weekly treatments of *Bacillus thuringiensis* for a period of five weeks on the shoot and this approach, besides being cost-effective, reduced the indiscriminate use of the insecticide (Sivapragasam and Loke 1992).

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Session 3

Insect plant interactions, host plant resistance and chemical ecology of diamondback moth and other crucifer pests

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Potential of *Barbarea* as a Trap crop for the Diamondback Moth: Developmental Changes in Glucosinolate and Saponin Content in Relation to Insect Behaviour and Performance

Badenes-Pérez, F.R., Reichelt, M.[1], Parrado Márquez, B.[2], Gershenzon, J.[3] and Heckel, D.G.[4]

Institute of Agricultural Sciences (CSIC), 28006 Madrid, Spain
E-mail: frbadenes@ica.csic.es
[1]Max Planck Institute for Chemical Ecology, 07745 Jena, Germany
E-mail: reichelt@ice.mpg.de
[2]Institute of Agricultural Sciences (CSIC), 28006 Madrid, Spain
E-mail: frbadenes@ica.csic.es
[3]Max planck Institute for Chemical Ecology, 07745 Jena, Germany
E-mail: gershenzon@ice.mpg.de
[4]Max Planck Institute for Chemical Ecology, 07745 Jena, Germany
E-mail: heckel@ice.mpg.de

Abstract

Glucosinolates are plant secondary metabolites used in plant defense. For the diamondback moth, *Plutella xylostella* L. (Lepidoptera: Plutellidae), glucosinolates are, however, essential in host plant recognition and act as oviposition stimulants. Some species and types of plants in the genus *Barbarea* (Brassicaceae) contain, besides glucosinolates, saponins that act as feeding deterrents and prevent survival of *P. xylostella* larvae. The concentration of glucosinolates and saponins in foliage can be very different depending on the species, variety, and type of *Barbarea*. Also, within a *Barbarea* plant, the distribution of glucosinolates and saponins is highly heterogeneous. Glucosinolates, but not saponins, are present on the leaf surface of *Barbarea* plants in concentrations that can be detected by *P. xylostella* females. Ontogenetic changes affect glucosinolate and saponin content in *Barbarea* plants. Younger *Barbarea* leaves contain higher concentrations of glucosinolates and saponins than older leaves. The overall glucosinolate and saponin content of plant foliage also changes with plant age. Changes in glucosinolate and saponin content in foliage affect both attraction and resistance to *P. xylostella* and other herbivores.

***Keywords*:** Host-plant resistance, oviposition, plant secondary metabolites, larval survival

Glucosinolates are plant secondary metabolites used for plant defense in the Brassicaceae (Halkier and Gershenzon 2006). In the case of herbivorous insects, glucosinolates are generally toxic for generalists and are used in host plant recognition by specialists (Hopkins *et al.* 2009). For the diamondback moth (DBM), *Plutella xylostella* L. (Lepidoptera: Plutellidae), glucosinolates and their hydrolysis products act as feeding and oviposition stimulants (Reed *et al.* 1989; Spencer *et al.* 1999; Renwick *et al.* 2006; Badenes-Pérez *et al.* 2010, 2011).

Like glucosinolates, saponins can provide protection against herbivores (Osbourn 1996; Nielsen *et al.* 2010; De Geyter *et al.* 2012a, 2012b). These compounds are found within some species of the genus *Barbarea* (Brassicaceae), in addition to glucosinolates and act as feeding deterrents for DBM larvae (Shinoda *et al.* 2002; Agerbirk *et al.* 2003a; Badenes-Pérez *et al.* 2010, 2014a). The saponins responsible for the lack or reduced survival of DBM on some species and types of *Barbarea*are 3-*0*-â-cellobiosylhederagenin (saponin 1) and 3-*0*-â-cellobiosyloleanolic acid (saponin 2) (Shinoda *et al.* 2002; Agerbirk *et al.* 2003a; Badenes-Pérez *et al.* 2010). Compared to several crucifer crops, *Barbarea vulgaris* R. Br. is highly attractive to ovipositing DBM females, making it suitable as a trap crop for this insect pest (Idris and Grafius 1996; Lu *et al.* 2004; Shelton and Nault 2004; Badenes-Pérez *et al.* 2004, 2014a;Shelton and Badenes-Pérez 2006). Trap crops like *B. vulgaris*, highly attractive to insects, but on which they cannot survive, are known as dead-end trap crops (Shelton and Nault 2004; Shelton and Badenes-Pérez 2006). Here we compare different *Barbarea* spp., varieties, and types to test which ones could have the highest potential as a dead-end trap

crop for DBM. We also show how glucosinolates and saponins vary with plant development in *Barbarea* and how this can affect resistance and within-plant preference of DBM.

Materials and Methods

Culture of insects and plants, analysis of glucosinolates and saponins, and bioassays were conducted as described in Badenes-Perez *et al.* (2011, 2014a, 2014b). The *Barbarea* spp. used in the experiments were *B. rupicola*, *B. verna*, and *B. vulgaris*. Within *B. vulgaris*, we tested the varieties *arcuata* and *variegata* and the types G, P, BAR and NAS (Agerbirk *et al.* 2003b; van Leur *et al.* 2006). Larval survival and oviposition preference for DBM was compared among these *Barbarea* lines and between each of the *Barbarea* lines and each of three crucifer crops (cabbage, *Brassica oleracea* L. *capitata*cv. Gloria; broccoli, *Brassica oleracea* L. *italica* cv. Marathon; and Chinese cabbage, *Brassica rapa* L. *pekinensis* cv. Kantonner). Plants used in the experiments comparing glucosinolate and saponin content among different *Barbarea* lines and comparing abaxial and adaxial leaf surfaces were 5-weeks old at the time they were used in the experiments. Plants used in the experiments comparing glucosinolate and saponin content in leaves of different size were 10-weeks old. Glucosinolate and saponin content was also compared in the foliage of plants that were 4-, 8- and 12-weeks old.

Results and Discussion

There were significant differences in the content of glucosinolates and saponins among the different *Barbarea* plants examined (Pd≤0.05) (Badenes-Pérez *et al.* 2014a). Plants of G-type *B. vulgaris* var. *arcuata* and plants of *B. rupicola* had, respectively, the lowest and highest glucosinolate content among all the plants analyzed (Pd≤0.05). G-type *B. vulgaris* plants contained more than 3 times higher content of saponin 1 and more than 50 times higher content of saponin 2 than *B. rupicola* and *B. verna* (Pd≤0.05). *B. verna* plants contained approximately 15 times more saponin 1 than *B. rupicola* plants (Pd≤0.05). Saponin 2 was not found in *B. rupicola*and *B. verna* contained only traces. Saponins 1 and 2 were associated with the lack of survival of DBM larvae (Badenes-Pérez *et al.* 2014a).

A significant negative relationship was found between leaf size and content of glucosinolates for both G- (y=13.01-0.95x; n=100; r=0.39; $F_{1,98}$=17.01; Pd≤0.001) and P-type *B. vulgaris* (y=14.81-0.86x; n=20; r=0.44; $F_{1,19}$=4.20; P=0.050) (Badenes-Pérez *et al.* 2014b). In G-type *B. vulgaris*, a significant negative relationship was also found between leaf size and content of saponins 1 (y=7.50-0.61x; n=100; r=0.51; $F_{1,98}$=34.89; Pd≤0.001) and 2 (y=2.50-0.23x; n=100; r=0.47; $F_{1,98}$=27.99; Pd≤0.001). There was also a significant negative relationship between leaf size and number of eggs laid by DBM per leaf area (y=1.84-0.17x; n=84; r=0.58; $F_{1,83}$=42.15; Pd≤0.001). The number of eggs laid by DBM per unit leaf area was positively correlated with glucosinolate content (y=0.32+0.04x; n=84; r=0.34; $F_{1,83}$=10.40; P=0.002).

When analyzing the whole plant foliage, there were significant differences in glucosinolate and saponin content among plants of different age in both G- and P-type plants (Pd≤0.05) (Badenes-Pérez *et al.* 2014b). The content of glucosinolates and saponins 1 and 2 washighest in 8-week-old plants and lowest in 4-week-old plants. The simultaneous presence of high concentrations of glucosinolates and saponins in small/young leaves of *Barbarea*, which are the most attractive to ovipositing DBM, protects the plant against this herbivore. The association between glucosinolates and saponins could indicate that, from an evolutionary point of view, saponins might have appeared after glucosinolates, enabling *Barbarea* plants to be defended against insects that had adapted to glucosinolate-defended plants. Saponins would then be what has been called a “second line of defense”, appearing as a response to herbivores that have overcome the “first line of defense” provided by glucosinolates (Shinoda *et al.* 2002).

Glucosinolates, but not saponins, were found on the leaf surface of *Barbarea* spp. in sufficient concentrations to be perceived by DBM females (Badenes-Pérez *et al.* 2011). There were also significant differences in glucosinolate content between abaxial and adaxial leaf surfaces within *B. rupicola and B. verna* plants (Pd≤0.05)(Badenes-Pérez *et al.* 2011). Glucobrassicin concentrations on the leaf surface were, respectively, 8 and 3 times higher on the abaxial compared to the adaxial side of *B. rupicola* and *B. verna*. Gluconasturtiin concentrations on the leaf surface were, respectively, 2.3 and 2.2 times higher on the abaxial compared to

the adaxial side of *B. rupicola* and *B. verna*. This higher concentration of glucosinolates on abaxial compared to adaxial leaf surfaces wasnot associated with a preference of ovipositing DBM (P>0.05) (Badenes-Pérez *et al.* 2011).

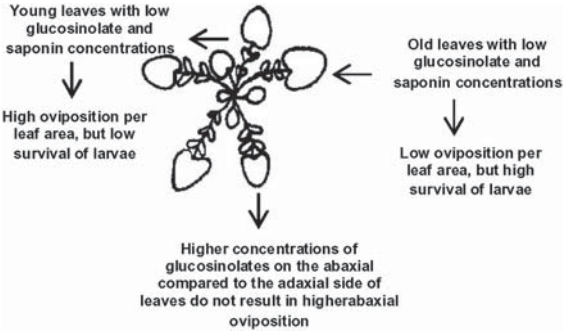

FIGURE 1

Summary diagram showing the distribution of defense compounds in Barbarea *leaves and its effect on DBM oviposition and survival of larvae.*

CONCLUSION

In *Barbarea*, content of glucosinolates and saponins is variable among different species and types. Within a *Barbarea* plant, the distribution of glucosinolates and saponins is highly heterogeneous (Fig. 1). Younger *Barbarea* leaves contain higher concentrations of glucosinolates and saponins than older leaves. Abaxial surfaces can have higher glucosinolate concentrations than adaxial surfaces of the same leaf. Oviposition preference by DBM is negatively correlated with leaf size, but does not seem to differ between the abaxial and the adaxial side of leaves. The simultaneous presence of high amounts of glucosinolates and saponins in small/young leaves of *Barbarea* provides protection against DBM and other herbivores. Further research in the field is necessary to study how changes in plant phenology can affect the potential use of *Barbarea* spp. as a trap crop for DBM and in conservation biological control.

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Attraction of Naive *Diadegma semiclausum* Hellen (Hymenoptera: Ichneumonidae) Females and *Cotesia vestalis* (Haliday) (Hymenoptera: Braconidae) to Cabbage (*Brassica oleracea*) Plant Volatile Organic Compounds Using the Olfactometer and Screen-Cage Methods

Ismail A., Mansour S.1, Mohamed Roff M.N.[2], Nadia M. Kermani[3] Mohd Hanaifh Yahaya[4], Idris A.B.[5]

School of Environmental and Natural Resource Science, Faculty Science & Technology, Universiti Kebangsaan Malaysia, 43600 Bangi, Selangor, Malaysia
E-mail: iabozid@yahoo.com
[1]Omar Al-Mukthar University. Zoology Department, P.O. Box. 919 Al-Bidya Libya.
E-mail: mansour_sm2001@yahoo.com
[2]Deputy Director/ Senior Principal Research Officer, Pest and Disease Management Programme, Horticulture Research Centre, MARDI HQ, P.O. Box 12301, GPO, 50774 Kuala Lumpur, Malaysia.
E-mail: roff@mardi.gov.my
[3]School of Environmental and Natural Resource Science, Faculty Science & Technology, Universiti Kebangsaan Malaysia, 43600 Bangi, Selangor, Malaysia.
E-mail: nadia.khermani@yahoo.com
[4]Horticulture Research Centre and Strategic Resources Research Centre MARDI Headquarter Persiaran, MARDI-UPM, 43400, Serdang Selangor, Malaysia
E-mail: hanifah58@yahoo.com
[5]School of Environmental and Natural Resource Science, Faculty Science & Technology, Universiti Kebangsaan Malaysia, 43600 Bangi, Selangor, Malaysia.
E-mail: idrisgh@ukm.edu.my

Abstract

Volatile organic compounds (VOCs) released by plant when attacked by herbivores could play a very important role in the indirect defense of host plants by attracting natural enemies of the attacking herbivore. The hymenopteran parasitoids, *Diadegma semiclausum* (Hellen) (Hymenoptera: Ichneumonidae) and *Cotesia vestalis* (Haliday) (Hymenoptera: Braconidae) are important biological control agents of the diamondback moth (DBM), *Plutella xylostella* (L.) (Lepidoptera: Plutellidae), the most important pest of cruciferous crops worldwide. These parasitoids use plant volatiles in their in-flight searching behavior. The objectives of this work were to determine the effects of the VOCs emitted by differently damaged Cabbage (*Brassica oleracea* L.) plants on the attracting response behavior of *C. plutellae* and *D. semiclausum* adult females using olfactometer and screen cage methods. Results showed that the naive females for each parasitoid discriminated separately between odors from plants damaged mechanically, by aphids, by cabbage leaf webber (*Crocidolomia pavonana* Zeller) larvae or by larvae of DBM. No comparison study between parasitoid species was detected. The plants that fed by *C. pavonana* larvae or mechanically were more attracted to the adult females *D. semiclausum* than intact plants in the olfactometer study. In the screen cage method, however, the plants damaged by DBM larvae were more attractive to the *Diadegma* females than to all other plants damaged by other types of damage. For *C. vestalis*, the females tested using olfactometer was also showed high significant preference to plants damaged by *C. pavonana* larvae, while plants damaged by DBM larvae were significantly preferred than to the *C. pavonana* by the naive females. Understanding the attraction of the VOCs emitted by host plants damaged by host or non-host larvae of these parasitoids may help us to discover the possibility of using plant-pest-parasitoid interactions for improving an integrated DBM management.

***Keywords*:** *Diadegma semiclausum, Cotesia vestalis, Plutella xylostella, Brassica oleracea,* volatile organic compounds

DIAMONDBACK moth (DBM), *Plutella xylostella,* has become the most destructive insect of cruciferous plants throughout the world (Talekar *et al.* 1993; Bhalla and Dubey 1986). *Plutella xylostella* feeds exclusively on plants of Cruciferae (Idris and Grafius 1996; Dosdall *et al.* 2011). Annual pest management costs for controlling DBM are approximately US$ 4-5 billion. (Zalucki *et al.* 2012). Chemicals are playing an important role in the production of cruciferous vegetables and many of them are insecticide products used for the control of DBM. (Sakai 1986). Due to unselective use of pesticides, DBM has developed resistance and has been very difficult to control (Mari 2012). However, maximizing the role of Biological Control Agents (BCA) is one of the ways to reduce dependence on chemical insecticides used for controlling DBM (Grzywacz *et al.* 2010). Numerous parasitoids and predators attack all developmental stages of *P. xylostella* (Kfir 2005). The natural enemies of DBM are important for biological control and the larval parasitoids are most commonly used as BCA of DBM. It could be true that an IPM program would be better if there were more species of parasitoids attacking different stages of DBM (Ooi 1990). Therefore, some parasitoid species have been introduced to Southeast Asia with various degrees of success in the biological control programs of *P. xylostella* (Lim 1986). The ichneumonid, *Diadegma semiclausum* has been recorded in many parts of the world as an important parasitoid of *P. xylostella* (Khatri *et al.* 2012). *D. semiclausum* and *Cotesia vestalis* as specialist parasitoids of DBM, are believed to be the best parasitoids in highlands and lowlands, respectively, and are keeping DBM population under control (Talekar *et al.* 1992; Murthy *et al.* 2013). The parasitoid impact on its host population depends upon its ability to find and parasitize it (Khatri *et al.* 2012). According to Soyelu (2014) the parasitic wasps can perfectly locate its host or food by using olfactory and visual signals. In general, natural enemies like parasitoids use herbivore induced plant volatile organic compounds (VOCs) to locate their herbivore host (Harris *et al.* 2012). Plant VOCs emissions differed between undamaged plants and plants under attack by herbivores (Kessler and Baldwin 2004). This is supported by the finding of Dicke (1999) that throughout the plant kingdom many species have been described as herbivore-induced semiochemical producers. An effective integrated insect pest management (IPM) program is thought to mainly be based on better understanding of the role of plant VOCs in the plant-insect interaction. As such, using the right plant at the right place and right time could guarantee the success of any IPM program. The aim of this study was to determine the effect of cabbage plants (*B. oleracea*) VOCs on the attraction of *D. semiclausum* and on *C. vestalis,* the two main parasitoids of DBM worldwide. It is hoped that the information from this study would contribute to betterment of IPM program for DBM and other insect pests in brassicas.

MATERIALS AND METHODS

Plants

Cabbage (*Brassica oleracea)* seeds were sown in plastic pots (12 cm diameter), filled with soil and grown in separate insect-proof cages (2.5 x 2 x 8 m), watered twice per day and fertilized 3x during the study (once per 2-weeks). The plants were grown under greenhouse conditions at the Malaysian Agricultural Research and Development Institute (MARDI), Serdang, Selangor, Malaysia and experiments were performed on 6-weeks-old plants (*i.e.* at the 7-8 leaf stage).

Insects

Cotesia vestalis (Haliday) (= C. plutellae) (Hymenoptera: Braconidae)

C. vestalis is a specialist solitary endoparasitoid preferably attacking *P. xylostella***.** Presumably parasitized earlier larval instar of DBM were field-collected and placed with few cabbage leaves for a week in plastic-cages (15 × 15 × 20 cm) in a climate-controlled room (27±2°C, 60±5% RH 16 h photoperiod) at the entomology laboratory of MARDI. Leaves were removed and replaced daily by fresh ones. The *C. vestalis* cocoons found daily were transferred into a plastic cage (15 × 15 × 20 cm) and incubated until the adults emerged. To obtain first generation (G1) *C. vestalis*, recently emerged and mated parasitoid females were placed with DBM larvae (second instar larvae offered for egg laying/ parasitism) on 1-3 small Chinese mustard leaves in four separate glass containers (30 cm high x 12 cm diameter) covered with mosquito netting (5 parasitoid females par container). The insects were given 20% honey solution as food. The parasitized larvae of DBM were transferred to small plastic cages (10 × 20 × 30 cm) and offered fresh Chinese mustard leaves

until the cocoon stage. After the emergence, mated *C. vestalis* females which were be used in the study were collected daily into metallic insect cages covered with mosquito-netting and fed on water-honey solution. One to 3-d-old females were used in the Y-tube assay.

Diadegma semiclausum Hellen (Hymenoptera: Ichneumonidae)

The parasitoid pupae were obtained from the entomology laboratory of MARDI Cameron Highland, Malaysia and further reared at the entomology laboratory at MARDI, Serdang, Selangor, Malaysia where the experiments were done.

Diamondback Moth (DBM), Plutella xylostella (L.) Lepidoptera: Plutellidae)

Adults and larvae of DBM were collected from small farms in Danau Desa at Kampong Batu Muda in Kuala Lumpur, Malaysia. No specific permits were required for the described field studies and permission was provided by the landowners. The field studies did not involve endangered or protected species. Small netting cages (40 x 35 x 45 cm) were used for rearing the insects on cabbage plants in the entomology laboratory at MARDI (27±2°C, 60±5% RH) and a photoperiod of 16:8 (L:D). The insects were fed with 20% honey solution through a cotton swab. Fresh mustard leaves were provided for the oviposition. One day later, leaves with the eggs were transferred to small plastic cages (20 x 15 x 30 cm) covered with mosquito netting. The larvae were kept and fed on cabbage leaves.

Cabbage Leaf Webber, Crocidolomia pavonana Zeller (Lepidoptera: Crambidae)

Larvae of the cabbage leaf webber were collected from MARDI premises at Jalan Kebun and from the SENR Research Centre of MARDI, Serdang, Selangor, Malaysia. The larvae were then reared on cabbage leaves in small cages (20 x 15 x 30 cm) covered with mosquito netting.

Aphid, Lipaphis erysimi (Kltb) (Homoptera: Aphididae)

Aphids were collected from Chinese mustard plants grown in glasshouses at MARDI, Serdang, Selangor, Malaysia. The insects were kept on Chinese mustard plants in small screen cages (45 × 45 × 50 cm) in the entomology laboratory at MARDI until further use in the experiments.

Y-olfactometer assay

The Y-shape olfactometer was made of transparent Plexiglas (2.5 cm diameter; stem 13 cm, arms 10 cm; angle of stem-arms 120°). Its arms were connected to two 22 L glass containers. An electric pump (Cole-Parmer Air cadet vacuum/pressure station, Illinois, U.S.A) was connected to the olfactometer to push the air into both containers through silicon tubes. Before being pushed into the glass containers, air was filtered through an activated-charcoal. Airflow in each arm of the Y-tube was adjusted with a flow meter to 600 ml/min. To investigate whether *C. vestalis* and *D. semiclausum* females respond differently to different type of damage, the parasitoid females were tested separately and individually per treatment. Plants from only one species (*B. oleracea*) were used and four treatments namely the undamaged plant versus infested with approximately 25-30 2nd and 3rd instar DBM larvae, undamaged plant versus infested with 15 2nd instar *C. pavonana* larvae, undamaged plant versus plants infested with 150 aphids and undamaged versus mechanically damaged plants. Each treatment was repeated five times. Each insect was allowed to respond for eight minutes and was used only once (25 insects per treatment, 100 insects in total per species). All experiments were carried out in daylight at 26±2 °C and 60 % relative humidity

Screen-cage assay

To investigate the effect of the VOCs of the cabbage plants (*B. oleracea*) on parasitic wasps *C. vestalis* and *D. semiclausum*, we used six-leaved stage cabbage plants which were damaged mechanically, by aphids, by cabbage leaf webber (*C. pavonana*) larvae or by DBM larvae plus five intact plants as control. Experimental and control plants were placed randomly in the screen-cage measuring 2.2 × 4.0 × 4.0 m. To do this, plants were firstly planted in pots and enclosed in rectangular screen cages measuring 2.5m x 1m x 0.60m. Six leaf-stage potted cabbage plants maintained in screen-houses for 24 h were exposed for 25 mated naïve parasitoid females (*C. vestalis, D. semiclausum*) of 2 and 4 d old. The parasitoid females were released from small plastic cage (15 × 15 × 5 cm), at 0800 h, at height of one meter in the middle of the screen house. After one, two, three, six and 24 h from releasing moment, the number of parasitoid females visually observed on each plant in the screen-house was counted and recorded to determine the preference of *C. vestalis* and *D. semiclausum*.

Results and Discussion

Time of exposures and type of damage significantly interacted to influence the number of *C. vestalis* parasitoids. The parasitoid, *C. vestalis* was found significantly more on plants damaged by DBM than by other means (Table 1)**.** Time or damage types alone also significantly influenced the number of parasitoid attracted to the treated plant.

Table 1

Mean numbers of C. vestalis females found on treatment plants (B. oleracea) at different times after release in free-choice bioassays (screen-cage)

Source	df	Sum of squares value	F-value	P-
Time	1	52.81	11.03	0.001
Damages	4	300.93	15.72	0.000
Time × damages	4	79.16	4.13	0.005
Error	65	311.10		

There was no significant interaction between time and damage types in influencing *D. semiclausum* response (Table 2). As for *C. vestalis*, time and type of damages significantly influenced the number of *D. semiclausum* responded or observed on treatment plants. The number of *D. semiclausum* females observed on plants damaged by DBM or *C. pavonana* larvae was more significant. No significant difference between the females' number found on plants damaged by other types of damage.

Table 2

Mean numbers of D. semiclausum *(DS) females found on treated plants (*B. oleracea*) at different times after release in free-choice bioassays (screen-cage)*

Source	df	Sum of squares value	F-value	P-
Time	1	90.69	25.15	0.000
Damages	4	437.88	30.35	0.000
Time × damages	4	47.74	3.31	0.018
Error	50	180.32		

The *C. vestalis* females attracted significantly more to VOCs from plants damaged by DBM and *C. pavonana* larvae than VOCs from the undamaged plants (Figure 1). No significant difference was observed between the *C. vestalis* females number responded to plants damaged by aphid, mechanically-damaged and *C. vestalis* female number that responded to undamaged plants.

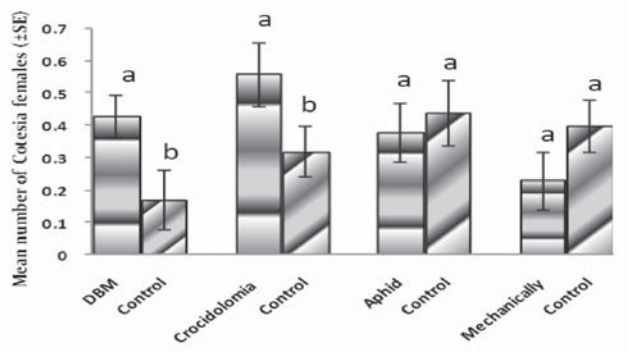

Figure 1

Mean number (±SE) of C. vestalis *females attracted to VOCs from undamaged (control) Cabbage plants (B. oleracea) and damaged plants as tested using Y-olfactometer*

There was no significant difference observed between the *D. semiclausum* female number responded to plants damaged by DBM larvae than female number responded to undamaged plants (Figure 2). However, *D. semiclausum* significantly attracted to plants damaged by *C. pavonana* larvae than undamaged plants.

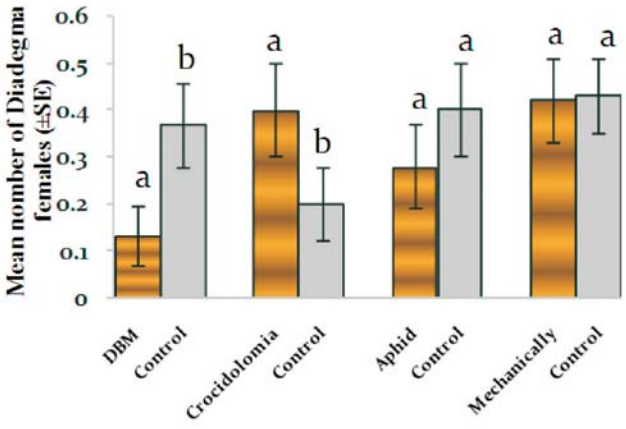

Figure 2

Mean number (±SE) of D. semiclausum *females attracted to VOCs from undamaged (control) cabbage plants (B. oleracea) and damaged plants tested using Y-olfactometer*

Results of this study demonstrated that the orientation response of parasitoid females to plants damaged by their hosts depends on the information sent by the host or by other herbivores infesting the plant (Han Baoyu *et al.* 2001; Reddy and Guerrero 2004). These results indicated the relative importance of different VOCs emitted by infested plants damaged by different herbivores. Different types of damage could cause a variation or difference in the VOCs components emitted by the same host (Dicke *et al.* 2000), which contributed to differences in parasitoid responses. In this study, the *C. vestalis* was found to

be only attracted to VOCs emitted by plants damaged by its insect hosts. However, the *D. semiclausum* significantly preferred the plant damaged by another chewing insect that is not its host. Producing and sprinkling of these VOCs could induce typical response in *D. semiclausum* females, which varied with where their hosts are. Strangely, in this study, regardless of the type of damages, both *C. vestalis* and *D. semiclausum* females responded according to the VOCs information they got from both damaged and undamaged plants (Ohara *et al.* 2003). It has been suggested that *D. semiclausum* females will prefer damaged plants over undamaged ones because of the attractive VOCs emanated by damaged plants (including its host) or the VOCs emitted by damaged plants by any type that might be attractive. Similarly, our result also shows that there are no significant differences between the number of *D. semiclausum* females responded to the undamaged plants and those attracted to plants infested/injured by aphids or damaged mechanically. Interestingly, *D. semiclausum* responded significantly less to plants with DBM damage than the control. Except between the VOCs plant damaged by *C. pavonana* and the control, *D. semiclausm* responded more to the control plants than plants damaged by other types. This indicates that the parasitoid preferred to use other signal such as the VOCs of plants damaged by *C. pavonana* (Figure 2) and/or the kairomones produced by the hosts such as frass and facces. In general, the plants probably emitted different VOCs that attracted or repelled the tested insects to different extent. We can explain that by:

- The cabbage plants infested by *C. pavonana* larvae produce VOCs that are attractive to *D. semiclausum* females than VOCs emitted by DBM-damaged plants.
- The cabbage plants attacked by DBM larvae do not produce enough VOCs compared with those attacked by *C. pavonana*, which make the females to choose those attacked by *C. pavonana*.
- The preference of *D. semiclausum* is mediated by host plant signals, associated with crucifers and/or the kairomones from it host and not by herbivore feeding.

The purpose of this research was to investigate whether or not if the changes in herbivore (non host) could induce different plant volatiles that would positively affect the olfactory response of *D. semiclausum* and *C. vestalis* females.

So, it is most probably that VOCs emitted by DBM-damaged cabbage seem not to be used by *D. semiclausum* as primary cues for host location. Whereas, the undamaged host plant volatiles have a strong influence on parasitoids. In our study, the volatiles emitted by plants damaged by chewing insects were attractive to *D. semiclausum* females as the females reacted evenly when they were offered *Crocidolomia*- damaged plants (Figure 2). This suggests that the preference of *D. semiclausum* for *Crocidolomia*- damaged plants may be explained by the quantity of VOCs released from the plants which may be less in plants damaged by DBM larvae when taking into account the amount of damage caused by *C. pavonana* larvae. However, *C. pavonana* is not *D. semiclausum*'s host insect; plants damaged by *C. pavonana* may produce VOCs that are more attractive to *D. semiclausum* and that the presence of *C. pavonana* in the field may increase effectiveness of *D. semiclausum* in integrated DBM management.

Conclusion

The result clearly indicate that *Diadegma semiclausum* and *Cotesia vestalis* responded differently to VOCs released from cabbage (*Brassica oleracea*) plants that were injured by different ways. The potential of using it to improve IPM for DBM is great but further study is needed especially under field situation.

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Session 4

Insecticides and insecticide resistance in diamondback moth and other crucifer pests

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Mutations in *ace1* Associated with an Organophosphate Insecticide Resistant Population o*f Plutella xylostella*

Kim Juil, Joo Yoo Ree1, Kwon Min2, Kim Gil-Hah3 and Lee Si Hyeock4
Highland Agriculture Research Center, NICS, RDA
E-mail: forwweek@korea.kr
[1] Highland Agriculture Research Center, NICS, RDA
E-mail: yooreenim@daum.net
[2] Highland Agriculture Research Center, NICS, RDA
E-mail: mkwon@korea.kr
[3]Department of Plant Medicine, Chungbuk National University
E-mail: khkim@chungbuk.ac.kr
[4]Department of Agricultural Biotechnology, Seoul National University
E-mail: Shlee22@snu.ac.kr

Abstract

Insensitive acetylcholinesterase (AChE) was determined to be involved in an EPN-resistant (ER) strain and a contaminated susceptible (CS) strain of diamondback moth (DBM, *Plutella xylostella* L.), as estimated by AChE inhibition assay using DDVP as an inhibitor in a nondenaturing electrophoresis gel. The ER strain exhibited very high AChE insensitivity, high resistance ratio, and two point mutations (G324A, A298S) in ace1-type AChE gene (*Pxace1*). The CS strain showed low AChE insensitivity, low resistance ratio, and it has only one point mutation (G324A). These findings suggest that the A298S mutation, along with reported G324A mutation (Baek *et al.* 2005), can be important in the development of organophosphate resistance. These results also suggest that the A298S mutation could be a good candidate for a molecular diagnosis marker for resistance monitoring. Three molecular diagnosis methods (Quantitative Sequencing; QS, PCR amplification of specific alleles; PASA and restriction fragment length polymorphism; RFLP) were developed which successfully detected specific resistance associated point mutations. Seven local populations of DBM were surveyed and showed high insecticide resistance levels and an A298S mutation in *Pxace1*. These methods can be used to monitor the resistance allele in field populations of DBMs and be used for resistance management strategy.

***Keywords*:** insensitive acetylcholinesterase, molecular diagnosis, PCR amplification of specific alleles, restriction fragment length polymorphism

Acetylcholinesterase (EC 3.1.1.7, AChE) terminates nerve impulse by catalyzing the hydrolysis of the neurotransmitter acetylcholine (ACh) to acetic acid and choline in the synapse and neuro-muscular junction in most invertebrates and vertebrates. It is a key enzyme in the insect nervous system in which the cholinergic system is essential (Fournier *et al.* 1992) and a target for the action of organophosphate (OP) and carbamate (CB) insecticides.

Insect AChE is encoded by one or two ace genes. *Drosophila melanogaster* was confirmed to have only one *ace* (later named as *ace2*) after full genome sequencing (Myers *et al.* 2000). However, many insect species have recently been found to have two genes. Two AChEs, differing in their substrate and inhibitor specificity, were first proposed to be encoded from two different loci in *Culex pipiens* (Bourguet *et al.* 1996). The second insect *ace* (later named as *ace1*) that is paralogous to the *ace2* was also cloned from Hemipteran insects (Li and Han, 2002), Lepidopteran species (Baek *et al.* 2005) and Blattodea (Kim *et al.* 2006) etc. In spite of the co-existence of two AChEs in many insect species including *Culex* spp. (Weill *et al.* 2003; Nabeshima *et al.* 2004), *Myzus persicae* (Nabeshima 2003) and *Plutella xylostella* (Baek *et al.* 2005), the *ace1*-type AChE only has been shown to be responsible for the AChE insensitivity-mediated resistance to OP and CB insecticides. More than 33 insect and acari species have developed resistance to OP and CB insecticides through the decreased sensitivity of AChE (Fournier and Mutero 1994).

Diamondback moth (DBM, *Plutella xylostella*) is a serious pest of cruciferous crop throughout the world including Korea (Kim *et al*. 2001). The nucleotide sequences of the ace1 and ace2 from the DBM were previously reported (Ni *et al.* 2003; Baek

et al. 2005). Initial studies on the mechanisms of prothiofos resistance in the resistant strain showed that insensitive AChE caused by three amino acid substitutions is likely the major factor in resistance (Seo and Boo 2004; Baek *et al.* 2005; Lee *et al.* 2007). The functional role of each mutation, however, still remains to be elucidated.

In this study, the EPN resistance mediated by AChE insensitivity mechanism in the DBM was investigated. AChE mutations associated with reduced sensitivity to OPs and CBs were identified from two strains of DBM with differential level of resistance and we want to solve the question which is the main factor among the mutations. Finally, molecular diagnostic methods were developed for the detection of the AChE mutations as alternative resistance monitoring tools.

Materials and Methods

Insects and bioassay

The susceptible (S) strain of DBM was initially obtained from Korea Research institute of Chemical Technology (KRICT) and has been maintained in an insect rearing room at Chungbuk National University. The EPN resistance (ER) strain was collected from Cheongwon gun, Chungbuk province, South Korea in 2004 and has been selected by LC_{50} concentration of EPN in every generation. A third strain (contaminated susceptible CS) strain possessing a low level of OP resistance originated from a selected subpopulation of the ER strain. It has been selected by LC_{50} concentration of EPN in 2008 for five generations. Local populations of DBM were collected from Bong-wha, Heng-seong, Hong-cheon, Je-ju, Mu-ju, Pyeong-chang and Yang-gu region during July to August 2008 in Korea. The three laboratory strains and seven local populations of DBM were reared on Chinese cabbage in plastic cages under the conditions of 25 ± 2 °C, 16L: 8D photoperiod, and 50~70% relative humidity. The susceptibility of DBM to some insecticides (EPN, DDVP and Thiodicarb) was determined by leaf-disk dipping method where commercial formulations were dissolved in distilled water to appropriate concentrations. Chinese cabbage leaf disks (5.5 cm diameter) were dipped into the test solution for 1 min and dried in a fume hood. The dried leaf disk was transferred to a petri dish, onto which 10 third instars were introduced. Mortality was recorded 48 hrs after treatment and LC_{50} value was estimated by probit analysis (Finney, 1971).

Enzyme preparation

Enzyme was extracted from the head part of 100~200 adult DBMs in each strain. Adult heads were prepared using the liquid nitrogen and each sample was homogenized with 0.3% Triton X-100 (v/v) containing 100 mM Tris-HCl (pH. 7.8) buffer using the glass-glass tissue grinder on ice. The homogenate was centrifuged at 10,000 g for 15 min and the supernatant was filtered through glass wool to remove lipids. The filtered homogenate was used immediately as the enzyme source in the AChE assay. Protein concentration was determined according to the method of Bradford (1976) using the bovine serum albumin as a standard protein.

Native PAGE and native IEF

Native PAGE was performed in a vertical electrophoresis unit (Novex® mini cell, Invitrogen, Carlsbad, CA, USA) by using the 7.5% T/2.67% C separating and 5% T/2.67% C stacking gel with a discontinuous Tris–glycine buffer system. Gels and running buffer contained 0.3 or 0.1% Triton X-100. Equal amounts (10g) of preparations were electrophoresed for 90 min at 120 V in a cold chamber. Following electrophoresis, the gel was activity-stained to visualize AChE bands according to the method of Lewis and Shute (1966). Native IEF was performed in a same vertical electrophoresis unit which

used in native PAGE by using the pH. 3~7 pre-cast IEF gel (Invitrogen). Following the focusing, the gel was activity-stained to visualize esterase bands incubated with 1mM alpha naphthyl acetate as a substrate.

Amplification of partial Pxaces *and point mutation survey*

Partial cDNA fragments of the *ace1* type acetylcholinesterase gene in DBM (*pxace1*) and *ace2* type acetylcholinesterase gene in DBM (*Pxace2*) were amplified from the first strand cDNA or genomic DNA with a set of primers (Table 1) designed from Genbank database. All PCRs were performed by EX Taq Polymerase (Takara, Shiga, Japan) and DNA Engine Dyad Peltier Thermal Cycler (Biorad, Hercules, CA, USA).

Molecular diagnosis

Based on previous investigation, we modified the quantitative sequencing (QS) method (Kwon *et al.*, 2008). A 484-bp genomic DNA fragment was amplified from individual genomic DNAs obtained from both S and ER strains using DBM ace p-F and R primer set. Individual PCR products were purified using a PCR purification kit (Qiagen) and sequenced to confirm the genotypes at mutation sites (NICEM Sequencing Facility, Seoul National University, Seoul, Korea). To identify any sequence polymorphisms in the mutation region, the genomic DNA template quantized by using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). The standard genomic DNA templates were mixed in the following ratios: 0:10, 1:9, 3:7, 5:5, 7:3, 9:1, and 10:0 (resistant allele : susceptible allele at each mutation site). Standard DNA template mixtures (50ng) were amplified by EX Taq Polymerase (Takara) and DNA Engine Dyad Peltier Thermal Cycler (Biorad) with following program: 94 °C for 5 min, 35 cycles of 94 °C for 20 sec, 60 °C for 20 sec, 72 °C for 30 sec, followed by final extension at 72 °C for 5 min. PCR products were purified using a PCR purification kit (Qiagen). The nucleotide signal intensities of the resistant and susceptible alleles at each mutation site were measured from the sequence chromatogram by using Chromas version 2.31 software (Technelysium Pty Ltd., Tewantin, Australia) and the signal ratios were calculated. Partial *Pxace1* was amplified using DBM ace p-F and R primer set from the susceptible (S), contaminated susceptible (CS) and EPN-resistant (ER) strains of DBM for restriction fragment length polymorphism (RFLP). PCR was performed by EX Taq Polymerase (Takara) and DNA Engine Dyad Peltier Thermal Cycler (Biorad) with following program: 94 °C for 10 min, 45 cycles of 94 °C for 10 sec, 60 °C for 10 sec, 72 °C for 20 sec, followed by final extension at 72 °C for 5 min. The amplified PCR products were digested by Eag¥°for 2 hours at 37 °C. Results were analyzed by electrophoresis on 2% agarose gel in 1X TBE buffer.

PCR amplification of specific allele (PASA) was performed by susceptible allele specific or resistance allele specific primer, EX Taq Polymerase (Takara) and DNA Engine Dyad Peltier Thermal Cycler (Biorad) with following program: 94 °C for 5 min, 45 cycles of 94 °C for 5 sec, 57 °C for 5 sec, 72 °C for 10 sec, followed by final extension at 72 °C for 5 min. PCR products were analyzed by electrophoresis on 2% agarose gel in 1X TBE buffer.

Results and Discussion

AChE inhibition assay and mutation survey in ace1

The ER strain exhibited 314-fold resistance to EPN compared to the S strain as judged by LC_{50} values (Table 2). Moreover, the ER strain showed cross-resistance to DDVP and thiodicarb. The CS strain was ca 5-fold resistant to thiodicarb and 1~2 fold resistant to OP insecticides such as EPN and DDVP. Therefore, the ER strain showed a high level of resistance whereas the CS strain showed low level of resistance to OP or CB insecticide. The enzyme preparations of the three strains from head parts were differentially inhibited by 100 or 200μM DDVP on native polyacrylamide gel (Figure 1). As shown in the inhibitor-untreated lanes, almost identical levels of AChE activity were detected in all strains. When inhibitor was treated, the AChE of the S strain was almost completely inhibited. In contrast, the AChE of the ER strain was ca. 30% inhibited whereas that of the CS strain was ca. 80% inhibited (Fig. 1A and C). Based on IEF result, there was no significantly highly expressed esterase isozyme (Fig. 1B). However, strain specific polymorphic esterase isozyme banding patterns showed in three strains. And all esterase isozymes were clearly inhibited by DDVP (Fig. 1B).

Partial cDNA sequence of Pxace1 was obtained from three strains (S, CS and ER) by PCR with sequence-specific primer sets (Table 1). Two point mutations (G324A and A298S) reported previously (Baek *et al.* 2005; Lee *et al.* 2007) were also found. Only the G324A (GGA to GCA) mutation was found in the CS, whereas both G324Aand A298S (GCC to TCC) mutations were observed in the ER strain and confirmed from pooled genomic DNA amplification (Fig. 2). Thirteen and five silent mutations were identified in the ER and CS strains, respectively. Three exons and two introns (1st intron, 293 bp in S versus 283 bp in CS and ER; 2nd intron, 413 bp) were predicted within the partial *Pxace1* genomic DNA (2.187 kb in S versus 2.177 kb in CS and ER) (Kim *et al.* 2012). Compared to the sequences of S strain, 37 and 30 nucleotide polymorphisms were found in the CS and ER strains, respectively, and eleven nucleotide deletions and a single nucleotide insertion were observed in the 283 bp intron of the CS and ER *Pxace1* (cf. 293bp of S *Pxace1* intron). Additionally, five and two nucleotide polymorphisms were observed in the 413 bp intron of *Pxace1* CS and ER strain, respectively. Based on intron sequence

comparison, the ER and CS AChEs appeared to be more closely related each other compared to that of S strain. From these results, the G324A and A298S mutations, when present together, are likely to confer high levels of EPN resistance in DBM whereas the G324A mutation alone only provides a baseline resistance. A summary of the correlations among insecticide resistance ratio, AChE insensitivity and mutation(s) is shown in Table 3.

Quantitative sequencing (QS)

When sets of standard DNA mixtures with different ratios of resistant and susceptible alleles were amplified and sequenced, the signal intensity of each resistant allele increased as the resistance allele frequency increased (Fig. 3A) The A298S mutation was detected in all of the seven local populations of DBM examined (Fig. 3B) and the G to A mutation was saturated in all of the local populations (data not shown). Bioassay revealed that over 50% larvae survived following the treatment of recommended dose (400 ppm) of thiodicarb in all tested local populations (data not shown).

Restriction fragment length polymorphism (RFLP)

RFLP method was used to determine the presence or absence of the A298S mutation in the *Pxace1*. Partial *Pxace1* fragments were amplified using DBM ace p-F and R primer set from the S, CS and ER strains of DBM. Although the S and CS strains were digested by EagI, the ER strain was not digested because of the presence of the A298S mutation (Fig. 4A). The seven local populations of DBM showed the same result, confirming the presence of the A298S mutation (Fig. 4B).

PCR amplification of specific alleles (PASA)

Several factors, such as annealing temperature, primer, and template concentrations were tested to determine the optimum conditions of the PASA that enable complete allele-specific detection of the A298S mutation. The critical annealing temperature that ensures no allele-nonspecific amplification was determined to be 57 °C. Considering both the level and specificity of amplification, about 10 ng template DNA per 20μl reaction was determined to be the optimum template concentration. To determine optimum concentrations for respective allele-specific primers, equal amount (50 nM) of each primer were needed (Fig. 4C). Therefore, following conditions were considered to be optimal while performing PASA: 10 ng template DNA, 57 °C annealing temperature, 50 nM of allele specific primers. Allele-specific PCR products were amplified by susceptible allele-specific primer in the S and CS strains but not in the ER strain and seven other local populations. When the resistance allele-specific primer was used, however, opposite results were obtained. The PASA results matched completely with those of PCR-RFLP in all the laboratory strains and local populations of DBM (Fig. 4C and D).

DISCUSSION

Involvement of insensitive AChE in the OP and CB resistance was confirmed by the inhibition assay on native PAGE gel (Fig. 1). The ER strain showed high level of AChE insensitivity whereas the moderately resistant CS strain showed a low level of AChE inhibition. The Gly324Ala (GGA to GCA) mutation observed in CS and ER strains (Fig. 3A). The A298S mutation was only observed in the ER strain. Based on DBM local population genotyping data, the G324A mutation was almost saturated but the A298S mutation was mixed in Heng-seong and Je-ju populations. From these data, the A298S mutation might be an additive factor and substantially increase to the AChE insensitivity. To date, no DBM individual with the A298S mutation alone has been found, further suggesting that DBM may acquire the G324A or other mutation(s) first prior to the A298S mutation perhaps due to the fitness disadvantage of having A298S mutation alone. This resembles the case of the super kdr mutation in para-type voltage sensitive sodium channel, where M918T mutation does not exit alone but is always accompanied by another L1014F mutation, most likely due to the fitness disadvantage associated with the M918T mutation (Lee at al. 2001).

The A298 residue is located in the characteristic 'FGESAG' motif surrounding the active serine, and lies in the oxyanion hole that can contributes to stabilizing the tetrahedral molecule during catalysis. The alanine to serine substitution changes the side group from –CH3 to –CH2OH, which is believed to alter the conformation of the adjacent serine of the catalytic triad and to affect the interaction between AChE and both substrates and inhibitors (Jiang *et al.* 2009). This site corresponds to A201 in *T. californica* AChE, and the same amino acid substitution has been reported to confer resistance in the cotton aphid, *Aphis gossypii* (Andrews *et al.* 2004; Li and Han 2004; Toda *et al.* 2004).

TABLE 1

Primers used for Pxace1, 2 amplifications, Quantitative sequencing (QS), restriction fragment length polymorphism (RFLP) and PCR amplification of specific allele (PASA)

Name	Sequence
For Pxace1 amplification	
DBM ace1 F	CGCGCCAAGTACGCCGACAA
DBM ace1 R	GCGAGCGATCGTATCGGCATTT
DBM ace1 F1	GGGACGCTCGGCATTTGTGAAT
DBM ace1 R1	CCGGAAAAGCGAGATTCAAACTTGAA
For Pxace2 amplification	
DBM ace2 F	GCGGATTATTTCCGATTACACGCACA
DBM ace2 R	GGTGCCTTCATCTTGATTGCTTCCAA
DBM ace2 F1	GGGGAGTGGATGCGAAGACCATCT
DBM ace2 R1	CGAGGGCGGTGAGCAGCACTAT
For Pxace1 point mutation survey, QS and RFLP	
DBM ace p-F	GGCACGGCCACGCTAGATGTTT
DBM ace p-R	CCGAGCGTCCCCCACTCATTATTT
For PASA (A298S)	
Dace1PASAR	GTCACATTGTTTGGAGAATCGT
Dace1PASAS	GTCACATTGTTTGGAGAATCGG
Dace1 PASA	GAGCGTCCCCCACTCATTATTT

TABLE 2

Resistance ratio of susceptible (S), contaminated susceptible (CS) and EPN resistance (ER) strains of P. xylostella *against EPN, DDVP, Thiodicarb*

Insecticide	**Strain**	**LC_{50}(ppm) (95%CL[a])**	**RR[b]**
EPN	S	1.8 (0.9~3.0)	1.0
	CS	2.1 (1.3~3.3)	1.2
	ER	565.5 (417.4~725.8)	314.2
DDVP	S	3.4 (2.4~4.9)	1.0
	CS	5.8 (3.8<“8.7)	1.7
	ER	208.2 (173.4<“245.4)	61.2
Thiodicarb	S	37.2 (27.7~47.9)	1.0
	CS	185.1 (136.3<“240.6)	5.0
	ER	1376.7 (915.0<“3504.6)	37.0

[a] 95% Confidence limits

[b] Resistance ratio: LC_{50} value of CS or ER strain/LC_{50} value of susceptible strain

TABLE 3

Correlations of strain and insecticide resistance ratio or AChE insensitivity or mutation(s)

Strains	Resistance ratio	AChE insensitivity	Point mutation(s)
S	Low	Extremely Low	No
CS	Low	Low	G324A
ER	High	High	G324A, A298S

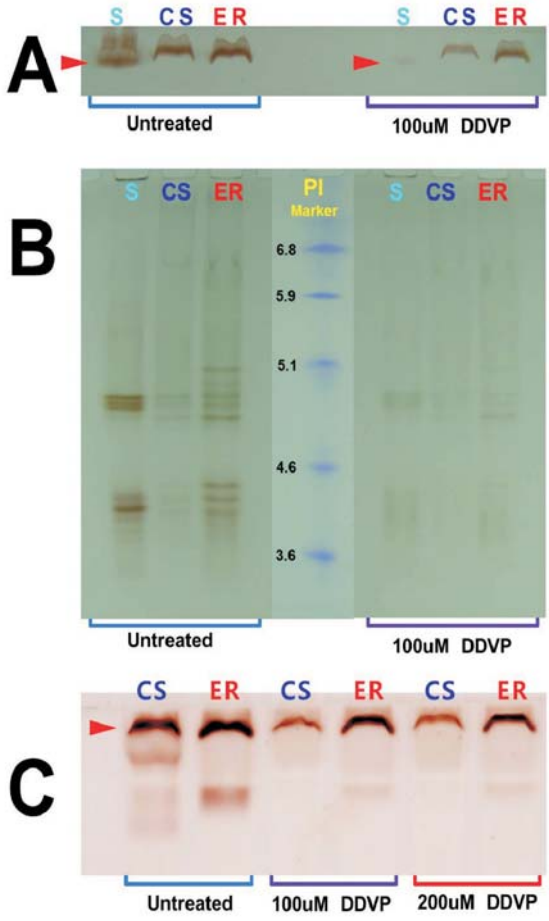

FIGURE 1

Native PAGE of AChE (A, C) and native IEF esterase (B) results from susceptible (S), contaminated susceptible (CS) and EPN resistance (ER) P. xylostella *with or without 100 or 200uM DDVP (Dichlorvos) as inhibitor. Identical amount (10 ìg) of enzyme preparations from each strain of adult head part were run in the presence of 0.3% Triton X-100 in the native gel and running buffer (A, C) or focused in the absence of Triton X-100 (B) in a cold chamber. Red triangle indicated major AChE band*

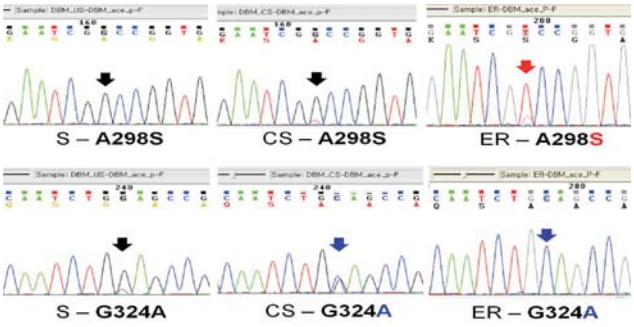

FIGURE 2

Nucleotide sequence chromatograms of the two mutation sites of Pxace1 from the susceptible (S), contaminated susceptible (CS) and EPN-resistant (ER) strains of DBM. The point mutation site is indicated with arrows. Partial fragment of Pxace1 amplified using DBM ace p-F, R set and each strains of pooled gDNA which extracted from over 30 DBMs

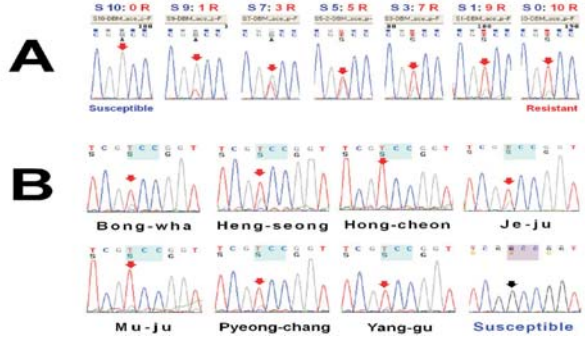

FIGURE 3

Sequencing chromatograms of the standard template DNA mixtures with different ratios of resistant and susceptible alleles at the A298S mutation site (A) and that of local population (B). Partial fragment of Pxace1 amplified using DBM ace p-F, R set. The point mutation site is indicated with arrows

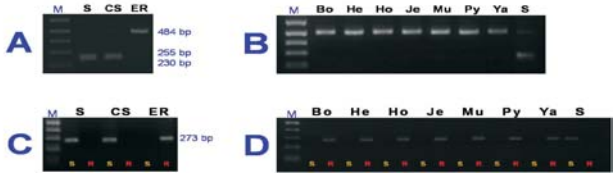

FIGURE 4

Result of the RFLP (A and B) and PASA (C and D) for the diagnosis of insensitive AChE as determined by the presence or absence of the A298S mutation in the Pxace1. *Partial* Pxace1 *amplified using DBM ace p-F and R primer set from the susceptible (S), contaminated susceptible (CS) and EPN-resistant (ER) strains of DBM. After than 484bp of PCR product digested by Eag¥°for 2 hours at 37¡É. Although S and CS strains were digested two fragments, ER strains did not digest because of point mutation (A). Seven local populations of DBM showed same result and S strain used as positive control (B). S, CS ER strains of pooled DNA amplified 273bp fragment using universal PASA primer and susceptible allele specific primer or resistant allele specific primer (C). PASA also showed same results from surveyed seven local populations of DBM (D). M: size marker, Bo: Bong-wha, He: Heng-seong, Ho: Hong-cheon, Je: Je-ju, Mu: Mu-ju, Py: Pyeong-chang, Ya: Yang-gu and S: susceptible strain of DBM*

CONCLUSION

G227A mutation was proposed to be the main factor in insensitive AChE (Baek *et al.* 2005). Lee *et al.* (2007) also reported three different mutations (D229G, A298S and G324A, amino acid number based on deduced amino acid sequence from open reading frame, among which the A298S and G324A mutations were suggested to be involved in prothiofos-resistance through three-dimensional modeling. Sequence comparison of partial fragment of *Pxace1* revealed that the two point mutations (A298S and G324A) were almost saturated in the highly resistant ER strain, demonstrating that these two mutations are the main contributing factor to AChE insensitivity.

Molecular diagnosis methods such as quantitative sequencing (QS), Restriction fragment length polymorphism (RFLP) and PCR amplification of specific alleles (PASA) were developed to detect the A298S mutation. The three methods showed high sensitivity and accuracy. QS can detect all nucleotide polymorphism within sequenced fragment. However, QS needs 2 or more days and about $30 (US). On the other hand PASA and RFLP, though they can only detect single nucleotide substitution, take only ca 2-4 hours and $5-10 (US), respectively. Many parameters were tested to determine the optimal conditions of PASA that enable complete allele-specific detection. From these results it was concluded that the two point mutations identified contributed to develop the EPN resistance in DBM (G324A; basic and A298S; additive) and molecular diagnosis methods were developed. QS, RFLP and PASA showed high sensitivity and accuracy and hence these methods can be used for DBM resistant genotyping and resistant management strategy.

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- Some of tables and figures were modified from Kim *et al.* 2012

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Effectiveness of New Anthranilic Diamide Insecticide Cyantraniliprole 10% OD Against Diamondback Moth and Sucking Insect Pests of Cruciferous Vegetables

KODANDARAM, M.H., RAI, A.B.[1] AND JAYDEEP HALDHAR[2]
Division of Crop Protection
ICAR-Indian Institute of Vegetable Research, Varanasi, India
E-mails: kodandaram75@gmail.com
[1] abraiiivr@gmail.com
[2] jaydeep.haldhar@gmail.com

ABSTRACT

Cyantraniliprole 10 OD (Cyazpyr) is a second generation anthranilic diamide insecticide representing the ryanodine receptor modulators. Unlike other diamide insecticides, this new insecticide has broad spectrum of activity against both chewing and sucking insect pests. Diamondback moth (DBM), *Plutella xyllostella* is the most serious lepidopteran pest and flea beetle, *Phyllotreta cruciferae*, painted bug, *Bagrada hilaris*, aphids, *Brevicoryne brassicae, Myzus persicae* and *Lipaphis erysimi* are other major leaf feeding and sucking insect pests contributing to significant yield losses in crucifer vegetables. The present study was undertaken to determine the effectiveness of cyantraniliprole 10 OD in comparison to other commonly used insecticides against DBM and sucking pests of crucifers by different bioassay methods. The three doses of cyantraniliprole @ 60, 70, 90 g a.i./ha evaluated were most effective and similar to the effectiveness of another novel insecticide, emamectin benzoate, with 100 % mortality against third instar *P. xyllostela* at 48 hours after treatment. Spinosad was the next best treatment with 82.67 % mortality. Different species of aphids, *B. brassicae, L. erysimi* and *M. persicae* were highly susceptible to cyantraniliprole @ 60 g a.i./ha and recorded the highest mortality at 80, 86 and 75% respectively. Flea beetle was less susceptible to cyantraniliprole, as compared to other commonly used insecticides, however maximum mortality of 71.25% was obtained with the highest dose of cyantraniliprole @ 90 g a.i./ha. Cyantraniliprole was in effective against the painted bug at all three doses. The present findings indicate that cyantraniliprole 10 OD @ 60 g a.i./ha was highly effective against *P. xyllostella* and all aphid species infesting crucifers. This new insecticide can strengthen integrated pest management (IPM) and insecticide resistance management (IRM) programmes in cruciferous vegetables.

***Keywords*:** anthranilicdiamide, cyantraniliprole, new insecticide, DBM, aphids

INDIA is the second largest vegetable producing country in the world after China. Cabbage, cauliflower and radish are major cruciferous vegetables grown across the country. There are 400,100 ha of cabbage and 433,900 ha of cauliflower area, which accounts for a 4.3 and 4.6% share of the total vegetable area, respectively (NHB, 2014). The diamondback moth (DBM), *Plutella xylostella*, is a major chewing pest and causes losses up to 50% in India (Sandur, 2004). Globally, it is estimated to cost US$ 4–5 billion annually in terms of direct losses and control costs (Furlong *et al.* 2013). Aphids (*Brevicoryne brassicae, Myzus persicae* and *Lipaphasis erysimi*) are major sucking pests causing 8-24% loss (Badenes-Perez and Shelton 2006). Flea beetle and bagarda bugs are minor pests but they are of significant importance in late season brassica vegetable and seed production.

In India, pesticides use in vegetables crops is around 13-14%, cole crops is the fourth highest crop for pesticide usage (Kodandaram *et al.* 2013). Most farmers rely on the use of synthetic insecticides for controlling insect pests in cruciferous vegetables, especially DBM, *Spodoptera* spp, aphids and flea beetles. Extensive use of these conventional groups resulted in pest resistance, outbreaks of secondary pests, pesticide residues, direct hazard to the users and adverse effect on environment and non-target organisms. DBM has developed field resistance to 93 insecticides representing all major classes of insecticides (APRD 2015). It is ranked second in the

Arthropod Pesticide Resistance Database (APRD). This resulted in huge demand for new insecticide products by farmers.

Cyantraniliprole (Cyazpyr) is a second generation new anthranilic diamide molecule with broad spectrum activity against chewing and sucking insect pests (Selby *et al.* 2013). This new molecule selectively binds to the ryanodine receptors (RyRs) in insect muscle cells, resulting in activation of RyRs and causing an uncontrolled release of Ca^{+} ions from internal stores which leads to depletion of calcium, muscle paralysis and death (Cordova *et al.* 2006; Sattelle *et al.* 2008). Due to unique mode of action it is classified by the Insect Resistance Action Committee (IRAC) as a ryanodine receptor modulator, in Group 28, and shares this mode of action with two other commercial diamides, chlorantraniliprole and flubendiamide. Cyantraniliprole causes rapid feeding cessation and protects foliage and developing fruits. It is highly suitable for greenhouse vegetable crops and the translaminar activity makes it suitable for foliar and soil applications. Therefore, the present investigation was carried out to determine the laboratory effectiveness of cyantraniliprole 10 OD in comparison to other commonly used novel insecticides against DBM, flea beetle, painted bug and aphid pests of crucifer vegetables.

Materials and Methods

Insect cultures

Larvae of DBM, *P. xylostella* were collected from the cabbage fields of ICAR-Indian Institute of Vegetable Research (IIVR), Varanasi and were reared in laboratory at 27±1°C and 60±5% RH. Similarly the flea beetle, *Phyllotreta cruciferae*, painted bug, *Bagrada hilaris*, different aphids viz., *Brevicoryne brassicae, Myzus persicae* and *Lipaphis erysimi* were collected from infested leaves of cabbage and mustard from the farm of ICAR-IIVR. Third instar F1 larvae of DBM, adults of flea beetle, bagrada bug and apterous, viviparous adult aphids were used for bioassay experiments.

Insecticides Used

The proprietary formulations of insecticides used in this study were obtained from their respective source of supply and their recommended doses were used for bioassays (Table 1). Insecticide formulations were diluted with deionized water containing spreader/sticker (Excel Crop Care) at 0.1%.

Table 1

Insecticides used for effectiveness studies with DBM and sucking pests of cruciferous vegetables

Insecticides & Formulation	Product Name	InsecticideGroup (IRAC Code)	Application Rate (g.ai/ha)	Dose used (ml/g) perLitre of Water	Source of Supply
Cyantraniliprole 10 OD	Benevia	Diamide (28)	60 75 90	1.3 1.5 1.8	E .I. Dupont
Chlorantraniliprole18.5SC	Coragen	Diamide (28)	50	0.3	
Flubendamide 40 SC	Fame	Diamide (28)	18.24	0.5	Bayer Crop Science
Emamectin benzoate 5SG	EM-1	Avermectins (6)	10	0.5	Dhanuka Agritech
Spinosad 2.5 SC	Success	Spinosyns 5 A	17.5	1.5	Dow Agro Science
Indoxacarb 15.8 EC	Avuant	Oxadiazines (22 A)	40	0.5	E .I. Dupont.
Novaluron 10 EC	Rimon	Benzoylureas (15)	75	1.5	Indofil
Imidacloprid 18.5 SL	Confidor	Neonicotinoid (4A)	25	0.35	Bayer Crop Science
Imidacloprid 70 WG	Admire	Neonicotinoid (4A)	25	0.10	
Imidacloprid 30 SC	Confidor super	Neonicotinoid (4A)	26.25	0.15	
Thiamethoxam 25 WG	Actara	Neonicotinoid (4A)	25	0.35	Syngenta
Thiacloprid 21.7 SC	Alanto	Neonicotinoid (4A)	180	0.65	Bayer Crop Science
Chloropyriphos 20	Tafaban	Organophosphate (1B)	400	2.0	Tata Chemicals
Dimethoate 30 EC	Tafgor	Organophosphate (1B)	200	2.0	Tata Chemicals
Cypermethrin 25 EC	Krycip	Synthetic pyrethroid (3A)	50	0.5	Krishi Rasayan

Laboratory Bioassays

Direct spray method

The test insects were randomly selected and placed in the petri dishes and one ml of each concentration of the insecticides was directly sprayed at a pressure of 340 g cm^2 using a Potter spray tower. The sprayed petri dishes containing treated DBM larvae, aphids and flea beetles were air dried for about 5 minutes and fresh untreated cabbage leaves were given as food. The control insects were sprayed with distilled water.

Leaf residue method

Cabbage leaf discs of approximately 5 cm diameterwere dipped in the different insecticide solutions for 20 seconds and then air-dried. For control the leaf discs were dipped in the distilled water. The treated leaf discs were then transferred to petri dishes and test insects were released in each petri dish and were allowed to feed on the treated leaf.

Dry film residue

A film of each insecticide was prepared on both the surfaces of petri dish by depositing 1 ml of each test insecticide by gentle swirling and later dried for 10 minutes under an electric fan. Test insects were introduced into one paired petri dish and untreated cabbage leaves were given as food. For control the petri dishes were treated with the distilled water.

In all the three bioassay methods each treatment including control was replicated thrice. Twenty test insects were released in each treatment. All the treated insects were kept at 27±1°C and 70±5 % RH. The mortality was assessed after 24 and 48 h after treatment. Morbid insects showing no sign of movement were scored as dead.

Statistical Analysis

The data on per cent mortality obtained in all the treatments were corrected by Abbott's formula (Abbot 1925) and analyzed using one way analysis of variance (ANOVA) after transformation by using SPSS Version 16.0 software.

Results and Discussion

All the three doses of cyantraniliprole @ 60, 70, 90 g a.i./ha evaluated against third instar DBM were effective with 100% mortality when tested by leaf dip and direct spray methods. Similar effectiveness was observed in emamectin benzoate in leaf dip bioassay with 100 % mortality of *P. xyllostela* at 48 hours after treatment. Spinosad was next best treatment with 82.67 and 95 % mortality in leaf dip and direct spray assays, respectively at 48 hours after treatment (Figure 1). High susceptibility of *P. xyllostela* to cyantraniliprole may be attributed to the unique mode of action of this new molecule which activates ryanodine receptors (RyRs) and causes an uncontrolled release of Ca^+ ions which leads to muscle paralysis and death (Sattelle 2008). The results of this investigation are in conformity with other laboratory bioassays on brinjal shoot and fruit borer *Leucinodes orbonalis*, where the third instar was found to be more susceptible to cyantraniliprole @ 90 g a.i./ha and recorded the highest mortality at 24 and 48 hrs after treatment (Kodandaram *et al.* 2015). Populations of *P. xylostella* were highly susceptible to another ant ranilic diamide insecticide, chlorantraniliprole when tested by direct spraying in a Potter spray tower (Wang *et al.* 2010; da Silva *et al.* 2012).

In case of different species of aphids infesting crucifers, cyantraniliprole @ 60 and 90 g a.i./ha recorded highest mortality of *B. brassicae* with 80 and 100 % in leaf dip and direct spray bioassays, respectively. Cyantraniliprole @ 60 g a.i./ha was most effective aganist *L. erysimi* with 86.67 and 100 % mortality of *L. erysimi*in leaf dip and direct spray methods, respectively. In case of *M. persicae* cyantraniliprole @ 60 and 90 g a.i./ha was most effective with 80 and 85 % mortality in leaf dip and direct spray methods, respectively (Figure 2.). This variation in the effectiveness of cyantraniliprole to different species of aphids infesting crucifers may be due to their differential susceptibility and translaminar movement of cyantraniliprole in the treated leaves (Barry *et al.* 2014). However, the present findings on aphid species were in conformity with the results of Mandal (2012) and Patel *et al.* (2014), who reported that higher doses of cyantraniliprole @ 90 and 105 g a.i./ha were highly effective in managing the aphid *Aphis gossypii*.

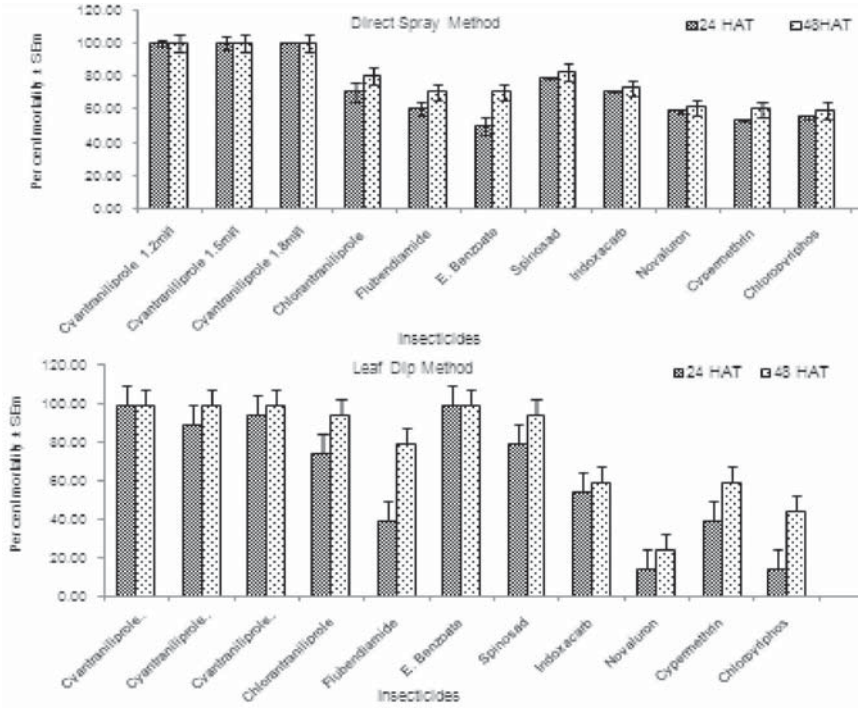

FIGURE 1

Efficacy of cyantraniliprole and other novel insecticides against P. xyllostela

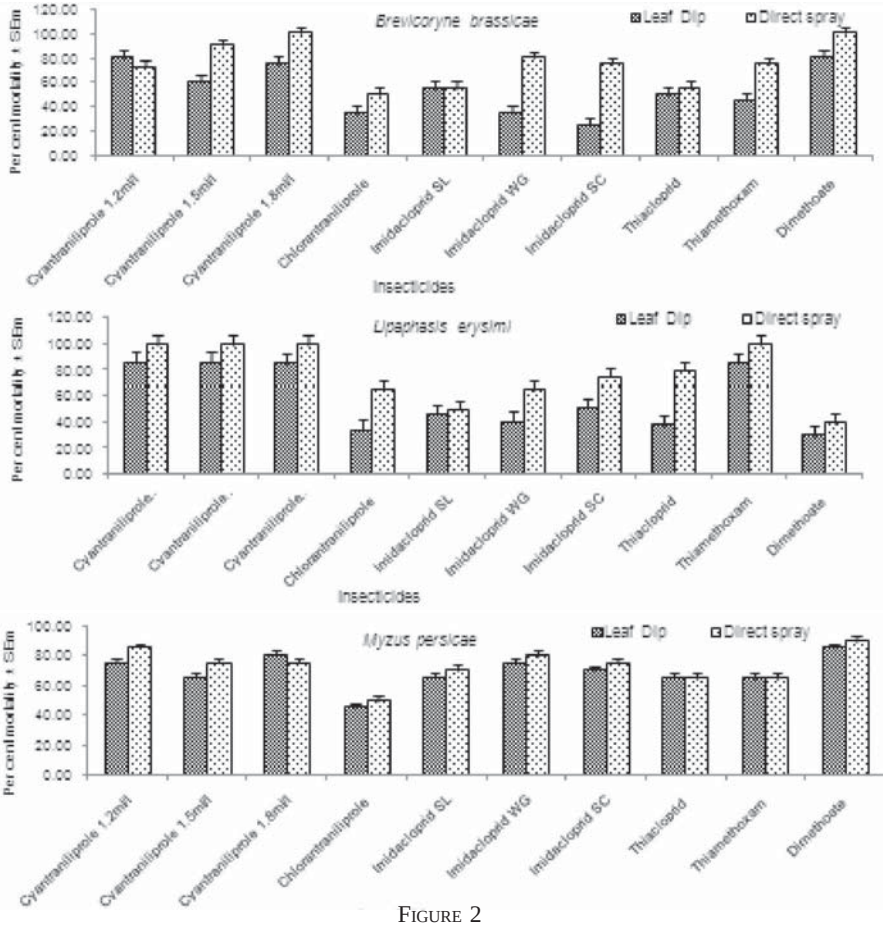

FIGURE 2

Efficacy of cyantraniliprole and other novel insecticides against different species of aphids infesting cruciferous vegetables

Adult flea beetle was less susceptible to all the three doses of cyantraniliprole as compared to other commonly used insecticides, however maximum mortality of 71.25 % was observed at the highest dose of cyantraniliprole (Figure 3.) Cyantraniliprole was ineffective against adults of painted bug at all the three doses and recorded only 0-10 % mortality. The other insecticides including indoxacarb, imidacloprid, thiamethoxam and dimethoate were most effective with 95, 94, 80 and 100 % mortality of treated bugs (Figure 4.). Similarly, Yadav *et. al.* (2012) reported that cyantraniliprole @ 80 g a.i./ha showed the highest leaf damage reduction by flea beetle, *Scelodonta strigicollis* in grapes. Also, Mishra and Mukherjee (2012) revealed that cyantraniliprole @ 105 and 90 g a.i. (pq)ha to be most effective against red pumpkin beetles *Aulacophora foveicollis* on gherkins. This new molecule should be extensively utilized as a new tool for management of DBM and sucking insect pests and which can be taken advantage for developing effective integrated pest management (IPM) programs. Also it can be used in spray windows as insecticide partners for rotation in resistance management of DBM in cruciferous vegetables.

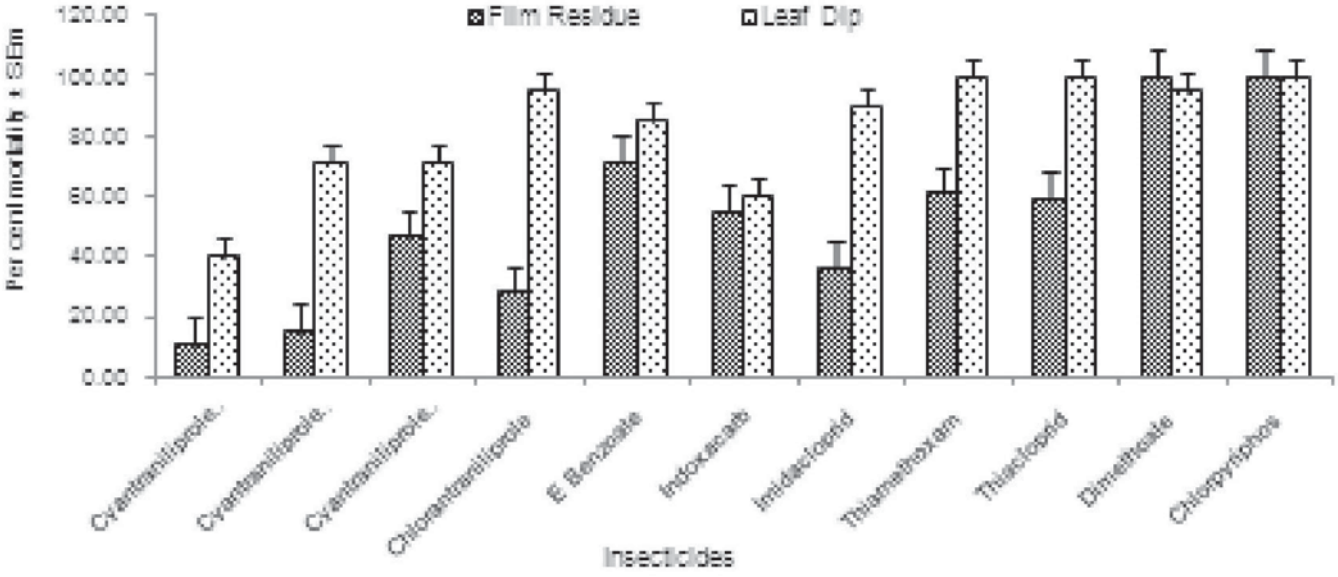

FIGURE 3

Efficacy of cyantraniliprole and other novel insecticides against flea beetle

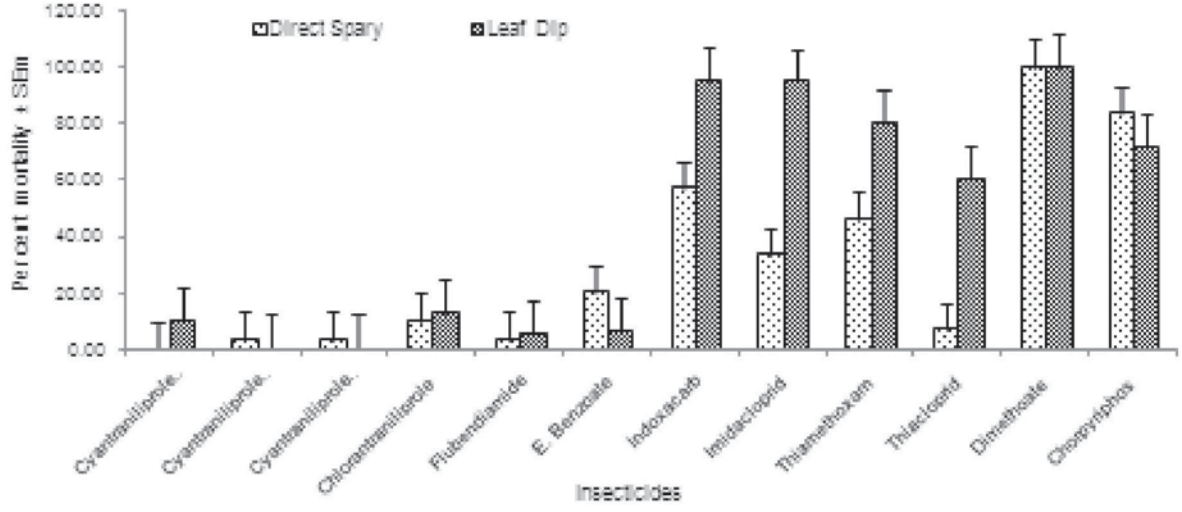

FIGURE 4

Efficacy of cyantraniliprole and other novel insecticides against painted bug

Conclusion

Cyantraniliprole is the third generation diamide insecticide but the first that has activity on both chewing and sucking insect pests of crucifers @ 60 g a.i./ha. This new molecule will be crucial for strengthening integrated pest management (IPM) and remain an effective insecticide partner for rotation in insecticide resistance management (IRM) programs for DBM in India.

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Synergistic Effects of Oils with Flubendamide and Indoxacarb Against Diamondback Moth, *Plutella xylostella* (L.) (Lepidoptera: Plutellidae) on Cabbage

Onkaranaik, S., Sridhar, V., Jayashankar, M. and Chakravarthy, A.K.
Division of Entomology and Nematology, ICAR-Indian Institute of Horticultural Research
Hessaraghatta Lake P.O, Bengaluru - 560 089, India
E-mail: onkar632@gmail.com

Abstract

Laboratory and field studies were conducted to assess the synergistic effect of two plant oils *viz*., neem and pongamia and a fish oil on the bioefficacy of Flubendiamide 480 SC and Indoxacarb14.5% SC against diamondback moth (DBM), *Plutella xylostella* (L.) on cabbage during 2010-11 at Alambagiri-Hosahalli, Chintamani Taluk, Chikkaballapur District of Karnataka. Under laboratory conditions a combination of Indoxacarb 14.5% SC 0.3 ml + Neem oil 3 ml + Pongamia oil 3 ml + Fish oil 3 ml/L was the most effective (83 % mortality) followed by Flubendiamide 480 SC 0.3 ml + Neem oil 3 ml + Pongamia oil 3 ml + Fish oil 3 ml/ L (78 % mortality) against DBM larvae. Indoxacarb 14.5 % SC 0.75 ml/Lalone resulted in 48 % mortality of DBM larvae followed by Flubendiamide 480 SC 0.3 ml/L (45 % mortality). Under field conditions, Indoxacarb along with synergists (Indoxacarb 14.5% SC 0.3 ml + Neem oil 3 ml + Pongamia oil 3 ml + Fish oil 3 ml/L) gave 73 % mortality of DBM larvae followed by Flubendamide 480 SC 0.3 ml + Neem oil 3 ml + Pongamia oil 3 ml + Fish oil 3 ml/L with 68 % mortality. Treatments of Indoxacarb 14.5% SC 0.75 ml/L and Flubendiamide 480 SC 0.3 ml/L alone gave 53% and 45 % mortality of DBM larvae respectively. The study indicated the potential of plant oils and fish oil as synergists with synthetic insecticides for the management of DBM on cabbage.

***Keywords*:** Fish oil, neem oil, *Plutella xylostella*, pongamia oil, synergist.

Diamondback moth (DBM), *Plutella xylostella* (L.) (Lepidoptera: Plutellidae), is considered the most destructive insect pest of crucifer crops worldwide. DBM larvae feed on leaves of cruciferous crops such as cabbage, broccoli, cauliflower, collards, kale, kohl rabi, Chinese cabbage and Brussels sprouts. It is a specialist, phytophagous pest and the diet is exclusively restricted to crucifers due to the presence of glucosinolates. The crop loss due to DBM can vary from 52 to 100% (Calderon and Hare 1986). The absence and reduction of effective natural enemies, especially parasitoids, as well as insecticide resistance, contribute to the status of DBM as a pest (Talekar and Shelton 1993; Capinera 2001; Shelton 2004; Sarfraz *et al.* 2005; Furlong *et al.* 2013).

Insects are becoming resistant to several insecticides of diversified chemical groups. Insecticidal resistance is a hereditary phenomenon and is governed by the developmental history, frequency of pesticide use and the selection pressure (Sayyed and Wright 2006). Detoxification enzyme based resistance occurs when enhanced levels or modified activities of esterases and glutathione s-transferase prevent the insecticide from reaching its site of action. The ability of broad-spectrum insecticides to control DBM has seriously declined due to resistance and cross resistance (Miyata *et al.* 1986; Talekar and Shelton 1993; Li *et al.* 2012). To address the risk of insecticide resistance, oils used as synergists on the bio-efficacy of Flubendiamide and Indoxacarb against DBM larvae was undertaken under laboratory and field conditions on cabbage, *Brassica oleracea* Var *capitata* L.

Materials and Methods

Laboratory bioassays

Laboratory assays were undertaken at the ICAR-Indian Institute of Horticultural Research (IIHR), Bengaluru. Healthy cabbage leaves were collected from the field and washed under running water. Air dried leaves were dipped in different combinations of insecticide concentrations and oils suspensions, air dried 8-10 minutes and fed to ten second instar *P. xylostella* larvae placed in plastic Petri dishes of 9 cm diameter (12 treatment combinations × 3 replications randomized in Complete Randomized Design (CRD)). Control larvae were fed with water-dipped and subsequently air-dried cabbage leaves alone separately to detect any natural mortality under similar conditions.

Field studies

The experiment was laid out at Alambagiri-Hosahalli (13.43°N,77.72°E), Chintamani Taluk, Chikkaballapur District of Karnataka during *rabi* and summer (*zaid*) seasons of 2010-11. Twelve treatments with three replications randomized in Randomized Block Design (RBD), following all the agronomical practices as per recommendations (Asia Farming 2015). Cabbage crop *cv.* unnathi was planted (0.5m X 0.6m) with subsurface drip irrigation. The experiment was designed to assess the synergistic effect of two plant oils *viz*., neem oil and pongamia oil and fish oil on the bioefficacy of Flubendiamide 480 SC and Indoxacarb14.5% SC against DBM larvae. Four sprays were applied at interval of 14 days after 20 days of planting. Observations were recorded on larval mortality based on five plants randomly selected under each treatment.

Detoxifying enzyme assays

The quantification of detoxifying enzymes assay was carried out at ICAR-IIHR, Bengaluru. The analysis were carried as the procedure described by Nauven and Stumpf (2002) for glutathione-S-transferases (GST), Devonshire *et al.* (1992) for esterases and De Sousa *et al.* (1995) for monooxygenases (mixed function oxidases – MFO cytochrome P^{450}) quantification.

Statistical analysis

The different observations recorded were number of larvae before and after treatment, and larval mortality. The data were subjected to analysis of variance and means were separated by Duncans Multiple Range Test using SAS V 9.3.

Results and Discussion

Laboratory observations

Synthetic insecticides used alone showed less impact than when used in combination with synergistic oils (Table 1). Indoxacarb 14.5% SC 0.75 ml/L alone provided 48 % mortality of DBM larvae followed by Flubendiamide 480 SC 0.3 ml/L with 45 % larval mortality. Indoxacarb 14.5% SC 0.3 ml + Neem oil 3 ml + Pongamia oil 3 ml + Fish oil 3 ml/L recorded the highest mortality (83 %) of larvae, followed by 78 % mortality by Flubendiamide 480 SC 0.3 ml + Neem oil 3 ml + Pongamia oil 3 ml + Fish oil 3ml/L. Combination of oils alone (Neem oil 3 ml + Pongamia oil 3 ml + Fish oil 3 ml) gave only 45 % larval mortality in comparison with Flubendiamide 480 SC 0.3 ml/L 48 h after treatment.

Field observations

The synthetic insecticides used alone showed less effectiveness on sucking pests when used in combination with oils (Table 2). Under field conditions during *rabi*, Indoxacarb along with synergists (Indoxacarb 14.5 % SC 0.3 ml + Neem oil 3 ml + Pongamia oil 3 ml + Fish oil 3 ml/L) gave 73 % mortality of DBM larvae followed by Flubendiamide 480 SC 0.3 ml + Neem oil 3 ml + Pongamia oil 3 ml + Fish oil 3 ml/L with 68 % mortality. Indoxacarb 14.5 % SC 0.75 ml/L and Flubendamide 480 SC 0.3 ml/L alone gave 53% and 45% mortality of DBM larvae, respectively. Combinations of oils (Neem oil 3 ml + Pongamia oil 3 ml + Fish oil 3 ml) were on par with Indoxacarb 14.5 % SC 0.75 ml/L and significantly higher larval mortality (17 %) in comparison with Flubendiamide 480 SC 0.3 ml/L with 16 % mortality during *rabi* season (Table 2). During summer, Flubendiamide 0.3 ml + Neem oil 3 ml + Pongamia oil 3 ml + Fish oil 3 ml caused 87 % mortality of DBM larvae followed by Indoxacarb 0.75 ml + Neem oil 3 ml + Pongamia oil 3 ml + Fish oil 3 ml/L with 83 % mortality (Table 3). The findings indicated the potential of plant oils and fish oil as synergists when used with synthetic insecticides for the management of DBM on cabbage.

Insecticide resistance and detoxifying enzyme activity

The most common resistance mechanisms in insects are increased levels or activities of esterase detoxification enzymes that metabolize a wide range of insecticides. Field collected *P. xylostella* populations were assayed for insecticide resistance, with insecticides belonging to different groups *viz*., Indoxacarb14.5 % SC 0.75 ml/L and Flubendiamide 480 SC 0.3 ml/L indicated no role of esterase in resistance, whereas GST (18.12 pg/mg) and MFO (19.04 pg/mg) had a positive impact (Figure 1). However, esterases and glutathione s-transferases (3.09 pg/mg) played a minor role compared to MFO in the metabolism of the insecticides for both strains of DBM. These results suggest that oils and synthetic insecticides can be used to suppress DBM infestation on cabbage in the field.

The present findings agree with the available literature on the utility of oils as synergists with synthetic insecticides. Gavi Gowda (1996) opined that Pongamia oil acted as an MFO inhibitor and the

inhibition of the oxidasesby Pongamia oil may be the reason for synergism of methomyl toxicity. Suneel Kumar and Sannaveerappa-navar (2003) recorded sesamum oil and Pongamia oils synergism with synthetic pyrethroids. Muralitharan (2008) found that among the detoxifying enzymes studied, MFO exhibited substantially higher activity. To combat resistance problem synergists can also be employed as essential components of IRM system to enhance the efficacy of safer insecticides, to conserve the natural enemies andto make treatmentscost effective. Since farmers need information on the proper means of combating pests, collaboration and co-operation among farmers, academics and industry researchers are strongly suggested in making any planned resistance management effective under field conditions.

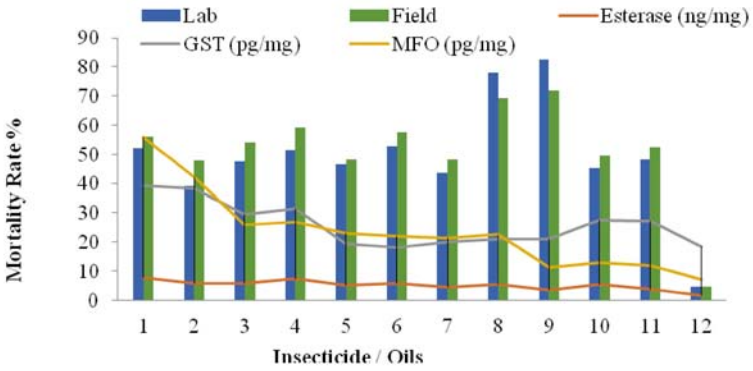

FIGURE 1

Relation between DBM larval mortality in response to enzyme activity. See the table for definition of Insecticide oil combinations

TABLE 1

Mean larval mortality of DBM under laboratory conditions at 48 and 72 h in bioassays

Sl. No	Treatments	Mean (no.) Mortality of larvae	Treatments	Mean (no.) Mortality of larvae
		48 hrs		72 hrs
1	Indoxacarb 0.75ml+Neem oil 3ml + Pongamia oil 3ml + Fish oil 3ml	[a]27.00	Indoxacarb 0.75ml+Neem oil 3ml + Pongamia oil 3ml + Fish oil 3ml	[a]28.00
2	Flubendamide 0.3ml + Neem oil 3ml + Pongamia oil 3ml + Fish oil 3ml	[a]26.00	Flubendamide0.3ml + Neem oil 3ml + Pongamia oil 3ml + Fish oil 3ml	[a]26.67
3	Fish oil 3ml+ Indoxacarb 0.3ml	[b]17.33	Indoxacarb 0.75ml	[b]16.33
4	Neem oil 3ml + Flubendamide 0.3ml	[cb]17.00	Pongamia oil 3ml + Indoxacarb 0.75ml	[b]15.67
5	Neem oil 3ml + Indoxacarb 0.75ml	[cb]16.67	Flubendamide 480 SC 0.3ml	[b]15.67
6	Indoxacarb 0.75ml	[cb]15.67	Neem oil 3ml + Indoxacarb 0.75ml	[b]15.67
7	Fish oil 3ml+ Flubendamide0.3ml	[cb]15.67	Fish oil 3ml+ Flubendamide 0.3ml	[b]15.33
8	Pongamia oil 3ml + Indoxacarb 0.75ml	[cb]15.33	Neem oil 3ml + Flubendamide 0.3ml	[b]14.67
9	Flubendamide 480 SC 0.3ml Fish oil 3ml	[cb]14.33	Neem oil 3ml + Pongamia oil 3ml +	[b]13.67
10	Neem oil 3ml + Pongamia oil 3ml + Fish oil 3ml	[cb]14.33	Fish oil 3ml+ Indoxacarb 0.3ml	[b]13.67
11	Pongamia oil 3ml + Flubendamide 0.3ml	[c]12.67	Pongamia oil 3ml + Flubendamide 0.3ml	[b]13.67
12	Control/ Check	[d]1.33	Control/ Check	[c]1.00
CV		2.79	CV	2.06
LSD @0.05		4.61	LSD@0.01	3.07

*Means with the same letter are not significantly different by DMRT

TABLE 2

Mean larval mortality under field conditions during rabi

Sl. No	Treatments	Mean (no.) Mortality of larvae/3 plants	Treatments	Mean (no.) Mortality of larvae/3 plants
		7 days		14 days
1	Indoxacarb 0.3ml+Neem oil 3ml + Pongamia oil 3ml + Fish oil 3ml	[a]23.67	Indoxacarb 0.3ml+Neem oil 3ml + Pongamia oil 3ml + Fish oil 3ml	[a]24.00
2	Flubendamide0.3ml + Neem oil 3ml + Pongamia oil 3ml + Fish oil 3ml	[a]22.33	Flubendamide0.3ml + Neem oil 3ml + Pongamia oil 3ml + Fish oil 3ml	[ba]21.66
3	Fish oil 3ml+ Indoxacarb 0.3ml	[b]18.67	Fish oil 3ml+ Indoxacarb 0.3ml	[bc]19.33
4	Neem oil 3ml + Indoxacarb 0.75ml	[b]18.33	Neem oil 3ml + Indoxacarb 0.75ml	[dc]18.33
5	Fish oil 3ml+ Flubendamide0.3ml	[b]18.00	Neem oil 3ml + Pongamia oil 3ml + Fish oil 3ml	[dce]17.33
6	Neem oil 3ml+Flubendamide0.3ml	[b]17.67	Indoxacarb 0.75ml	[dce]17.33
7	Neem oil 3ml + Pongamia oil 3ml + Fish oil 3ml	[b]17.33	Fish oil 3ml+ Flubendamide0.3ml	[dce]17.33
8	Indoxacarb0.75ml	[b]17.33	Neem oil 3ml + Flubendamide0.3ml	[dce]17.33
9	Pongamia oil 3ml + Flubendamide0.3ml	[b]16.33	Flubendamide 480 SC 0.3ml	[de]16.33
10	Pongamia oil 3ml + Indoxacarb0.75ml	[b]16.00	Pongamia oil 3ml + Indoxacarb0.75ml	[e]15.33
11	Flubendamide 480 SC 0.3ml	[b]15.67	Pongamia oil 3ml + Flubendamide0.3ml	[e]15.33
12	Control/ Check	[c]1.00	Control/ Check	[f]1.33
CV		2.82	CV	2.82
LSD@0.01		4.17	LSD@0.01	2.58

*Means with the same letter are not significantly different by DMRT

TABLE 3

Mean larval mortality under field conditions during summer

Sl. No	Treatments	Mean (no.) Mortality of larvae/3 plants	Treatments	Mean (no.) Mortality of larvae/3 plants
		7 days		14 days
1	Flubendiamide0.3ml + Neem oil 3ml + Pongamia oil 3ml + Fish oil 3ml	[a]23.000	Flubendiamide 0.3ml + Neem oil 3ml + Pongamia oil 3ml + Fish oil 3ml	[a]26.00
2	Indoxacarb 0.75ml+Neem oil 3ml + Pongamia oil 3ml + Fish oil 3ml	[a]22.67	Indoxacarb 0.75ml+Neem oil 3ml + Pongamia oil 3ml + Fish oil 3ml	[a]25.00
3	Neem oil 3ml + Indoxacarb 0.75ml	[ba]21.00	Neem oil 3ml + Indoxacarb 0.75ml	[b]18.67
4	Neem oil 3ml + Flubendiamide0.3ml	[bc]19.33	Neem oil 3ml + Flubendiamide 0.3ml	[b]18.50
5	Fish oil 3ml+ Indoxacarb 0.3ml	[bcd]18.67	Indoxacarb 0.75ml	[b]17.33
6	Fish oil 3ml+ Flubendiamide 0.3ml	[cd]17.67	Fish oil 3ml+ Flubendiamide0.3ml	[b]16.67
7	Indoxacarb 0.75ml	[ecd]17.00	Flubendiamide 480 SC 0.3ml	[b]16.33
8	Flubendiamide 480 SC 0.3ml	[ecd]16.67	Pongamia oil 3ml + Indoxacarb 0.75ml	[b]16.00
9	Pongamia oil 3ml + Indoxacarb0.75ml	[ed]16.00	Pongamia oil 3ml + Flubendiamide0.3ml	[b]16.00
10	Pongamia oil 3ml + Flubendiamide 0.3ml	[ed]15.67	Neem oil 3ml + Pongamia oil 3ml + Fish oil 3ml	[b]15.33
11	Neem oil 3ml + Pongamia oil 3ml + Fish oil 3ml	[e]14.33	Fish oil 3ml+ Indoxacarb 0.3ml	[b]15.00
12	Control/ Check	[f]1.33	Control/ Check	[c]1.00
CV		2.07	CV	2.07
LSD@0.05		3.00	LSD@0.05	4.22

*Means with the same letter are not significantly different by DMRT

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Changing Trends in Resistance of *Plutella xylostella* Field Population to Chlorantraniliprole in Yunnan Province, P. R. China

YIN, Y.Q., ZHAO, X.Q.[1], CHEN, A.D.[2], LI, X.Y.[3], WU, Q.J.[4] AND ZHANG, Y.J.[5]
Institute of Agricultural Environment and Resources, Yunnan Academy of Agricultural Sciences
Kunming 650205, P. R. China
E-mail: yinyq1977@sina.com
[1]Institute of Agricultural Environment and Resources, Yunnan Academy of Agricultural Sciences
Kunming 650205, P. R. China
E-mail: snow.xue-5@163.com
[2]Institute of Agricultural Environment and Resources, Yunnan Academy of Agricultural Sciences
Kunming 650205, P. R. China
E-mail: shenad68@163.com
[3]Institute of Agricultural Environment and Resources, Yunnan Academy of Agricultural Sciences
Kunming 650205, P. R. China
E-mail: lxybiocon@163.com
[4]Institute of Vegetables and Flowers Chinese Academy of Agricultural Sciences, Beijing 100081, P. R. China
E-mail: wuqj@mail.caas.net.cn
[5]Institute of Vegetables and Flowers Chinese Academy of Agricultural Sciences Beijing 100081, P. R. China
E-mail: zhangyoujun@caas.cn

ABSTRACT

In order to understand the current situation and development of resistance in field population of DBM *Plutella xylostella* (L.) to chlorantraniliprole in Yunnan Province, the sensitivity of three DBM population from Tonghai County of central Yunnan, Midu County of west Yunnan and Linxiang district of southwest Yunnan to chlorantraniliprole was determined by the leaf dipping method in laboratory condition. Meanwhile, synergism effects of triphenyl phosphate (TPP), piperonyl butoxide (PBO) and diethyl maleate (DEM) to the F1 generation of Tonghai population were tested. The results showed that the resistance levels of Tonghai population of *P. xylostella* to chlorantraniliprole showed an increasing tendency yearly, with resistance index increased from 2.30 in 2009 to 47.79 in 2012, and to 43.05 in 2013, which reached middle level of resistance; Midu population of *P. xylostella* showed low level of resistance except in 2010 when the resistance index was 12.35; Linxiang population of *P. xylostella* showed low level of resistance, with resistance index remained between 1.01- 4.60 during 2009-2013. The three compounds had no significant synergistic effects on the toxicity of chlorantraniliprole to *P. xylostella.*

Keywords: *Plutella xylostella*, chlorantraniliprole, insecticide resistance, synergistic effect

DIAMONDBACK moth (DBM), *Plutella xylostella* L. is an important pest of cruciferous vegetables worldwide. It occurs throughout the year in Yunnan Province of P. R. China, with large population intensity (Li *et al.* 2011b), resulting in increasing insecticide resistance. Currently, DBM has developed resistance against more than 90% of insecticides, belonging to organochlorine, organophosphorus, carbamates, pyrethroids, acylurea and microbial pesticides such as *Bacillus thuringiensis* (Bt) (Feng *et al.* 2011). As a new anthranilic diamide pesticide, chlorantraniliprole acts on ryanodine receptor and achieves insecticidal purpose through activating ryanodine (Cordva *et al.* 2006), which has advantages of high efficiency, low toxicity and safety to non-target organisms (Yang *et al.* 2012). Chlorantraniliprole can control almost all important lepidopteran pests and parts of pests belonging to Coleoptera, Diptera and Hemiptera, and can effectively control pests that have already developed resistance against other insecticides (Marcon *et al.* 2007). Since 2009, the insecticides with chlorantraniliprole as the main ingredient, such as Coragen, Virtako, Prevathon, *etc*. have gradually become major agents against DBM in Yunnan Province. To understand the resistance level of DBM against new insecticide chlorantraniliprole, we had monitored resistance of DBM populations in three

different vegetable producing areas for five consecutive years from 2009 to 2013, and experimentally screened synergists, in order to provide scientific basis for resistance assessment and resistance management of chlorantraniliprole.

Materials and Methods

Insect sources and rearing

The insects tested in the study were collected from perennial planting areas of cruciferous vegetables in Tonghai County of Yuxi City (central Yunnan), Midu County of Dali Prefecture (western Yunnan) and Linxiang district of Lincang City (southwest Yunnan). Three to five representative plots in local area were chosen and grown up larvae and pupae were collected randomly from multi-points of each plot. More than 200 larvae and/or pupae were collected from each field. Mature larvae were moved into worm bags with vegetable leaves and brought back to laboratory for pupation. The emerged pupae were stored in 4 °C refrigerator for less than 15 d before the measurement commenced. When all insects pupated, they were placed in rearing cages together and reared under the conditions of temperature 25±1 °C, RH 65-70 %, L: D = 16:8 h for emergence, and the emerged adults were fed with 15% honey water. Three days after emergence, the dried aluminum foils (10 × 2 cm) that had been soaked with cabbage juice were hung at four corners of rearing cage, and the spawning foils were collected every 24 h. Eggs were stored in 4 °C refrigerator for less than 15 d before the measurement commenced. All eggs were transferred on to radish seedlings under the conditions briefed above on the same day. After 11-12 d, when larvae developed to third instar, the larvae in F1 generation, with approximately equal sizes and development progress, were selected as test insects.

Insecticides and reagents

5.22 % Chlorantraniliprole EC was prepared with its original active ingredient by Plant Protection Research Institute, Guangdong Academy of Agricultural Sciences, and its effective content was analyzed.

The reagents used in the study included 90 % piperonyl butoxide (PBO) (Aldrich Chemical Co. Ltd.), 99 % triphenyl phosphate (TPP) (Shanghai No. 1 Chemical Reagent Factory) and 97 % diethyl maleate (DEM) (Beijing J & K Scientific Ltd.).

Experimental methods

Bioassay

5.22 % Chlorantraniliprole EC was diluted into five series of concentrations with the distilled water containing 0.05 % Triton X-100 according to geometric progression dilution method, and the volume of each concentration was 200 mL. Clean cabbage (*Brassica oleracea*) leaves were cut into round discs with the diameter of 6.5 cm (avoiding main veins and edges). The leaf discs were soaked in different concentrations of solution for 10 s, and then dried. The dried leaves were placed in the petri dish with the diameter of 6.5 cm, and inoculated with ten third instar larvae. Each treatment was replicated for four times, and the leaves soaked with water and 0.05 % Triton X-100 were set as control. After mulching with double layers of suction paper, the petri dish was finally covered. The petri dishes were cultured in the RXZ-380B type incubator with front side upward under the conditions of temperature (25±1 °C), RH 65-70 %, L: D = 16:8 h. The larvae were checked after 48 h for mortality, and those without coordinate movements when touched with small brush were regarded as death.

Synergist test

Synergists (PBO, TPP and DEM) were mixed with chlorantraniliprole according to the ratio of 5:1 (active ingredient), and the other steps were the same as described in bioassay. Synergists had no direct insecticidal effect within the measurement range.

Calculation methods

Calculation of resistance ratio: the slope, LC_{50} value and its 95 % confidence limit of toxicity and regression equation for measurement results was calculated by POLO software (Guo *et al.* 2003). Resistance ratio was also calculated through comparison with LC_{50} of indoor sensitive strains.

Resistance ratio = LC_{50} of field population / LC_{50} of relative susceptible population

The sensitive baseline LC_{50} of chlorantraniliprole was 0.23 mg/L, which was derived from DBM resistance monitoring technical regulation in cruciferous vegetables (NY / T 2360-2013).

Resistance grading standards referred to DBM resistance monitoring technical regulation in cruciferous vegetables (NY / T 2360-2013) (2013): resistance ratio RR < 10.0 was low resistance; resistance ratio 10.0 d" RR <100.0 was moderate resistance; resistance ratio RR e" 100.0 was high resistance.

Synergistic ratio (SR) was calculated according to the method by Brindley (Brindley 1977): SR = LC_{50} of single agent / LC_{50} of synergist treatment.

Results and Discussion

Resistance of DBM population to chlorantraniliprole and its annual changes

The resistance of DBM populations to chlorantraniliprole was determined in three main vegetable areas of Yunnan Province since 2009, and the results were as follows. In 2009, the resistances in three monitoring points were all sensitive; LC_{50} was between 0.52 and 1.01 mg/L and resistance index was less than 10, belonging to low resistance. In 2010, the resistance index of Midu population was 12.35, belonging to moderate level, while the other two populations were still sensitive to chlorantraniliprole, belonging to low level. In 2011, the resistances of Midu and Tonghai populations were rising; LC_{50} was 4.67 and 6.37 mg/L, and resistance index was 20.66 and 28.19, respectively, reaching moderate resistance; Linxiang population was still at low resistance level, and LC_{50} and resistance index were 0.49 mg/L and 2.17, respectively. In 2012, LC_{50} of Tonghai population was 10.80 mg/L, while its resistance index increased to 47.79, belonging to moderate resistance; the resistances of Midu population decreased to 5.18, belonging to low resistance; Linxiang population remained at low resistance level. In 2013, the resistance ratios of populations in three vegetable areas slightly decreased, but the resistance levels remained the same as that in 2012 (Table 1).

Since 2009, the resistances of Tonghai DBM population continued to increase from 2.30 in 2009 to 47.79 in 2012 and 43.05 in 2013, reaching moderate resistance; the resistances of Midu population were basically at a low level except that in 2010 reaching 12.35 (belonging to moderate resistance); the resistances of Linxiang DBM were between 1.01 and 4.60, belonging to low resistance.

Synergistic effect of PBO, TPP and DEM on chlorantraniliprole

Synergist test was commenced on F1 generation of Tonghai DBM population in 2013, and measurement results showed that TPP, DEM and PBO had no obvious synergistic effect on chlorantranili-prole, with synergistic ratios of 1.28, 1.47 and 1.94, respectively; 95 % confidence limit of LC_{50} that added with synergists overlapped with the 95 % confidence limit of LC_{50} without synergists, indicating that the toxicity of chlorantraniliprole had no significant changes after adding three kinds of synergists (Table 2).

Yunnan is one of the earliest vegetable areas using chlorantraniliprole to control DBM on brassicas in P. R. China, and the insecticide has been applied in field early in 2009. Resistance monitoring data for five consecutive years showed that the resistance of Yunnan DBM population to chlorantraniliprole was increasing year by year, and the resistance had developed from low level to moderate level. Specifically LC_{50} in Tonghai vegetable area was exponentially increasing, and resistance ratios in 2011 and 2012 were 8.98 and 20.77 times higher than that of 2009, respectively. Hu *et al.* (2012) monitored resistance of DBM against chlorantraniliprole in major vegetable areas in southern regions including Lianzhou, Huizhou, Foshan and Guangzhou of Guangdong, Liuzhou of Guangxi and Xinfeng of Jiangxi, and found that populations in these vegetable areas were sensitive in 2009, which were at low resistance level in 2010, but populations in Zengcheng vegetable area reached extremely high resistance level in 2011, and resistance increased significantly. Xing *et al.* (2011) screened chlorantraniliprole for five consecutive generations, and found that LC_{50} of Tianjin susceptible strains significantly increased by 2.34 and 3.10 times, respectively. These studies have shown that there is higher risk for DBM producing resistance against chlorantraniliprole.

Resistance development of chlorantraniliprole is affected by many factors such as application background, cultivation mode and application habits. Linxiang vegetable area in southwest Yunnan is hot in summer with more rainfall, and its geographical environment does not allow large-scale cultivation or continuous cropping of cruciferous vegetables; besides, the occurrence of DBM is lighter in spring, and hence chlorantraniliprole is rarely used as the

prevention insecticide, and five years of monitoring showed that the resistance in DBM population to chlorantraniliprole is in sensitive level. In Midu vegetable area, there is certain application amount of chlorantraniliprole only in first two years of its promotion and application amount is decreasing in last few years. So resistance also changes. Tonghai vegetable area in central Yunnan is a developed area with strong ability to accept new insecticides, coupled with large-scale cultivation and continuous cropping of cruciferous vegetables, and thus resistance increases exponentially every year. Hu et al (2010) investigated DBM in fields, and the results also showed that application frequency of chlorantraniliprole in different regions was directly proportional to resistance ratios.

Metabolic resistance of insects is mainly due to increased activity of various detoxification enzymes, so inhibiting activity of metabolic enzymes by synergists to improve control effects of agents is one of the effective ways to overcome and delay resistance development of pests. Studies have shown that carboxylesterase (CarE) and mixed function oxidases (MFO) are related to decreased sensitivity of DBM to chlorantraniliprole. Xingjing et al (2011) treated DBM with sublethal concentrations continuously for five years, and found that the specific activities of CarE and cytochrome P450 O-deethylase (ECOD) were 1.40 and 1.56 times of control group, respectively, while enzyme activity continuously increased with the prolongation of treatment time. Leaves treated with chlorantraniliprole had greater inhibition on growth of DBM. In this study, three synergists including PBO, TPP and DEM have no significant synergistic effect on DBM population that has produced moderate resistance against chlorantraniliprole.

Resistance monitoring is the basis for scientific evaluation of resistance management strategy and guidance of rational use of insecticides. Resistance level and its distribution can be measured timely and accurately through resistance monitoring, in order to make clear decisions on pesticides to be protected (Zhang and Jiang 1998; Pan and Li 2006). Zhao *et al.* (1996) found that when resistance of DBM was at a low level, it is unstable, but it will be stabilized after reaching an extremely high level. The resistance ratio of Midu population in western Yunnan was 4.47 in 2009, which increased to 12.35 in 2010, but the resistance level had gradually declined in subsequent years, indicating that resistance of DBM against chlorantraniliprole had unstable field performance in its initial production. Therefore, continuous resistance monitoring of novel agent chlorantraniliprole, timely analysis of resistance production and developing scientific and rational application strategy are very important to protect and prolong service life of novel agents.

TABLE 1

Annual changes of resistance of Yunnan DBM population to chlorantraniliprole

Year	Population	LC_{50} (mg/L)	95% Confidence limits	Slope of toxicity regression equation (b±SE)	Resistance index	Resistance ratio
2009	Tonghai	0.52	0.39-0.70	1.73±0.27	2.30	1.00
	Midu	1.01	0.71-1.34	1.45±0.20	4.47	1.00
	Linxiang	0.78	0.62-0.96	2.36±0.31	3.45	1.00
2010	Tonghai	0.32	0.13-0.55	1.47±0.24	1.42	0.62
	Midu	2.79	1.74-7.46	1.48±0.31	12.35*	2.76
	Linxiang	0.73	0.59-0.89	2.68±0.35	3.23	0.94
2011	Tonghai	4.67	3.43-6.08	1.66±0.25	20.66*	8.98
	Midu	6.37	3.99-15.96	1.09±0.27	28.19*	6.31
	Linxiang	0.49	0.15-0.84	1.17±0.26	2.17	0.63
2012	Tonghai	10.80	6.93-21.40	1.37±0.33	47.79*	20.77
	Midu	1.17	0.86-1.52	0.88±1.52	5.18	1.16
	Linxiang	1.04	0.48-2.43	2.31±0.31	4.60	1.33
2013	Tonghai	9.73	6.47-23.88	1.54±0.61	43.05*	18.71
	Midu	0.45	0.18-0.71	1.40±0.29	1.99	0.45
	Linxiang	0.23	0.15-0.32	1.47±0.22	1.01	0.29

Note: "*" indicates significant difference (95% confidence limits of LC_{50} of sensitive and resistant populations do not cross).

TABLE 2
Synergistic effect of TPP, PBO, DEM on chlorantraniliprole

Agents	LC_{50} (mg/L)	Slope of toxicity regression equation (b±SE)	95% Confidence limits	Chi-square	Synergistic ratio
Chlorantraniliprole	10.49	1.40±0.26	7.20-19.96	2.17	–
Chlorantraniliprole + PBO	5.40	1.70±0.25	4.12 - 7.70	1.26	1.94
Chlorantraniliprole + TPP	8.16	1.43±0.26	5.75 - 14.43	1.01	1.28
Chlorantraniliprole + DEM	7.14	1.07±0.24	4.59 - 16.37	2.00	1.47

CONCLUSION

The resistance of DBM population in Tonghai in central Yunnan to chlorantraniliprole was increasing significantly after using this insecticide for two years, and has developed from low level to moderate resistance, but Midu population in western Yunnan, and Linxiang in southwestern Yunnan was at low level, although the Midu population had changes in 2012. The changing trends of the resistance were varying, depending on the vegetable cultivation system, insecticide use technology, DBM population occurrence characteristics and other control methods, and also indicated that resistance of DBM against chlorantraniliprole was unstable in its initial stages. In this study, three synergists including PBO, TPP and DEM had no significant synergistic effect on DBM population that has showed moderate resistance against chlorantraniliprole.

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Synergistic Effect of *Neem* and *Pongamia* Oils on the Bioefficacy of Deltamethrin Against Diamondback Moth, *Plutella xylostella* (L.) (Lepidoptera: Plutellidae) on Cabbage

Sridhar V. and Chakravarthy A. K.
Division of Entomology and Nematology, ICAR-Indian Institute of Horticultural Research
Hessaraghatta Lake P.O, Bengaluru - 560 089, India. E-mail: vsridhar@iihr.res.in

Abstract

Diamondback moth (DBM), *Plutella xylostella* (L.) has a history of developing resistance to a majority of insecticides. Insecticide resistance and control failures are now common and in certain cases economical production of crucifers has become increasingly difficult. One option for the control of insect strains which are resistant to insecticides has been the combined use of insecticides and synergists. Seed oils *viz*., cotton seed oil, neem oil, *Pongamia* oil, mahua oil (*Madhuca longifolia*) and sesamum oil can synergise the toxicity of the insecticides and can be used in resistance monitoring studies as inhibitors of MFO's in place of costly synthetic synergists like piperonyl butoxide. Synthetic synergists' like piperonyl butoxide are expensive and have to undergo the cumbersome formalities for registration. Considering the availability and economy of the synergists, two plant origin oils *viz*., neem and *Pongamia* oils (@ 0.1% and 0.2%) were tried as synergists with deltamethrin 2.5 EC, a commonly used insecticide against *P. xylostella* on cabbage both under laboratory and field conditions at Indian Institute of Horticultural Research, Bengaluru, India. Under laboratory conditions, deltamethrin @ 0.5 mL/L + *Pongamia* oil (0.2 %) resulted in the maximum efficacy (86.67 %), followed by deltamethrin (0.5 mL/L) + *Pongamia* oil (0.1%). However, under field conditions, *Pongamia* oil (0.2 %) with deltamethrin (1 mL/L) showed good synergism both in terms of reducing *P. xylostella* and in realizing marketable yields of cabbage compared to all other treatments.

***Keywords*:** Deltamethrin, diamondback moth, neem oil, *Pongamia* oil, synergists

The diamondback moth, *Plutella xylostella* (L.) (Lepidoptera: Plutellidae) is a major pest of cruciferous vegetables worldwide. In the tropics and subtropics, where cruciferous plants are grown year-round, this pest can be present at any time (Talekar & Shelton 1993) and has a history of developing resistance to most of the insecticides (Talekar 1992). Insecticide resistance and control failures are now common and often profitable production of cruciferous vegetables has become increasingly difficult (Walker *et al.* 2012). One of the options for the effective control of insect strains that are resistant to insecticides is to use synergists with insecticides. Seed oils *viz*., cotton seed oil, neem oil, *Pongamia* oil, mahua oil (*Madhuca longifolia)* and sesamum oil were reported to synergise the toxicity of the insecticides to varying levels and can also be used in resistance monitoring studies as inhibitors of Mixed Function Oxidases (MFO's) in place of costly synthetic synergists like piperonyl butoxide (Suneel Kumar & Sannveerappanavar 2003). Synthetic synergists like piperonyl butoxide are expensive and not available locally. Keeping in view of the availability and economy of the synergists, two plant origin oils *viz*., neem and *Pongamia* oils were tried as synergists along with deltamethrin, a commonly used insecticide against *P. xylostella* on cabbage under laboratory and field conditions.

Materials and Methods

The efficacy of two plant oils *viz*., neem and *Pongamia* as synergists with deltamethrin 2.5 EC was tested under laboratory conditions during 2007 with deltamethrin at 0.5 mL/L and both oils at 0.1 and 0.2 per cent concentrations. Other treatments included neem and *Pongamia* oils at 0.1, 0.2 and 1 per cent alone and deltamethrin at 0.5 mL/L along with control. The experiment was conducted in Completely Randomised Design. Ten, third instar larvae of diamondback moth were released in the petri plates (14 cm diameter) containing cabbage leaf disc. The larvae were exposed to the insecticide alone and insecticide + oil mixtures under Potters's spray tower. Liquid soap of 0.1% was added to the spray fluid as an emulsifier. Two ml of each treatment (please refer

Table 1) was sprayed directly on the larvae held in Petri dishes at 10 lbs / sq. inch pressure and the treated larvae were maintained at 25 ± 1 °C. Each treatment was replicated thrice with 10 larvae per replication. The treated insects were observed for mortality at 48 h after treatment. Abbott's corrections were carried out and the data were subjected to analysis of variance (ANOVA) and treatment means were compared using DMRT. Field trials were conducted on cabbage in the research farm of the Indian Institute of Horticultural Research, Bengaluru (12° 58' N; 77° 35'E), India during 2008 and 2009. For field evaluation, same treatments tested in the laboratory, except oils at 0.1 per cent, were used besides neem soap (1.0%) (Table 2). The experiments were carried out using the cabbage *cv.* Unnati. The experiment was laid out in a Randomized Block Design with three replications. The seedlings were transplanted at 45 cm x 45 cm in a plot of 2.5 m x 3 m during the second week of February, both the years. The recommended package of practices was followed for cultivation of the crop. Three sprays of each treatment were given starting from the crop primordial stage at 15 d interval.

Observations on the number of diamondback moth larvae were recorded on three randomly selected plants from each replication, two days after each spray. Marketable head yield was recorded on plot basis and converted to t/ha. Data on the number of larvae/plant was transformed to square root of x + 0.5 values, before subjecting them to ANOVA and treatment means were compared using DMRT. For the purpose of estimation of detoxifying enzyme activity in the treated larvae (plant oils + deltamethrin), carboxyl esterases, glutathion-S-transferase and mixed function oxidases were estimated as per the methods followed by Gong *et al.* (2013) and Qian et al. (2008). Single larva of *P. xylostella* was used for estimating the detoxifying enzymes.

Results and Discussion

Laboratory studies

All the treatments tested for the management of diamondback moth, *P. xylostella* except *Pongamia* oil (0.1 % and 0.2 %) were effective over control. Deltamethrin (0.5 mL/L) + *Pongamia* oil (0.2 %) was the most effective treatment (86.67 % mortality) followed by deltamethrin @ 0.5 mL/L + *Pongamia* oil @ 0.1 per cent (80.00 % mortality). Deltamethrin alone resulted in 43.33 per cent mortality of *P. xylostella* (Table 1).

Field Evaluation - First Year (2008)

Of different treatments evaluated in the field for *P. xylostella* management, the lowest number of larvae was recorded in deltamethrin @1 mL/L + *Pongamia* oil (0.2 %) (0.56/ plant), neem soap (1.0 %) (0.78/ plant), deltamethrin @1 mL/L + 0.2 % neem oil (1.11/ plant), deltamethrin 0.5 mL/L + 0.2 % *Pongamia* oil (1.22/plant) after the first spray. Pooled averages over three sprays revealed deltamethrin 1 mL/L + 0.2 % *Pongamia* oil (0.44/plant) as the best treatment followed by deltamethrin 1 mL/L + 0.2 % neem oil (2.22 mL/L) (Table 2). However, deltamethrin @ 1 mL/L + *Pongamia* oil (0.2 %) resulted in highest yield of 30.32 t/ha followed by deltamethrin @ 1mL/L + 0.2 % neem oil (29.09 t/ha) and neem soap 1 % (27.65 t/ha) compared to control (15.35 t/ha).

Field Evaluation – Second Year (2009)

Results followed more or less a similar trend in this year also. The lowest number of larvae was recorded in deltamethrin 1 mL/L + *Pongamia* oil 0.2 % (0.22/plant), deltamethrin 0.5 mL/L + 0.2 % *Pongamia* oil (0.44/plant) and neem soap 1 % (1.22/ plant) after the first spray. After second and third spray also, deltamethrin 1 mL/L + 0.2 % *Pongamia* oil resulted in highest efficacy (1.11 and 0.11 larvae/ plant). Pooled averages over three sprays revealed deltamethrin 1 mL/L + 0.2 % as the best treatment for the management of *P. xylostella* (0.48 larvae/plant). Deltamethrin @ 1 mL/L + 0.2 % *Pongamia* oil resulted in the highest yield of 29.76 t/ha followed by deltamethrin 1 mL/L + 0.2 % neem oil (28.00 t/ha) and neem soap 1 % (26.56 t/ha) when compared to control (16.12 t/ha) (Table 3).

Laboratory assessment of detoxifying enzymes in *P. xylostella* from different treatments revealed that esterases and glutathione-S-transferase levels were reduced when deltamethrin was added with neem oil.

However, *Pongamia* oil reduced the levels of the mixed function oxidases (MFO's) when applied with deltamethrin resulting in more mortality of *P. xylostella*.

Suneel Kumar and Sannaveerappanavar (2003) recorded Sesamum oil and *Pongamia* oil synergism with synthetic pyrethroids *viz*., fenvalerate to the tune of 84.79 and 88.52 per cent, respectively whereas in deltamethrin it was 77.19 and 85.65 per cent over pyrethroids alone under laboratory conditions. Similarly, the toxicity of lambda-cyhalothrin was synergized to a greater extent by Sesamum oil followed by *Pongamia* oil. Similar observations for synergism of fenvalerate toxicity to field populations of *Helicoverpa armigera* were reported by Sundaramoorthy and Chitra (1992) and Manoharan and Uthamasamy (1993). Gavi Gowda (1996) opined that *Pongamia* oil acted as an MFO inhibitor and the inhibition of the oxidases by *Pongamia* oil may be the reason for synergism of methomyl toxicity. In our study also *Pongamia* oil inhibited the MFO in *P. xylostella* when added with deltamethrin.

TABLE 1

Effect of neem and Pongamia oils in enhancing the bio-efficacy of deltamethrin against P. xylostella

Treatments	**Percent mortality of DBM at 48 HAT**	**Per cent over Reduction control**
Deltamethrin 2.5 EC @ 0.5 mL/L	43.33* (41.15)^{de}	30.00
Deltamethrin 2.5 EC @ 0.5mL/L+ Neem oil (0.1%)	53.33 (46.92)^{cd}	50.00
Deltamethrin 2.5 EC @ 0.5mL/L + Neem oil (0.2%)	70.00 (57.70)^{bc}	56.67
Deltamethrin 2.5 EC @ 0.5mL/L + *Pongamia* oil (0.1%)	80.00 (63.72)^{ab}	66.66
Deltamethrin 2.5 EC @ 0.5ml/l + *Pongamia* Oil (0.2%)	86.67 (68.85)^{a}	73.34
Pongamia oil (0.1%)	23.33 (28.78)^{f}	10.00
Pongamia oil (0.2%)	26.67 (30.78)^{ef}	13.33
Neem oil (0.1%)	23.33 (28.78)^{f}	10.00
Neem oil (0.2%)	46.67 (43.08)^{d}	33.34
Neem oil (1.0%)	60.00 (50.65)^{cd}	46.67
Pongamia oil (1.0%)	53.33 (46.92)^{cd}	40.00
Control	13.33 (21.15)^{f}	-
CD (*p=0.05*)	**10.19**	
CV (%)	**13.74**	

* Mean of 3 replications; ** Figures in parentheses are arc sine transformed values; *** Treatments denoted with same alphabet in a column are statistically non-significant.

CONCLUSION

Under field conditions, *Pongamia* oil (0.2 %) with deltamethrin (1 mL/L) showed good synergism both in terms of reducing *P. xylostella* and in realizing higher marketable yield of cabbage compared to all other treatments in the current study.

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TABLE 2

Efficacy of plant oils as synergists with Deltamethrin for P. xylostella *management (First Year)*

Treatments	No. of *P. xylostella* larvae/3 plants			Pooled average	Yield (Kg/plot)	Yield (t/ha)
	I spray	II spray	III spray			
Deltamethrin 2.5 EC @ 0.5 mL/L	11.00 (3.39)^{d}	13.00 (3.67)^{cb}	6.67 (2.68)^{cb}	10.22	16.35	21.80
Deltamethrin 2.5 EC @ 1 mL/L	5.87 (2.48)^{c}	5.00 (2.35)^{ba}	1.33 (1.29)^{ba}	4.06	18.64	24.84
Deltamethrin 2.5 EC @ 0.5 mL/L + Neem oil (0.2%)	3.67 (2.42)^{cb}	2.67 (1.77)^{e}	3.33 (1.95)^{e}	3.22	19.47	25.95
Deltamethrin 2.5 EC @ 0.5 mL/L + *Pongamia oil* (0.2%)	3.67 (2.03)^{ba}	6.33 (2.61)^{b}	0.00 (0.71)^{a}	3.33	17.15	22.86
Deltamethrin 2.5 EC @ 1 mL/L + Neem oil (0.2%)	3.33 (1.95)^{a}	2.33 (1.68)^{dc}	1.00 (1.22)^{dc}	2.22	21.83	29.09
Deltamethrin 2.5 EC @ 1 mL/L + *Pongamia oil* (0.2%)	1.67 (1.72)^{a}	1.67 (1.46)^{ed}	0.67 (1.05)^{ed}	1.33	22.75	30.32
Neem oil (0.2%)	14.00 (3.81)^{e}	14.67 (3.89)^{dc}	7.33 (2.80)^{d}	12.00	13.53	18.03
Pongamia oil (0.2%)	12.33 (3.58)^{ed}	16.67 (4.14)^{d}	4.00 (2.11)^{c}	11.00	15.60	20.79
Neem soap (1.0%)	2.33 (1.68)^{a}	7.33 (2.80)^{b}	3.67 (2.04)^{c}	4.44	20.75	27.65
Control	14.33 (3.85)^{e}	14.33 (3.85)^{e}	11.67 (3.48)^{e}	13.44	11.52	15.35
CD (*p=0.05*)	**0.39**	**0.26**	**0.37**		**5.13**	
CV (%)	**8.49**	**5.28**	**11.11**		**16.84**	

* Mean of 3 replications; ** Figures in parentheses are square root x + 0.5 transformed values;
*** Treatments denoted with same alphabet in a column are statistically non-significant.

TABLE 3

Efficacy of plant oils as synergists with deltamethrin against P. xylostella *(Second year)*

Treatments	No. of *P. xylostella* larvae/3 plants			Pooled average	Yield (Kg/plot)	Yield (t/ha)
	I spray	II spray	III spray			
Deltamethrin 2.5 EC @ 0.5 mL/L	9.33 (3.13)^{d}	16.33 (4.10)^{ef}	4.67 (2.28) ^{bc}	10.11	15.31	20.40
Deltamethrin 2.5 EC @ 1 mL/L	6.33 (2.62)^{c}	11.67 (3.48)^{d}	4.67 (2.27)^{bc}	7.56	17.54	23.38
Deltamethrin 2.5 EC @ 0.5 mL/L + Neem oil (0.2%)	6.33 (2.62)^{c}	6.67 (2.68)^{c}	3.33 (2.04)^{bc}	5.44	16.91	22.54
Deltamethrin 2.5 EC @ 0.5 mL/L + *Pongamia oil* (0.2%)	1.33 (1.00)^{a}	4.67 (2.27)^{b}	0.66 (1.17)^{c}	2.22	18.42	24.55
Deltamethrin 2.5 EC @ 1 mL/L + Neem oil (0.2%)	10.33 (3.26)^{d}	7.67 (2.80)^{e}	0.67 (1.05)^{c}	6.22	21.01	28.00
Deltamethrin 2.5 EC @ 1 mL/L + *Pongamia oil* (0.2%)	0.67 (1.05)^{b}	3.33 (1.95)^{a}	0.33 (0.88)^{c}	1.44	22.33	29.76
Neem oil (0.2%)	10.67 (2.42)^{bc}	17.67 (4.26)^{f}	5.33 (3.34)^{d}	11.22	12.80	17.06
Pongamia oil (0.2%)	9.67 (3.26)^{d}	16.00 (4.06)^{e}	5.00 (2.35)^{bc}	10.22	14.86	19.80
Neem soap (1.0 %%)	3.67 (2.04)^{b}	7.33 (2.04)^{b}	3.67 (1.97)^{c}	4.89	19.93	26.56
Control	12.00 (3.53)^{d}	21.00 (4.63)^{g}	6.00 (2.55)^{c}	13.00	12.10	16.12
CD (*p=0.05*)	**0.39**	**0.18**	**0.50**		**4.03**	
CV (%)	**8.75**	**3.22**	**15.30**		**13.73**	

* Mean of 3 replications; ** Figures in parentheses are square root x + 0.5 transformed values; *** Treatments denoted with same alphabet in a column are statistically non-significant

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Assessment of Resistance Development in Field Populations of Diamondback Moth, *Plutella xylostella* (L.) from Andhra Pradesh to Insecticides and Cry2Ab Toxin

Sunitha, V., Singh, T.V.K.[1] and Satyanarayana, J.[2]
A.I.N.P. on Agril. Ornithology, Rajendranagar, Hyderabad, Telangana State - 500 030.
E-mail: vanamsunitha10@gmail.com
[1]P.J.T.S.A.U., Rajendranagar, Hyderabad, Telangana State - 500 030.
E-mail: prof.tvksingh@yahoo.com
[2]Dept. of Entomology, College of Agriculture, Rajendranagar, P.J.T.S.A.U. Hyderabad - 500 030.
E-mail: snjella@gmail.com

Abstract

Insecticidal resistance studies against third instar larvae of diamondback moth (DBM), *Plutella xylostella L*. from Andhra Pradesh were carried out from F_1 to F_3 against acephate, cypermethrin, spinosad, cartap hydrochloride and Cry2Ab using leaf dip bioassay method. The LC_{50} values deduced were used to quantify the resistance in *P. xylostella* of parental generation (F_0). Resistance development was assessed with a concentration fetching 80.00 % mortality in every generation from F_1 to F_3 generations against all the tested insecticides and Cry2Ab toxin. The results revealed that 2.57-, 1.00-, 1.00-, 1.33- and 1.98-fold resistance against acephate, cypermethrin, spinosad, cartap and Cry2Ab toxin, respectively in F_3 generation. The levels resistance from F_1 to F_3 generations increased against all the test insecticides and Cry toxin, except against cypermethrin and spinosad from F_1 to F_3 generations.

***Keywords*:** Insecticide resistance, DBM, Andhra Pradesh, toxin

India is the world's largest cauliflower (*Brassica oleracea* var. *botrytis* L.) grower and second largest cabbage (*B. oleracea* var. *capitata* L.) grower next to China occupying an area of 372,000 ha and 402,000 ha, respectively. Diamondback moth (DBM), *Plutella xylostella* (L) (Lepidoptera: Plutellidae), is the most important pest of cruciferous crops and is very destructive in nature (CIE 1967). In India, DBM was reported in 1914 on cruciferous vegetables and is now the most devastating pest of cole crops in the states of Punjab, Haryana, Himachal Pradesh, Delhi, Uttar Pradesh, Bihar, Andhra Pradesh, Tamil Nadu, Maharashtra and Karnataka (Uthamasamy *et al*. 2011). The infestation of the pest increases gradually from first fortnight of August and leads to total loss of the crop (Dhaliwal *et al.* 2010). In India it causes significant economic losses of up to 50% with an estimate of US$ 168 million per year. Absence of effective natural enemies and rapid development of insecticide resistance to many classes of insecticides, which account for 30-50% of the total cost of production are considered to be the major causes of increasing pest status of DBM in most parts of the country.

DBM occupies second position in being resistant to 82 compounds of insecticides (APRD 2012) and to be the first species to develop field resistance to *Bacillus thuringiensis (Bt)* Cry toxins, and one amongst three insect species to have developed field resistance to Bt based spray products (Talekar and Shelton 1993). It is documented that resistance is inevitable within a span of two to three years after the introduction of a new insecticide (Sayyed and Wright 2006; Wang and Wu 2012; Zhao *et al.* 2006). Recent examples of field resistance to relatively newer compounds, include indoxacarb, avermectins, spinosad, Bt- based products (Dipel), benzyl ureas and chlorantraniliprole (Furlong *et al.* 2013). Hence, the present study was undertaken for quantifying the resistance levels in DBM from Andhra Pradesh region against four commonly used insecticide groups with diverse modes of action and one *B. thuringiensis* toxin.

Materials and Methods

Laboratory investigations were carried out during 2011-2012 in the Bt Lab, Department of Entomology, College of Agriculture, Rajendranagar, Hyderabad.

Median lethal concentration (LC_{50}) calculation

Test insect population

Cabbage cultivar "Charmant" nursery was raised in the greenhouse and one month old seedlings were transplanted in the main field and raised without any insecticide application. DBM larvae were collected from farmers' cabbage fields in and around Hyderabad to establish insect culture. Leaves were harvested daily and washed with tap water to be used as feed for the larvae. Larvae were allowed to pupate in the jars (12x6 cm) and the pupae were placed in a cage for moth emergence. The emerged adult moths were allowed to lay eggs on mustard seedlings. Adults were provided with 10% honey solution fortified with multivitamins and proteinex on a cotton swab for better egg laying. Mustard seedlings with eggs of DBM were collected from the cage and kept in glass jars for hatching. The neonates were reared on insecticide free cabbage leaves. At every successive instar stage, the larvae were shifted to clean jars and fresh cabbage leaves were provided. Third instar larvae were used in bioassay studies.

Test insecticides and Cry toxin

To determine the LC_{50}valuesof insecticides and Cry toxin against DBM larvae, four insecticides *viz.*, acephate (Organophosphate), cypermethrin (Synthetic pyrethroid), spinosad (Spinosyn), cartap hydrochloride (Nereistoxin) and Cry2Ab were used. Hundred ml of one per cent stock solution of all the above test insecticides were used for the preparation of serial dilutions. Initially, broad range concentrations were tested for each test insecticide and toxin; depending on the 20 to 80 % mortality observed, narrow range concentrations were tested. A control was also maintained for each experiment and the mortality data was corrected by using modified Abbott's formula (Flemmings and Ratnakaran 1985). Bioassay was repeated for experiments in which control mortality exceeded 20%.

Stock solution preparation for Cry toxin

The technical formulation of Cry2Ab (3.93 mg/g) was supplied by Central Institute of Cotton Research (CICR), Nagpur. Hundred mg of the toxin was dissolved in 5 ml distilled water to obtain a stock solution of 60ppm concentration. The stock solution was subjected to serial dilutions to obtain different concentrations and a drop of emulsifier, Tween-80 was added. Similarly a drop of Tween-80 was also added to control.

Bioassay

Bioassays were conducted with third instar larvae of DBM by using a standard leaf dip method (Sayyed *et al.* 2000). A Bioassays was conducted to deduce the LC_{50} of for all the four test insecticides and Cry2Ab toxin, and the LC_{50} concentration deduced was used to assess the resistance in DBM from parental generation (F_0) to F_3 generation.The leaf discs (5 cm) were used for bioassay studies. The leaf discs were dipped in 10 ml of aqueous solution of various concentrations of test insecticides and Cry toxin, whereas control leaf discs were immersed in distilled water having a drop of Tween-80 for about 15 sec and shade dried before transferring onto a moistened filter paper in a petri plate. Ten third instar larvae were released on each treated leaf disc in each concentration. Each treatment was replicated thrice. Larval mortality was recorded at 24, 48 and 72 h after treatment (HAT) by counting the larvae as dead or moribund when they did not resume activity after repeated prodding. The mortality at 72 HAT was considered as end point for the assessment of toxicity of test insecticides and Cry toxin (Fisk and Wright 1992). LC_{50} values of all test insecticides and Cry2Ab toxin were determined by probit analysis (Finney 1971). The calculated LC_{50} was used in quantifying the resistance by inducing selection pressure.

Quantification of insecticidal resistance in Andhra Pradesh populations

To assess the resistant levels in DBM populations of Andhra Pradesh region, larvae were collected from Medak district of Andhra Pradesh and reared on insecticide free cabbage leaves in the laboratory and larvae in the third instar were used for bioassay studies.

Bioassay and lab selection

The DBM larvae obtained from the cabbage field were designated as F_0 population and the subsequent generations (obtained from previous generations) were designated as F_1 (first generation), F_2 (second generation) and F_3 (third generation). The process of selection pressure for insecticides and Cry toxin was

initiated in the parental generation (F_0) and continued up to F_3 generation. The calculated LC_{50} values of each insecticide and Cry2Ab toxin was subjected to preliminary bioassay for all the three populations separately. Individual DBM population was subjected to five concentrations ($LC_{50,}$ two concentrations higher than LC_{50} and two concentrations lower the value of LC_{50}) of each individual insecticide and Cry2Ab toxinand a control with ten third instar larvae per treatment and replicated thrice. Larval mortality was recorded at 24, 48 and 72HAT. The concentration (LC_{80}) that gave 80% mortality was selected from the preliminary bioassay and the survivors at other concentrations were rejected. Using this LC_{80} concentration of all the test insecticides and Cry2Abs subsequent bioassays were conducted with using 100 third instar larvae per treatment (individual insecticide and Cry2Ab) and replicating the same thrice for inducing selection pressure from the parental generation (F_0) onwards along with a control. The survivors in the bioassay were raised to first generation (F_1) and third instar F_1 larvae were subjected to bioassay as idicated above which was continued up to F_3 generation. The concentrations were adjusted in subsequent generations depending on the per cent larval survivors obtained in the previous generation.

Assessment of insecticidal resistance in P. xylostella

The degree of development of resistance through different generations was determined by working out LC_{50} values in each generation and thus computing the resistance ratio (RR) by dividing the LC_{50} value for F_n generation with LC_{50} value of the F_1 generation (Arora 2003).

$$\text{Resistance ratio (RR)} = \frac{LC_{50} \text{ value of } F_n \text{ generation}}{LC_{50} \text{ value of } F_1 \text{ generation}}$$

Results and Discussion

In general, the susceptibility of *P. xylostella* to conventional insecticides and toxin was low.

Resistance development in DBM against acephate

The concentrations of acephate which were used in bioassay varied from 0.01 to 0.20, 0.05 to 0.25 and 0.05 to 0.25% against the third instar larvae of DBM collected from Andhra Pradesh in three generations (F_1, F_2 and F_3). The LC_{80} which was used for inducing selection pressure varied in F_1, F_2 and F_3 generations. The documented LC_{80} against third instar larvae of DBM collected from Andhra Pradesh were 0.15, 0.20 and 0.20%. The calculated LC_{50} values obtained for Andhra Pradesh population in F_1, F_2 and F_3 generations were 0.033, 0.081 and 0.085%. Resistance ratios obtained in F_2 and F_3 generations in comparison to F_1 generation were 2.45 and 2.57 folds (Table 1).

TABLE 1

Toxicity of acephate to third instar larvae of P. xylostellain *different $F_1 - F_3$ generations*

Generation	Heterogeneity (X^2)	Regression equation	LC_{50} (%)	Fiducial limits	Resistance ratio	Slope ± S.E
F_1	5.81	Y = 6.79 + 1.21x	0.033	0.015 - 0.053	1.00	1.21 ± 0.26
F_2	0.50	Y = 7.46 + 2.25x	0.081	0.052 - 0.104	2.45	2.25 ± 0.48
F_3	1.29	Y = 7.66 + 2.49x	0.085	0.059 - 0.108	2.57	2.49 ± 0.49

The results showed that the LC_{50} values increased from F_1 to F_3, which indicated that DBM population from Andhra Pradesh had developed resistance against acephate because the resistance ratio was more than one. A high level of resistance to organophosphate insecticides in DBM has been reported from various parts of the world including - 2096 folds resistance to malathion in Malaysia (Sudderuddin and Kok 1978), 305 to 735 fold resistance to malathion in Thailand (Barroge *et al.* 1981), 20 to 75 fold resistance to chlorpyriphos, methyl parathion, malathion, methamidophos and diazinon in North Florida (Yu and Nguyan, 1992). Resistance ratios in the present study though are in conformity with the findings elsewhere, a very low level of resistance, to the tune of 2.57- folds has been documented for the DBM populations sampled from Andhra Pradesh in the F_3 generation.

Resistance is inevitable by inducing selection pressure for several generations with insecticides in insects even for the susceptible strains and laboratory

strains (Sasaki 1982; Cheng *et al.* 1985; Noppun *et al*. 1986; Hama 1989). However, low level of resistance documented in the present study can be attributed to the fact that selection pressure was induced at LC_{80} concentration and for only three generations. Lack of insecticidal exposure for several generations together can deplete resistance development in insects (Chen and Sun 1986; Miyata *et al.* 1986).

Though usage of acephate for the management of DBM has been replaced by new insecticides that are commercially available, recent studies of Shoji and Chikako (2010) is in confirmation with the present study where only 2.3-fold resistance was documented by the resistant *P. xylostella* strain over the susceptible strain against prothiofos. Calibration of resistance ratios would have been precise with that of laboratory strain maintained for several generations without insecticidal exposure as recorded elsewhere.

Resistance development in DBM against cypermethrin

LC_{50} concentration of 0.008% was obtained in bioassay using DBM population sampled from cabbage agro-ecosystem in and around Hyderabad and the same was used to obtain the survivors of DBM in Andhra Pradesh population with cypermethrin. The calculated LC_{80} for inducing selection pressures were 0.008, 0.016 and 0.008% for F_1, F_2 and F_3 generations, respectively. The LC_{50} recorded from the bioassays were 0.003, 0.004 and 0.003 % respectively for the three generation generally. Resistance ratios in F_2 and F_3 generations over the F_1 generation were 1.33 and 1.00 (Table 2).

TABLE 2

Toxicity of cypermethrin to third instar larvae of P. xylostella *in different generation*s

Generation	Heterogeneity (X^2)	Regression equation	LC_{50} (%)	Fiducial limits	Resistance ratio	Slope ± S.E
F_1	0.414	Y = 9.44 + 1.72x	0.003	0.001 - 0.004	1.00	1.72 ± 0.34
F_2	1.024	Y = 9.01 + 1.69x	0.004	0.003 - 0.006	1.33	1.69 ± 0.31
F_3	1.662	Y = 9.52 + 1.74x	0.003	0.001 - 0.004	1.00	1.74 ± 0.55

The median lethal concentration followed an increasing trend up to F_2 generation and later decreased in F_3 generation. Enzymatic role of mixed function oxidases coupled with target site nerve insensitivity (kdr) (Holden 1979; Gammon 1980) are considered as the most common mechanisms of resistance in DBM to synthetic pyrethroids, and reduced insecticide penetration through the cuticle is also cited to to be a reason. The low magnitude of resistance development in the present study might be due to the less usage of synthetic pyrethroids (cypermethrin) in cabbage agro-ecosystem. The resistance developed by DBM against cypermethrin is in accordance with other reports, for instance 144 fold resistance against cypermethrin in DBM at Panipat (Haryana) and 115 fold resistance to pyrethroids at Delhi and Karnataka (Saxena *et al.* 1989), 25 fold resistance against pyrethroids (Raju and Singh 1995), and 2814 folds resistance against cypermethrin by DBM population sampled from Bangalore (Sannaveerappanavar 1995). In the present study there was a decrease in susceptibility pattern up to F_2 generation, followed by an increase in the susceptibility in the F_3 generation showing a moderate level of resistance development. Further studies are required with regards to calibration of variation in enzyme titers *viz.*, mixed function oxidases, and glutathione S transferases using specific substrates that play vital role in resistance development against synthetic pyrethroids as reported in other insects as well. The present results are in corroboration with studies conducted elsewhere who reported resistance ratio of 21 fold for Peng Hu strain and 899 fold for Ban-Chu strain in China (Liu *et al.* 1982), Nicaragua (Perez *et al.* 2000), Taiwan (Liu *et al.* 1981), USA (Yu and Nguyen 1992) and Pakistan (Khaliq *et al.* 2007).

Resistance development in DBM against spinosad

The median lethal concentration of 0.003% for spinosad was recorded in the bioassay conducted with DBM larval population from Hyderabad. LC_{80} obtained was 0.0035% in all the three generations of Andhra Pradesh population. The LC_{50} calculated for Andhra Pradesh population was 0.003%, 0.002 % and 0.003 % in F_1, F_2 and F_3generations, respectively (Table 3).

TABLE 3

Toxicity of spinosad to third instar larvae of P. xylostella *in different generations*

Generation	Heterogeneity (X^2)	Regression equation	LC_{50} (%)	Fiducial limits	Resistance ratio	Slope ± S.E
F_1	1.445	Y = 23.99 + 7.40x	0.003	0.002 - 0.003	1.00	7.40 ± 1.28
F_2	1.442	Y = 19.72 + 5.64x	0.002	0.002 - 0.003	0.66	5.64 ± 1.15
F_3	5.697	Y = 27.64 + 8.81x	0.003	0.002 - 0.003	1.00	8.81 ± 1.46

Resistance ratios in DBM population of F_2 and F_3 generations in relation to F_1 generation were 0.66 and 1.00 for Andhra Pradesh population. The resistance ratios of F_2 and F_3 generation in relation to F_1 generation was less than one fold in study population, which indicated that resistance has not yet developed.

Resistance development studies for the DBM against spinosad showed neutral results. Susceptibility pattern of Andhra Pradesh population showed diverse results (as depicted by LC_{50} values) ultimately showing negligible resistance development. The reason for the *P. xylostella* populations either developing moderate resistance or no resistance may be due to the fact that spinosad being a novel insecticide and the usage pattern and selection pressure by this insecticide are relatively new in cabbage agro ecosystem in areas where the study population was collected, or alternatively the pest was never pre-disposed to spinosad sprays in these areas. The findings of the present studies are in corroboration with the findings of Peter *et al.* (2000), Dey and Som Choudhary (2001) and Vadodaria *et al.* (2000), who earlier reported the higher field efficacy of spinosad against *P. xylostella* in Gujarath.

The present data confirms the findings of Zhao *et al.* (2002) who determined the toxicity ratio of 1.3 to 1.2 from seven zones and 0.8 to 32 from six zones in Geneva. Kao and Cheng (2001) reported LC_{50} of 24.06 ppm and 26.77 ppm from Lu Chu and His-hu strain, respectively, in China during 2001. Shelton *et al.* (1996) showed tolerance ratio of more than 100 to spinosad in DBM population of California (USA), which indicated high levels of resistance than the present study. Walker *et al.* (2002) showed no significant resistance in field population of DBM in New Zealand,which is in conformity with the findings of the present study.

The present study indicated that *P. xylostella* from Andhra Pradesh remain susceptible to spinosad. However, resistance to spinosad occurred in Hawaii (2000), Georgia (2001) and California (2002) as a consequence of multiple years of extensive application. A major reason for the rapid resistance development to spinosad in Hawaii was the lack of suitable alternatives and the unsynchronized use of insecticide classes that led to continuous population exposure to spinosad as it happened in Southeast Asia (Sayyed *et al.* 2003, 2004) and North America (Zhao *et al.* 2002).

The propensity for the selection of spinosad resistance may have arisen from pre - existence of resistance alleles from the past use of organochlorine insecticides as the mode of action of organochlorine and spinosad as GABA/nicotinic acetyl choline receptor as a target (Ortells and Lunt 1995; Massol *et al.* 2000). However, the possibility of *P. xylostella* carrying spinosad resistance allele, which may have been introduced from other areas via transportation of cabbage also cannot be ruled out (Khaliq *et al.* 2007). Our results indicated that Andhra Pradesh populations of *P. xylostella* were so far susceptible to spinosad. Hence, spinosad can be used commercially as an alternative to particularly those insecticides against which *P. xylostella* has already developed resistance.

Resistance development in DBM against cartap hydrochloride

The median lethal concentration of 0.01 % for cartap was recorded in the bioassay conducted with DBM larval population from Hyderabad. LC_{80} obtained was 0.02 % for all three generations of Andhra Pradesh population. The LC_{50} calculated for Andhra Pradesh population was 0.009 %, 0.011 % and 0.012 % for F_1, F_2 and F_3 generations, respectively (Table 4).

TABLE 4

Toxicity of cartap hydrochloride to third instar larvae of P. xylostella *in different generations*

Generation	Heterogeneity (X^2)	Regression equation	LC_{50} (%)	Fiducial limits	Resistance ratio	Slope ± S.E
F_1	5.981	Y = 10.93 + 2.92x	0.009	0.007 - 0.011	1.00	2.92 ± 0.53
F_2	1.874	Y = 12.95 + 4.04x	0.011	0.008 - 0.013	1.22	4.04 ± 0.81
F_3	1.924	Y = 13.62 + 4.52x	0.012	0.010 - 0.014	1.33	4.52 ± 0.81

Resistance ratios in DBM population of F_2 and F_3 generations in relation to F_1 generation were 1.22 and 1.33 for Andhra Pradesh population. The results clearly showed that the resistance ratios were more than one in F_3 generation, which indicates that resistance has developed against cartap hydrochloride.

Studies pertaining to resistance development for the *P. xylostella* populations from Andhra Pradesh region showed considerable decrease in susceptibility pattern over the three generations against cartap hydrochloride. The development of resistance to cartap hydrochloride by *P. xylostella* is reported in India (Renuka and Regupathy 1996) and elsewhere globally *viz.*, Japan (Horikiri 1989; Ozawa *et al.* 1989; Hama *et al.* 1990), Taiwan (Cheng *et al.*1992), China (Chen *et al.* 1995) and Korea (Cho and Lee 1994). The present study is in accordance with that of Chandrasekharan and Reghupathy (1996) who found resistance levels (expressed as % survival) varied from 17.9 to 52.4 to cartap hydrochloride and Vastrad *et al.* (2002) who recorded the moderate survival percentage (1.11) of DBM treated with cartap hydrochloride. Sannaveerappanavar and Viraktamath (1997) reported the development of resistance to cartap hydrochloride at recommended field concentrations. The resistance ratio developed by the DBM populations in the present study are in accordance with Branco *et al.* (2002) who obtained resistance ratio in the range of 2.8 to 7.1for the most resistant strain that received multiple sprays of cartap hydrochloride. Likewise Vastrad *et al.* (2004) documented low to moderate levels of resistance to cartap hydrochloride against DBM populations from 3-12 locations of Karnataka. The results of the present study, by and large, fall in line with those obtained by Joia and Udeaan (1997) who obtained LC_{50} values 0.015 to 0.020 % with cartap against multi-resistant *P. xylostella* population from Punjab.

Resistance development in DBM against Cry2Ab

The median lethal concentration of 0.3 ppm for Cry2Ab was recorded in the bioassay conducted with DBM larval population from Hyderabad. LC_{80} obtained was 0.4, 0.5, 0.6 ppm in the F_1, F_2 and F_3 generations, respectively. The LC_{50} calculated for Andhra Pradesh population was 0.178 ppm, 0.239 and 0.353 ppm in F_1, F_2 and F_3 generations, respectively (Table 5).

TABLE 5

Toxicity of Cry2Ab to third instar larvae of P. xylostella *in different generations*

Generation	Heterogeneity (X^2)	Regression equation	LC_{50} (%)	Fiducial limits	Resistance ratio	Slope ± S.E
F_1	3.559	Y = 6.89 + 2.51x	0.178	0.124 - 0.224	1.00	2.51 ± 0.50
F_2	3.609	Y = 6.94 + 3.12x	0.239	0.162 - 0.290	1.34	3.12 ± 0.71
F_3	1.933	Y = 7.15 + 4.76x	0.353	0.283 - 0.401	1.98	4.76 ± 0.96

Resistance ratios in DBM population of F_2 and F_3 generations in relation to F_1 generation were 1.34 and1.98 for Andhra Pradesh population.The results clearly showed that in our study population, the resistance ratios were more than one in F_3 generation, which indicates that resistance has already developed against Cry2Ab.

The resistance development in *P. xylostella* populations sampled from Andhra Pradesh gradually increased from F_1 to F_3 generations. In general, the susceptibility of *P. xylostella* to *Bt* strains and their toxins was found to be significantly lower in populations that originated from Southern India followed by those from Western and Northern India

(Mohan and Gujar 2002). This suggests the possibility of DBM adaptation in the populations where *Bacillus thuringiensis kurstaki* (*Btk)* formulations are regularly used (Sawant 1998). However, in our studies the susceptibility patterns indicate some changes in susceptibility. This may be due to the fact that baseline susceptibility of local insect populations depend not only on the extent of selection pressure (amount of insecticide used) but also other factors like relative dominance of resistant alleles, level of immigration of susceptible individuals (gene flow), population structure and exposure to the pesticide application pattern. In addition, insect behavior also plays a major role (Roush and Daly 1990). Further studies are required in understanding the mechanism underlying the resistance through reduced binding of Cry toxin to brush border membrane vesicles (BBMV's). Reduced activation of proteases coupled with faster degradation of proteases are already documented to be the resistance mechanism in studies with regards to type of mid-gut proteases involved in activation of Cry toxin.

Conclusion

The rate of development of resistance from F_1 to F_3 generations increased against all the test insecticides and Cry toxin, except cypermethrin and spinosad. It can be construed from the current study that spinosad and cypermethrin can still be used as an alternative to particularly those insecticides against which *P. xylostella* has already developed resistance in Andhra Pradesh. However, proper pesticide windows should be adopted while using these pesticides, to avoid the development of resistance eventually.

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Mutation and Down-Regulation of nAChR-Beta1 Subunit is Associated with Imidacloprid Resistance in the *Aphis gossypii*

Kim Juil, Cha Deok Jea, Kwon Min, Kim Gil-Hah[1], Lee Si Hyeock[2]
Highland Agriculture Research Center, NICS, RDA. E-mail: forwweek@korea.kr
[1]Department of Plant Medicine, Chungbuk National University. E-mail: khkim@chungbuk.ac.kr
[2]Department of Agricultural Biotechnology, Seoul National University. E-mail: Shlee22@snu.ac.kr

Abstract

The cotton aphid, *Aphis gossypii* (Glover), is one of the main pests in various vegetable crops due to insecticide resistance in Korea. Neonicotinoid insecticide resistance in *Nilaparvata lugens, Bemisia tabaci* and *Myzus persicae* etc. has been associated with the over-expression of P450, particularly CPY6 family. However, *A. gossypii* is unique case having developed a non-P450 dependent resistance mechanism. Previously we reported that two point mutations (RtoT in nicotinic acetylcholine receptor, nAChR, beta 1 subunit and RtoT with LtoS in a transcript variant) contribute to imidacloprid resistance in *A. gossypii*. We surveyed the mutation(s) in various local field populations. Based on 3D modeling, we hypothesize that RtoT mutation can reduce the imidacloprid sensitivity. Stretch of 33 amino acids was deleted in the N-terminal region of original transcript of nAChR beta 1 subunit that contained RtoT with LtoS mutations in resistant strain. Among the transcripts, only nAChR beta 1 subunit original transcript differentially expressed between imidacloprid susceptible and resistant strain (resistant ratio = 3,800). Six alpha subunit (1~5, 7) transcript levels were not significantly different between two strains. Therefore mutation and down-regulation of nAChR beta 1 subunit is also associated with imidacloprid resistance in the *A. gossypii*.

Keywords: Insecticide resistance, nicotinic acetylcholine receptor

The cotton aphid, *Aphis gossypii* (Glover) (Hemiptera: Aphididae), is one of the most destructive pests in seed potato production and various vegetable cultivation, not only due to direct damage by feeding, but indirectly by virus transmission (Pan *et al.* 2009). Various insecticides, particularly neonicotinoids, have been intensively used to control this pest (Wang *et al.* 2002; Nauen and Elbert 2003; Shi *et al.* 2011).

Imidacloprid, the first marketed neonicotinoid insecticide exhibits strong insecticidal activity against sucking pests such as aphids, whiteflies and plant hoppers, and some coleopteran pests (Nauen and Denholm 2005; Millar and Denholm 2007; Nauen *et al*. 2008). Neonicotinoids act as agonists at the nicotinic acetylcholine receptor (nAChR), which mediates the signal transmission at cholinergic synapses in insects. Intensive use of imidacloprid has resulted in resistance development in several sucking insect species, including the green peach aphid *Myzus persicae* (Sulzer), the brown plant hopper *Nilaparvata lugens* (Stål) and the tobacco whitefly *Bemisia tabaci* (Gennadius) (Nauen *et al.* 2002; Foster *et al.* 2003; Liu *et al*. 2005; Szendrei *et al*. 2012). Increased detoxification by overexpressed P450s was responsible for neonicotinoid resistance in *B. tabaci*, *N. lugens* and *M. persicae* (Rauch and Nauen 2003; Puinean *et al*. 2010). In addition to the enhanced detoxification by P450s, target site insensitivity mechanism has been reported to be involved in neonicotinoids resistance. nAChR, the target site of neonicotinoids, is a typical member of the cys-loop ligand-gated ion channel superfamily consisting of five subunits (homo- or hetero-pentameric structure) arranged around a central ionophore (Sine and Engel 2006). In insects, about 10 genes are known to encode different subunits of nAChR (Bass *et al.* 2006; Jones *et al.* 2005; Jones *et al.* 2006; Jones and Sattelle 2010). A point mutation (Y151S) in two alpha subunits (Nlá1 and Nlá3) of nAChR was reported to be associated with imidacloprid resistance in a laboratory-selected strain of *N. lugens* (Liu *et al.* 2005; Liu *et al.* 2006; Shao *et al.* 2011) but it has not been detected in field populations. Later, a novel mutation in the beta subunit of nAChR was found to be involved in imidacloprid resistance in a field population of *M. persicae* as a major resistance factor (Bass *et al.* 2011). We investigated the molecular mechanisms of imidacloprid resistance in *A. gossypii* and found that the same mutation in the beta subunit of nAChR is associated with resistance phenotype as a major factor without P450-mediated detoxification being involved (Koo *et al.* 2014). However, the R81T mutation was

also detected in relatively low level of resistant populations (Koo *et al*. 2014). Subsequently, additional point mutation was found in nAChR beta subunit variant (Kim *et al*. 2015). Therefore we hypothesized that the R81T mutation is not the single factor which involved in imidacloprid resistance in the *A. gossypii*. And we tried to find other factors such as expression patterns of nAChR could be contributing to the high level of imidacloprid resistance in *A. gossypii.* Here we report the surplus factors which involve in imidacloprid resistance in *A. gossypii*.

Materials and Methods

Aphid strains and bioassay

The imidacloprid resistant (IR) strain of *A. gossypii* was initially collected from a hot pepper green house in Daejeon, Korea, in 2010. The IR strain has been maintained and selected by 20 ppm (8% SC) imidacloprid on a regular basis approximately every two weeks in Highland Agriculture Research Institute, Pyeongchang, Korea. The isogenic susceptible (IGS) strain was generated by maintaining a subpopulation of IR (imidacloprid LC_{50} was about 20 ppm in May, 2011) without any exposure to insecticide for about a year. Another susceptible strain (S) was obtained from Chungbuk National University, Chungju, Korea. These three laboratory strains of *A. gossypii* were reared on cucumber seedlings (*Cucumis sativus*) in plastic cages under the conditions of 25 ± 2 °C, 16L: 8D photoperiod, and 50~70% relative humidity. The susceptibility of *A. gossypii* to insecticides was determined by leaf-disk dipping method. In brief, technical grade imidacloprid (98.5%) was dissolved in distilled water with 0.2% triton X-100 to appropriate concentrations. In order to determine synergistic effects, diluted imidacloprid was combined with the same concentration and volume of three synergists; piperonyl butoxide (PBO, 98%), s,s,s-tributylphosphorotrithioate (DEF, 98%) and diethyl maleate (DEM, 98%). Cucumber leaf disks (5.5 cm diameter) were dipped into the test solution for 1 min and dried in a fume hood. The dried leaf disk was transferred onto a petri-dish, to which 10 adults were infested. Three replicates were used for each concentration tested and mortalities were recorded at 72 h post-treatment. LC_{50} value was estimated by probit analysis using SAS software (SAS institute 2009).

Protein preparation and two-dimensional electrophoresis (2DE)

Protein was extracted from 500 mg of wingless adult aphids of each strain. Each aphid sample was homogenized with 2DE rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 2.5% dithiothreitol, 10% isopropanol, 5% glycerol) using a glass-glass tissue grinder on ice. The resulting supernatant was purified using 2-D clean-up kit (Amersham Bioscience, Piscataway, NJ, USA) based on the manufacturer's protocol. For the first dimensional IEF, pH 3-10 IEF gel strips (7cm) were rehydrated overnight at room temperature after loading of 120μg of protein. Prior to the second dimensional SDS-PAGE, the IEF gels were equilibrated for 30min with shaking in equilibration solution (6 M urea, 2% SDS, 375 mM Tris-HCl, pH 8.8, 20 % glycerol and 2.5 % acrylamide) for the reduction of proteins. Each protein-separated IEF gel strip was loaded vertically onto the top of an 8-16 % gradient gel lacking the stacking layer. SDS-PAGE was performed at a constant power of 120V / gel. Gels were stained by coomassie blue G-250 and then distained by distilled water overnight. Images of the stained gels were scanned with densitometer. Analysis of the relative expression pattern of 2DE gels was performed with Melanie programs (Geneva bioinformatics, GenBio, S.A., Geneva, Switzerland). 2DE analysis was replicated three times.

Native IEF

Enzyme was extracted from the 100mg of wingless adult aphids of each strain as well as susceptible and multi-resistant strain of *M. persicae* which used as internal control (Kim *et al.* 2014). Each sample was homogenized with 100 mM Tris-HCl (pH. 7.8) buffer using the glass-glass tissue grinder on ice. The supernatant was transfer a column (Qiagen, Valencia, CA, USA) and centrifuged at 5,000 × g for 5 min at 4 °C to remove the lipid and remained cell debris. Protein concentration was determined according to the method of Bradford (1976) using the bovine serum albumin as a standard protein. Native IEF was performed in a vertical electrophoresis unit (Novex® mini cell, Invitrogen, Carlsbad, CA, USA) by using the pH. 3~7 pre-cast IEF gel (Invitrogen).

Equal amount (10 µg) of the preparations and 10 µg of pI marker (Sigma, St. Louis, MO, USA) were focused at 100 V for 1 h, 200 V for 1 h and 500 V for 30 min in a buffer system of Cathode buffer (20 mM Lysine, 20mM Arginine, Invitrogen) and IEF Anode buffer (7 mM Phosphoric acid, Invitrogen). Following the focusing, the gel was activity-stained to visualize esterase bands incubated with 1mM alpha naphthyl acetate (á-NA), butyrate (á-NB), propionate (á-NP), valerate (á-NV) as substrates in esterase.

Mutation survey in nAChR subunit genes, Transcriptome analysis and 3D structure modeling

Total RNA was isolated from about 500mg of adults of IGS and IR strains using Trizol according to the manufacturer's protocol (MRC, Cincinnati, OH, USA). The whole transcriptome shotgun sequencing (RNA seq) was performed at Macrogen Inc. (Seoul, South Korea) using the GS-FLX sequencer (Roche, Basel, Switzerland) with titanium chemistry following the GS-FLX manual. The adapter and primer sequences were removed and the *A. gossypii* transcriptome was assembled using the GS de novo assembler (Newbler v 2.6). All the contigs and singletons obtained were analyzed using the mega BLAST or BLASTn for more information. For comparative transcriptome analysis between IRS and IR strains, all contigs were mapped on *Acyrthosiphon pisum* genome database (Acyr_2.0). Following the pyrosequencing, assembly and annotation, nAChR subunit gene sequences were obtained. To acquire full-length sequences of each nAChR subunit gene, rapid amplification of cDNA ends (RACE) was carried out using the 5'-full core set (TaKaRa, Shiga, Japan) with some modification in cDNA synthesis. Total RNA extraction and cDNA synthesis steps were almost same with previously described (Koo *et al.* 2014). After preparing the cDNA samples from three strains, mutation survey and quantitative real-time PCR (qrtPCR) were performed using suitable primer sets (Table 1) with KOD polymerase (TOYOBO, Osaka, Japan) and SYBR qPCR mix (TOYOBO), respectively.

The 3D structure of nAChR created by the automated and aligned comparative protein modelling program of Swiss model server (http://swissmodel.expasy.org/) and modelling was performed by UCSF Chimera an extensible molecular modeling system v. 1.8.1 (University of California, San Francisco, CA, USA).

Results and Discussion

Bioassay and synergist test

The IR and IGS strains exhibited 3,856 and 15 folds resistance to imidacloprid compared to the S strain, respectively, as determined by LC_{50} values (Table 2). When compared to the IGS strain, an isogenic strain to IR the resistance level of IR was 257 folds. In the synergistic bioassay, no significant synergistic effects were observed in any of the synergist tested, suggesting that metabolic factors mediated by P450, esterase or GST are not likely involved in resistance.

Comparison of transcriptome and proteome profiles

Following the transcriptome analysis using genome sequencer FLX titanium (GS-FLX), a total of 293,303,458 bases (710,636 reads) and 290,745,940 bases (700,417 reads) were obtained from the IR and IGS strains, respectively. After contig assembly, 18,124 contigs were produced and mapped to the *A. pisum* genome database (Acyr_2.0). Among the contigs, 12,389 genes had matches to the *A. pisum* database. To avoid false-positive contigs, mapped genes were selected from contigs with more than 30 reads in a strain. Overall expression levels of several housekeeping genes, such as actin, tubulin and ribosomal protein 3 (RPS3), were almost the same as calculated by number of reads, indicating that the quality of RNA seq data were reliable (Kim *et al.* 2015). When compared the transcription levels of several gene families that are associated with metabolic resistance, such as P450, esterase, GST, ATP-binding cassette (ABC) transporter, and UDP-glucuronosyltransferase (UGT), no significant differences between the IR and IGS strains were noticed in the majority of genes except for a few cases. All other P450 genes, including CYP6 subfamily that is known as one of the main factors in imidacloprid resistance in *N. lugens* (CYP6ER1), *B. tabaci* (CYP6CM1) and *M. persicae* (CYP6CY3) (Puinean *et al.* 2010; Karunker *et al.* 2008; Bass *et al.* 2011), exhibited no apparent difference in their transcription levels between IR and IGS strains. In particular, both IR and IGS strain showed almost the same level of transcription in the 6a13-like and 6a14-like P450 genes, which are highly homologous to *M. persicae* CYP6CY3 that functions as a main detoxification factor in imidacloprid resistance

(Puinean *et al.* 2010; Bass *et al.* 2013). These findings suggest that P450 is not likely involved in imidacloprid resistance in IR strain, as did the synergist test results. To confirm the transcriptome analysis, five main CYP6 subfamily genes were checked in IR and IGS strains using qrtPCR (Fig. 1). However, the result was same. In case of the esterase family, FE4-like (LOC100163252) genes were mainly expressed in both strains and their transcription levels were almost alike. Although Wang *et al.* (2002) reported that esterase could be involved in the low levels of imidacloprid resistance and esterase can function as non-specific sequestration protein against imidacloprid (Philippou *et al.* 2010), our results suggest that esterase was not mainly associated with imidacloprid resistance in IR strain (Fig. 2). The esterase isozyme banding patterns and their intensities were almost identical between the S and IR strain in all tested model substrates (Figure 2). Only we confirmed the differences between S and MR strain from *M. persicae* as previously reported (Kim *et al.* 2014). The transcription levels of the GST and ABC transporter gene families were almost identical between the two strains. The UGT was reported to be an important detoxification enzyme, functioning as an insecticide resistance factor (Ahn *et al.* 2012). But UGT genes were transcribed in both strains at almost the same level. Taken together with synergistic bioassay results, detoxification factors are not likely involved in imidacloprid resistance in IR strain. Proteome profiles based on 2DE were also compared between the two strains (Fig. 3). 706 and 751 protein spots were detected in IGS and IR, respectively. Even though there was some replication variation, overall protein expression patterns were almost identical and no protein spots with differential expression level were detected. This finding confirms the whole transcriptome profiling results showing that the metabolic resistance mechanisms mediated by up or down-regulation of detoxification proteins is not responsible for imidacloprid resistance in IR strain. It further suggests that the target site insensitivity factor caused by the alteration of nAChR may be the major factor conferring imidacloprid resistance in IR strain of *A. gossypii*.

Two Mutations in nAChR subunit genes and down regulation putatively associated with imidacloprid resistance

Seven nAChR subunit genes (alpha 1~5, 7 and beta 1) were retrieved from the GS-FLX database of *A. gossypii*. The partial beta 1 subunit variant fragment was adventitiously amplified with that of original fragment in CA-GPA_b1-F, R primer sets (Fig. 4A and B). After qrtPCR, approximate transcript levels were compared via gel electrophoresis (Fig. 4B). Even though a smaller amount of transcript was expressed than that of original, the beta 1 subunit variant was expressed in all three strains. The genomic DNA sequences of original beta 1 subunit and its variant remains to be elucidated. Down regulation was detected in IR strain (Fig. 4C). Cross-comparison of all *A. gossypii* nAChR subunit gene sequences among three strains revealed that no non-silent mutations were found in alpha 1~5 and 7 subunit genes from IR strain. Non-silent mutations were detected in only beta 1 subunit (Fig. 4D). The R81T mutation was previously reported to be responsible for reduced sensitivity of nAChR to imidacloprid, thereby conferring resistance in *M. persicae*. Considering that the amino acid sequence identity of beta subunit *between M. persicae* and *A. gossypii* is extremely high (99.2%), the function of R81T mutation as a resistance factor is most likely conserved between these two aphid species.

Novel transcript variant of beta 1 subunit (KJ000006) was observed in all the three *A. gossypii* strains examined. The beta 1 subunit variant showed a 141-bp deletion in the N terminal region (Fig. 5A and B). Nevertheless, since the deletion in the N-terminus did not impair the conserved Loop D domain and all other critical domains were still retained, the truncated beta 1 variant is likely functional (Fig. 5B). We hypothesized that partial sequence of beta 1 subunit was truncated after a duplication event. Based on 3D modeling results, the R81T mutation could contribute to insensitive against imidacloprid (Fig. 5C and D). Interestingly, an additional point mutation, L80S (leucine to serine substitution, T to C), was detected in the beta 1 subunit variant and it was only found in IR strain but not in either IGS or S strain (Fig. 4D). Although the transcription level of the beta 1 subunit variant was significantly less than that of main transcript (Fig. 4C), the L80S mutation appears to function as an additional resistance factor in that its location is right next to the R81T mutation site within the loop D, which is crucial for ligand binding, and the leucine-to-serine substitution, which results in the conversion of polarity at the site, likely causes a dramatic functional alteration of beta subunit (Fig.5). However, more detailed investigation would be

required to confirm the functional role of this mutation in imidacloprid resistance. Since these two mutations were not found in IGS strain that had been generated from IR strain by being maintained without imidacloprid selection for about a year, they appear to be unstable in the absence of imidacloprid selection pressure. These two mutations are strongly linked to imidacloprid resistance phenotype, and could be utilized as molecular markers for the detection of imidacloprid resistance in field populations of *A. gossypii*.

Conclusion

A. gossypii has its own imidacloprid resistance mechanism; p450 independent and appears to involve two point mutations with down regulation of nAChR beta 1 subunit.

Acknowledgement

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Session 5

Biology and non-chemical methods of management of diamondback moth and other crucifer pests

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Biological Control of Diamondback Moth in A Climate of Change

M.J. Furlong, M.P. Zalucki[1], A. Shabbir[2] and D.C. Adamson[3]

School of Biological Sciences, The University of Queensland, Brisbane, Australia, 4072. E-mail: m.furlong@uq.edu.au
[1]School of Biological Sciences, The University of Queensland, Brisbane, Australia, 4072. E-mail: m.zalucki@uq.edu.au
[2]University of The Punjab,Quaid-E-Azam Campus, Lahore, Pakistan, 54590. E-mail:asad.iags@pu.edu.pk
[3]School of Biological Sciences, The University of Queensland, Brisbane, Australia, 4072. E-mail: d.adamson@uq.edu.au

Abstract

We have developed a CLIMEX model that describes the potential worldwide distribution of the diamondback moth (DBM) and also identifies regions where seasonal outbreaks of the pest could occur where it cannot persist year-round. IPM programs have been developed but the pest is still estimated to cost the world economy US$ 4-5 billion per annum. In parts of Southeast Asia, India, China, the Philippines, New Zealand and Australia biological control programs have established the Palearctic endo-larval parasitoid *Diadegma semiclausum* Hellén (Hymenoptera: Ichneumonidae) and it can make a significant contribution to IPM strategies if insecticide use is managed appropriately. Here we outline a CLIMEX model for *D. semiclausum* that describes its spatial and temporal distributions in areas where it has become established. We combine the CLIMEX models to investigate the possible impact of predicted climate change on the interactions between DBM and *D. semiclausum* at selected sites where the introduced parasitoid has become an important natural enemy of DBM. We then examine in detail the changes in growth indices for DBM and *D. semiclausum* that are predicted to occur across Australia as a consequence of climate change by 2070 and show that the host-parasitoid relationship is likely to be severely disrupted in some key vegetable growing regions. Finally we use measured levels of DBM population suppression by *D. semiclausum* and the costs of insecticidal control in the absence of the parasitoid to estimate the economic value of *D. semiclausum* in the region and the likely costs of predicted climate change for DBM management.

Keywords: CLIMEX, *Diadegma semiclausum*, economic value of biological control, climate change

Approaches to diamondback moth (DBM) management that rely on insecticides as the principal control tactic inevitably result in failure (Furlong et al. 2013) and integrated strategies for the sustainable management of the pest have been central to research for decades (Talekar and Shelton 1993; Grzywacz et al. 2010). Fundamental to all of these programs are the conservation of natural enemies, frequent pest and natural enemy sampling and threshold-directed interventions with selective insecticides, particularly Bt (Sivapragasam 2004; Furlong et al. 2008; Shepard et al. 2009). The benefits of IPM adoption can include greater profits and yields, reduced input costs and reduced exposure of farmworkers and the environment to hazardous broad-spectrum insecticides (Sivapragasam 2004; Furlong et al. 2008).

The solitary endo-larval DBM parasitoid *Diadegma semiclausum* (Hellén) (Hymenoptera: Ichneumonidae) has been widely introduced as a classical biological control agent of DBM and in much of East, South and Southeast Asia, Australia and New Zealand it is a key component of the IPM programs that have been developed. The populations of the parasitoid that have been established can all be ultimately traced back to the north of England in the United Kingdom where *D. semiclausum* and *D. fenestrale* (Holmgren) were collected from the field to initiate a mass rearing program for shipment to New Zealand in 1936 (Hardy 1938). *Diadegma semiclausum* and *D. fenestrale* can interbreed and Hardy (1938) noted that hybrid progeny exhibit morphological characteristics intermediate between the two species. However, *D. fenestrale*, for which *Cnephasia stephensiana* (Doubleday) (Lepidoptera: Tortricidae) is probably the main host (Fitton and Walker 1992), died out rapidly in culture and it is not clear what impact any hybrids between the two species had on the gene pool of the population that established in New Zealand. Similarly, the effects of hybridization between the introduced material and the native species *Diadegma novaezealandiae* (Azidah et al. 2000) have not been investigated. These issues notwithstanding, material subsequently collected from New Zealand has been directly introduced into Indonesia (Vos 1953), Australia (Wilson 1960), and Malaysia (Ooi 1992) where populations confirmed as *D. semiclausum* (Azidah et al. 2000) have established. In the 1980s the parasitoid was imported from Indonesia and established in Taiwan (Talekar et al. 1992), providing material for subsequent successful

introductions into the Philippines (Ventura 1997), India (Chandramohan 1994), Laos, Vietnam, China (Talekar 2004), and Kenya (Löhr et al. 2008). In 2005, *D. semiclausum* from Malaysia successfully established in the highland regions of Thailand (Upanisakorn et al. 2011). Hence, the provenance of the *D. semiclausum* populations that have established throughout South, Southeast and East Asia, Australasia, and parts of Africa is clear and well documented; all ultimately originate, via New Zealand, from the collections in the United Kingdom (Hardy 1938). As previously suggested (Fitton and Walker 1992), comparative studies between *D. semiclausum* material collected from New Zealand, the United Kingdom, continental Europe and the regions in which classical biological control programs have established the parasitoid would be instructive. Such studies could shed light on the genetic diversity of the various populations that are now established and would offer the possibility of identifying "biotypes" with improved characteristics for biological control. The recent report of a *D. semiclausum* population from Syria that might exhibit greater tolerance to higher temperatures than those typically endured by the introduced *D. semiclausum* populations studied to date (Kadirvel et al. 2011) make this an exciting proposition.

Studies on the effects of temperature on *D. semiclausum* populations introduced for biological control have been limited, but those that have been conducted indicate that the parasitoid does not perform well at temperatures 25ºC (e. g. Talekar and Yang 1991; Dosdall et al. 2012). In the tropics the introduced populations persist in apparent isolation in the highland regions into which they were introduced while in the subtropics populations are typically highly seasonal (e.g., Furlong et al. 2004a). Here we describe a CLIMEX model for *D. semiclausum* that is based on both published data and previously unpublished data from investigations into the thermal requirements of a population of *D. semiclausum* collected from southeast Queensland, Australia. We link the model to a previously published CLIMEX model for DBM (Zalucki and Furlong 2011). We then use the models to investigate the current interactions between the pest and its parasitoid and consider the possible impact of predicted climate change on the relationship in selected regions where introduced *D. semiclausum* is an important component of IPM programs.

Materials and Methods

Model description

We have previously described a CLIMEX model for DBM (Zalucki and Furlong 2008, 2011; Li et al. 2012). Here we summarize a CLIMEX model for *D. semiclausum* (Table 1) that was developed using a very similar approach. Most of the temperature related parameters for the *D. semiclausum* model were taken from Dosdall et al. (2012), while others were obtained from previously unpublished data. Unknown or poorly measured values were estimated by an iterative procedure in which the predicted distribution was compared with actual distributions and parameter values adjusted accordingly (see Zalucki and Furlong 2011).

Investigating the impact of predicted climate change

The possible impact of predicted climate change on the spatial and temporal distributions of DBM and *D. semiclausum* was investigated by running the CLIMEX models using the "CliMond" set of interpolated climate surfaces (30' resolution) for 2070 (Kriticos et al. 2012). The climate change surfaces provided by CliMond have been generated by integrating selected global climate models with plausible future greenhouse gas emission scenarios given current trends (Kriticos et al. 2012); the climate change surfaces can be downloaded from http://www.climond.org free of charge.

Estimating the economic impact of disruption of the DBM-D. **semiclausum** *relationship in southeast Queensland, Australia*

Previous research in southeast Queensland shows that an IPM program based on the strategic application of Bt can reduce the number of insecticide applications from an average of 8 crop^{-1} in conventionally managed crops to an average of 4 crop^{-1} in IPM managed crops (Furlong et al. 2004a). In IPM managed crops endemic natural enemies have a significantly greater impact on pest DBM populations than in conventionally managed crops (Furlong *et al.* 2004a, 2004b). We used the cost of insecticides in the different management scenarios to determine the

economic "value" of natural enemies by subtracting the average cost of Bt applications made to a crop in the IPM strategy from the average cost of insecticide applications made to a crop in the conventional strategy; see Furlong *et al.* (2004a) for details of insecticides used. We used data from detailed life-tables for DBM in *Brassica* crops in southeastern Queensland in 2011-2012 (Murtiningsih 2015) to estimate the impact of *D. semiclausum* on DBM populations relative to that of other natural enemies in order to estimate the economic "value" of *D. semiclausum.*

Results and Discussion

Investigating the impact of predicted climate change

Based on the model parameters (Table 1), the predicted worldwide distribution for DBM, plotted as the eco-climatic index (EI) (Figure 1a), is consistent with its known worldwide distribution (see Zalucki and Furlong 2011). Similarly, the model predictions for regions suitable for the persistence of *D. semiclausum* (Figure 1c) are consistent with the regions where classical biological control programs have been successful, e.g. highlands of Kenya, highlands of southern India, highland regions in Southeast Asia, Taiwan, southern and eastern China, highland regions of Papua New Guinea, eastern and southern Australia and New Zealand (Figure 1c). Note that *D. semiclausum* is not established in any parts of the Americas, despite climatic suitability in large areas of both continents (Figure 1c).

When driven by the CliMond climate surfaces, the CLIMEX model predicts that the suitability of much of the Earth's terrestrial surface for DBM persistence will change greatly by 2070 (cf. Figures 1a and 1c).

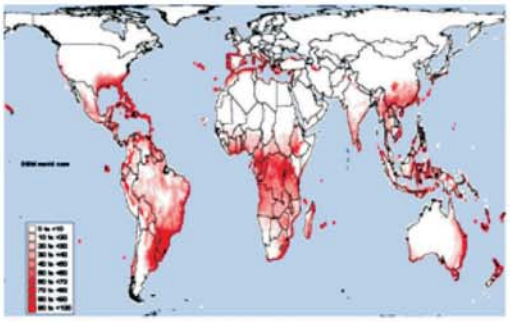
Table 1

Climex parameter values used to generate (DBM) and D. semiclausum *spatial and temporal distributions*

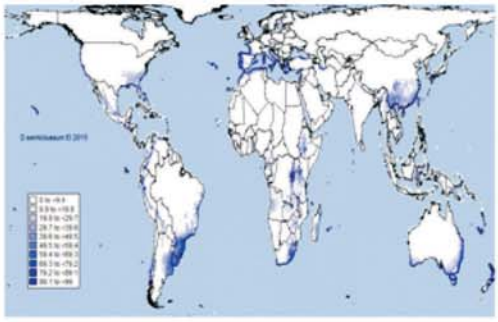
			Value	
Limiting process	**Parameter**	**Definition**	**DBM[1]**	***D. semiclausum***
Temperature	DV0	Lower temp threshold (°C)	7	5.9
	DV1	Lower optimal temp (°C)	14	14
	DV2	Upper optimal temp (°C)	28	23
	DV3	Upper temp threshold (°C)	38	30
Moisture	SM0	Lower soil moisture threshold (%)	0.05	0.05
	SM1	Lower optimal soil moisture (%)	0.5	1
	SM2	Upper optimal soil moisture (%)	1.25	1.5
	SM3	Upper soil moisture threshold (%)	1.75	2.5
Stresses				
Cold (CS)	TTCS	Temp threshold at which CS accumulates (°C)	4	4
	THCS	Rate at which CS accumulates below TTCS ($°C^{-1}d^{-1}$)	-0.0005	-0.0001
Heat (HS)	TTHS	Temp threshold at which HS accumulates (°C)	38	30
	THHS	Rate at which HS accumulates above TTHS ($°C^{-1}d^{-1}$)	0.001	0.005
Dry (DS)	SMDS	Soil moisture threshold at which DS accumulates (%)	0.05	0.05
	HDS	Rate at which DS accumulates above SMDS (d^{-1})	-0.005	-0.005
Wet (WS)	SMWS	Soil moisture threshold at which WS accumulates (%)	1.75	2.5
	HWS	Rate at which WS accumulates above SMWS (d^{-1})	0.05	0.002
	PDD	Number of Degree days (DD) >DV to complete development	268	

[1]See Zalucki and Furlong (2008, 2011) and Li *et al.* (2012).

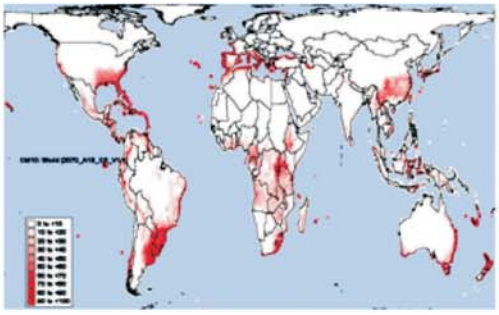
a) DBM: Eco-climatic Index 2015



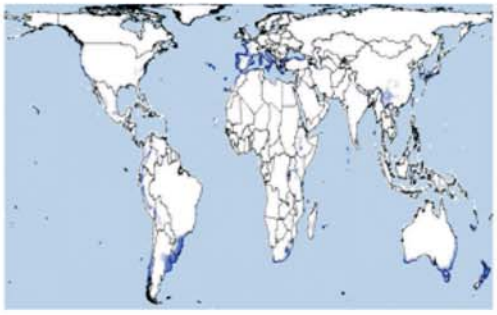
c) D. *semiclausum*: Eco-climate Index 2015



b) DBM: Eco-climate Index 2070

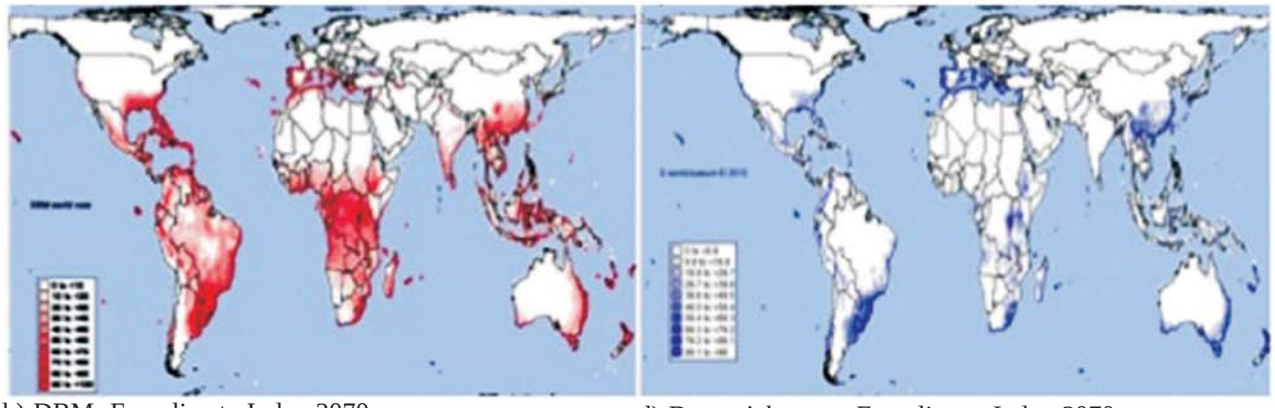


d) D. *semiclausum*: Eco-climate Index 2070



a) DBM: Eco-climatic Index 2015

c) D. *semiclausum*: Eco-climate Index 2015



b) DBM: Eco-climate Index 2070

d) D. *semiclausum*: Eco-climate Index 2070

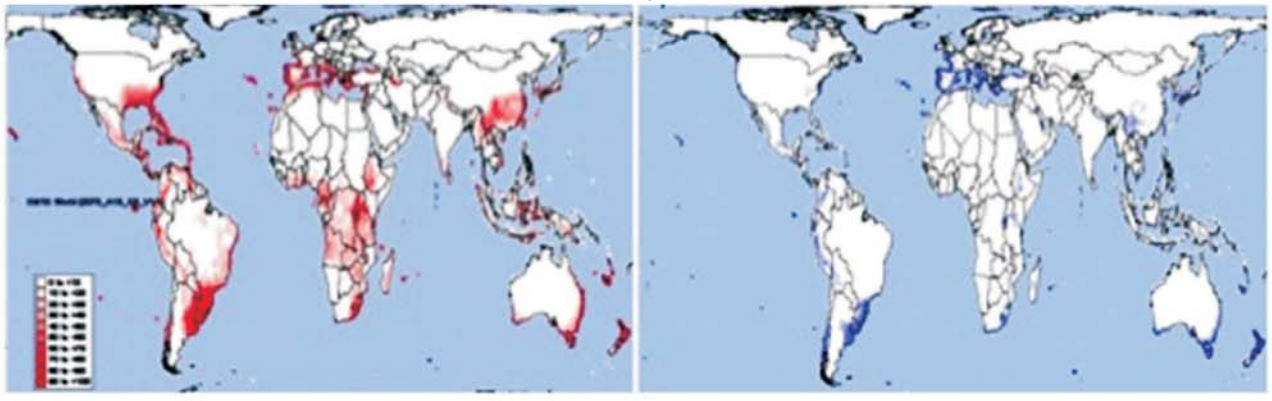
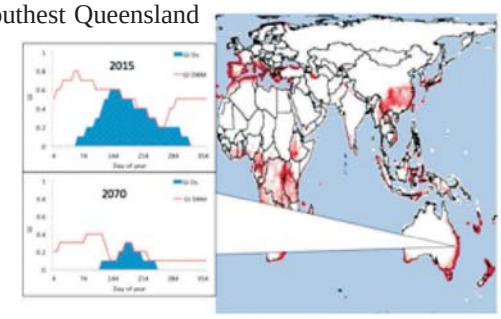


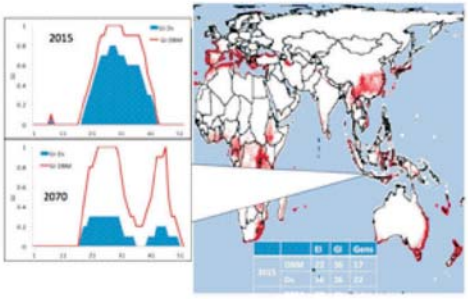
FIGURE 1

Predicted worldwide distributions for DBM and D. semiclausum *based on eco-climatic indices (EI) for a) DBM currently b) DBM in 2070 c)* D. semiclausum *currently and d)* D. semiclausum *in 2070*

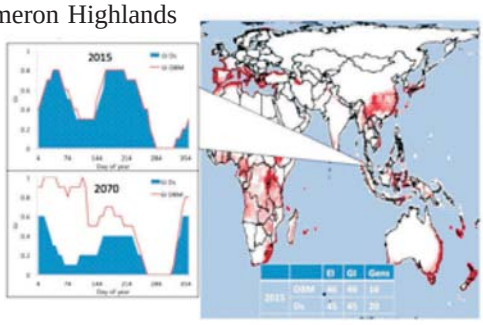
a) Current and predicted (2070) relationships: southest Queensland



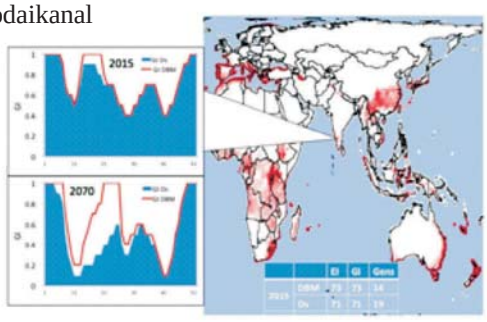
c) Current and predicted (2070) relationships: Lembang, West Java



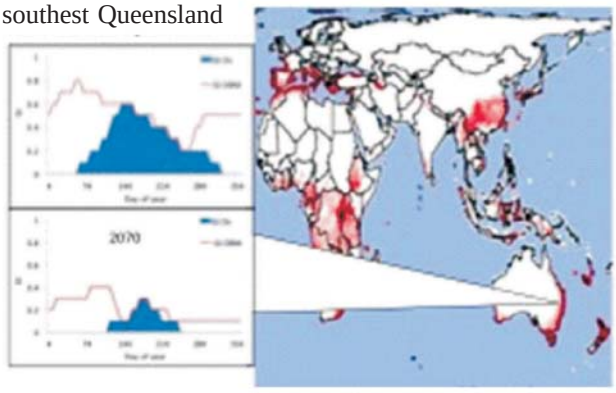
b) Current and predicted (2070) relationships: Cameron Highlands



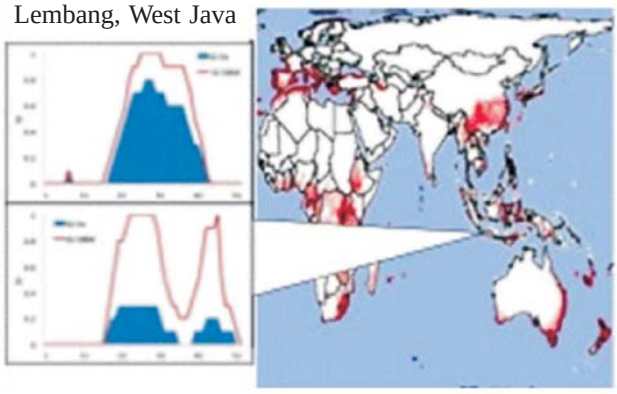
d) Current and predicted (2070) relationships Kodaikanal



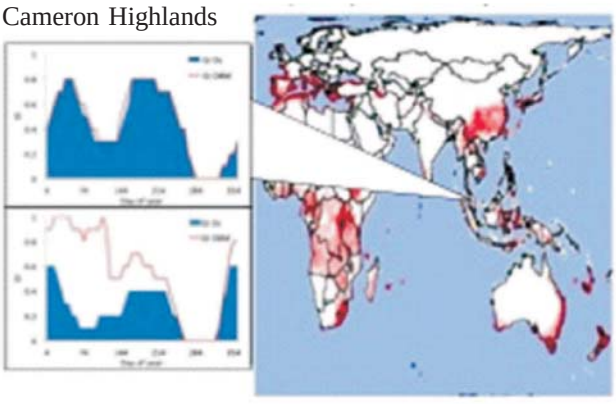
a) Current and predicted (2070) relationships: southest Queensland



c) Current and predicted (2070) relationships: Lembang, West Java



b) Current and predicted (2070) relationships: Cameron Highlands



d) Current and predicted (2070) relationships Kodaikanal

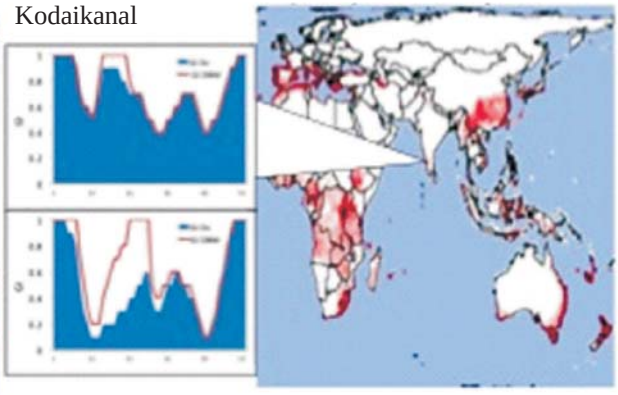


FIGURE 2

Predicted seasonal phenologies for DBM and D. semiclausum*based on growth indices (GI) at four locations where classical biological control programs have established introduced* D. semiclausum *populations. Predicted current and 2070 weekly GIs for DBM and* D. semiclausum *in a) Gatton, southeast Queensland, Australia b) Tanah Rata, Cameron Highlands, Malaysiac) Lembang, West Java, Indonesia and d) Kodaikanal, Tamil Nadu, India*

Much of South, East and Southeast Asia will become markedly less suitable for the pest, as will much of sub-Saharan Africa, northern regions of South America and inland regions eastern of Australia. However, changes are likely to be less pronounced in Europe, where northern expansion into the Iberian Peninsula is predicted, and in North America, where a similar slight northern expansion from the current distribution is to be expected. By 2070, the effect of predicted climate change on *D. semiclausum* populations established for classical biological control is likely to be more pronounced (cf. Figures 1c and 1d); regions in East Africa, China, Southeast Asia and eastern Australia with climate suitable for the persistence of *D. semiclausum* will contract markedly (Figure 1d).

At four locations where *D. semiclausum* has been established for DBM biological control investigation of current predicted weekly growth indices and those predicted by 2070 indicate that the suitability of all locations will change for both the pest and its parasitoid over time (Figure 2). While the conditions at two locations (southeast Queensland, Australia and Kodaikanal, Tamil Nadu, India) are predicted to become less suitable for DBM by 2070, those at the other locations are predicted to change little, or to increase in suitability (Figure 2). Conversely, the suitability of conditions for *D. semiclausum* is predicted to decline, but to varying degrees, at all sites. In southeast Queensland, Australia, it is predicted that conditions will change such that the region will be much less suitable for both DBM and *D. semiclausum* (Figure 2a), however the change is likely to have a greater impact on *D. semiclausum* (current annual GI= 23, 2070 GI= 6 [3.8-fold decline]) than on DBM (current GI= 52, 2070 GI= 20 [2.6-fold decline]) (Figure 2a). In the Cameron Highlands, Malaysia, conditions are predicted to become marginally more suitable for DBM (current GI= 46, 2070 GI= 56 [1.2-fold increase]) but less suitable for *D. semiclausum* (current annual GI= 45, 2070 GI= 27 [1.7-fold decline]) (Figure 2b). Conditions in West Java, Indonesia are also predicted to improve suitability marginally for DBM (current GI= 36, 2070 GI= 41 [1.1-fold increase]) but to decline more substantially for *D. semiclausum* (current annual GI= 26, 2070 GI= 11 [2.4-fold decline]) (Figure 2c). In Kodaikanal, Tamil Nadu, India conditions are predicted to decrease the suitability for DBM slightly (current annual GI= 73, 2070 GI= 64 [1.1-fold decline]) but have a greater impact on the suitability for *D. semiclausum* (current annual GI= 71, 2070 GI= 50 [1.4-fold decline]) (Figure 2d).

In order to understand the possible impact that the predicted effects of climate change might have on the relationship between DBM and *D. semiclausum* across Australia, we compared the current differences between the GIs for DBM and *D. semiclausum* across the continent with the differences predicted for 2070 (Figure 3a). Linear regression of the current differences in GIs versus the predicted differences in GIs in 2070 shows that the slope of the line (0.61) is <1, indicating that at the majority of sites the difference between the GI for DBM and the GI for *D. semiclausum* will increase by 2070 (Figure 3a). Thus overall, we predict that changed conditions expected by 2070 could lead to reduced biological control of DBM by *D. semiclausum*. However, this change is not predicted to be uniform and at some locations the difference between the GI for DBM and the GI for *D. semiclausum* is predicted to contract (Figure 3a). At these locations, we predict that biological control of DBM could improve. We further investigated the impact of the predicted changes by plotting the difference in the GI for DBM and the GI for *D. semiclausum* as a proportion of the GI for DBM across Australia. We plotted these data using current values (Figure 3b) and the values predicted for 2070 (Figure 3c). Using this proportion as a measure for the likely effectiveness of *D. semiclausum* as a biological control agent for DBM (at sites where DBM GI>0) current estimates suggest that *D. semiclausum* is least effective across northern Australia (Figure 3b, dark red shading). Its effectiveness is estimated to be greater in southeast Queensland and then it becomes more effective at increasing southerly latitudes through New South Wales and into Victoria (Figures 3b, lighter red and pink shading). In southern Victoria, southern South Australia and Tasmania the GI for *D. semiclausum* is currently > GI for DBM (Figure 3b, blue shading). By 2070, DBM is likely to be absent from large areas in central Australia (Figure 3c). The effectiveness of *D. semiclausum* is predicted to decrease in southeastern Queensland and through much of northern New South Wales (Figure 3 c, dark red shading) as the difference between GI for DBM and GI for *D. semiclausum* increases in these regions. Similarly, the effectiveness of the parasitoid is predicted to decline through southern regions of New South Wales (Figure 3c, previously pink shading (Figure 3b) changes to red shading) and into Victoria (Figure 3b) where the GI for DBM will increase to become > GI for *D. semiclausum* (Figure 3c, blue shading (Figure 3b) changesto pink shading). The predicted impact of the changed conditions by 2070 on *D. semiclausum* is likely to be least in Tasmania, where its GI is predicted to remain > GI for DBM (Figure 3c).

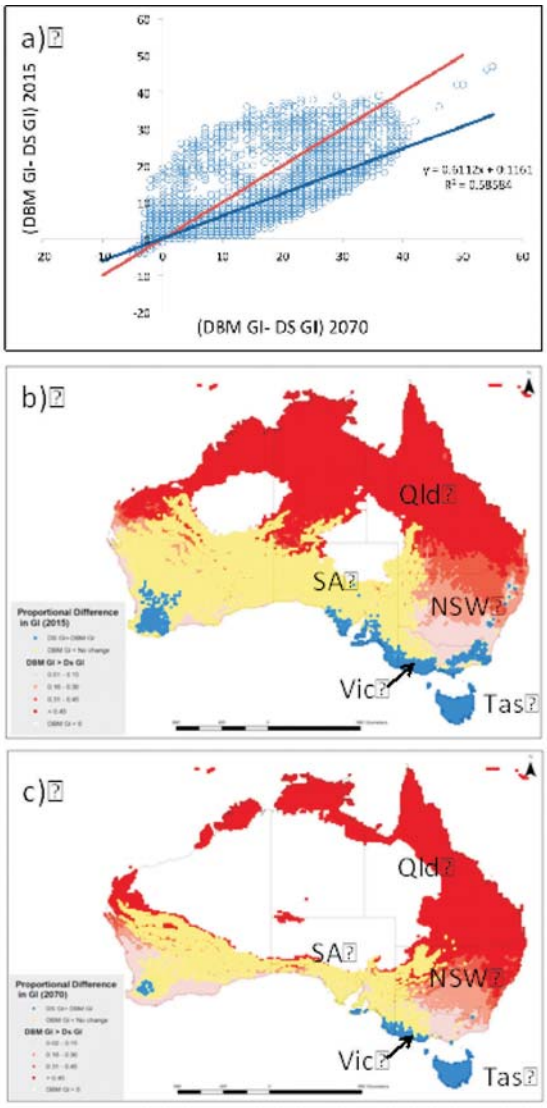


FIGURE 3

Effects of predicted climate change on the relationships between DBM and D. semiclausum *in Australia (Qld= Queensland; NSW= New South Wales; SA= South Australia; Vic= Victoria; Tas= Tasmania) a) Relationship between the current (2015) differences between DBM and* D. semiclausum *growth indices (GIs) and the predicted differences in these indices by 2070; slope <1 indicates that at most locations in Australia the difference between DBM GI and* D. semiclausum *GI will be greater than it is in 2015. b) Differences between DBM GI and* D. semiclausum *GI expressed as a proportion of DBM GI for 2015. c) Predicted differences between DBM GI and* D. semiclausum *GI expressed as a proportion of DBM GI for 2070. In both b) and c) dark red shading show locations where DBM GI>1 and differences are greatest, lighter red and pink shading show locations where differences are less pronounced; blue shading shows locations where* D. semiclausum *GI is > DBM GI and yellow shading shows location where DBM GI=* D. semiclausum *GI*

Estimating the economic impact of disruption of the DBM* - D. semiclausum *relationship in southeast Queensland, Australia

In IPM managed crops the average cost of insecticide input was $252 (±48) ha^{-1} while in conventionally managed crops the average cost of insecticide input was $498 (±48) ha^{-1}; see Furlong et al.(2004a) for details of insecticides used. As we have previously shown that yields under these different management programs are equal and that endemic natural enemies exert significantly greater impact on DBM populations in IPM-managed crops (Furlong et al. 2004a), we estimate that the economic value of natural enemies to a crop to be $498-$252= $246 ha^{-1}. Across series of lifetables for DBM constructed in *Brassica* crops in the Lockyer valley, southeast Queensland in 2011-2012, *D. semiclausum* was responsible, on average, for 35% of all natural enemy mortality inflicted on experimental cohorts of DBM (Murtiningsih 2015). We thus estimated the current economic value of the parasitoid to be 0.35* 246= $86 ha^{-1}. Currently the ratio of the *D. semiclausum* GI to the DBM GI in southeast Queensland is 0.44 but by 2070 it is expected to decline to 0.30, representing a change of 32%. Assuming that this directly affects the impact of *D. semiclausum* on DBM in the region this could reduce the economic value of the parasitoid to $58.5 ha^{-1}representing a cost of $27.5 ha^{-1}.

CONCLUSION

The CLIMEX models presented allow predictions of where DBM and *D. semiclausum* may be found currently and their relative abundance at those locations. They also enable the effects of predicted climate change on these distributions and the relative abundance of the host and its parasitoid across their distributions to be tested. The DBM CLIMEX model was parameterized using a range of laboratory and field collected data and it has been iteratively refined so that the distributions that it predicts fit well with current known distributions of the pest (Zalucki and Furlong 2011). The *D. semiclausum* CLIMEX model was parameterized using laboratory data for introduced *D. semiclausum* that have established in southeastern Queensland, Australia; iterative refining of the *D. semiclausum* CLIMEX model in the manner of the DBM model has not been possible due to a lack of data. Nevertheless, it describes the seasonal phenology of *D. semiclausum* in southeast Queensland

well and its prediction of the current distribution of the parasitoid correlates well with reports of where the parasitoid has established following classical biological control programs. There will always be an element of conjecture when using simulation models to investigate the predicted effects of climate change on the future distribution and abundance of organisms. That notwithstanding, the simulations indicate that if current predictions of climate change are correct then the worldwide distributions and local relative abundances of DBM are likely to change substantially by 2070. Given that the DBM CLIMEX model has been rigorously tested and that DBM populations that have been investigated show no evidence of adaptation to local tropical or temperate climatic conditions (Shirai 2000), we can be reasonably confident in these predictions. The *D. semiclausum* CLIMEX model can undoubtedly be iteratively refined. Nonetheless, it clearly identifies the possibility that if the sub-populations of parasitoids that have been established throughout Asia and parts of Africa from the small founder populations established in New Zealand 80 years ago have similar thermal requirements to the sub-population established in southeast Queensland from New Zealand 50 years ago then predicted climate change could substantially disrupt its associations with DBM in those regions. It has long since been suggested that investigations of the genetics and thermal requirements of the different sub-populations of *D. semiclausum* that have been established should be conducted and that populations from these regions should be compared with material from Europe (Fitton and Walker 1992). The recent report of a population of material identified as *D. semiclausum* from Syria that might exhibit a different thermal profile to *D. semiclausum* derived from the population established in New Zealand (Kadirvel et al. 2011) adds weight to this argument. Such a study would not only provide results that could improve future biological control of DBM but it would also contribute to our understanding of how founder populations change over time and provide valuable information on how genetic diversity might be maintained, or introduced into parasitoid populations established for biological control.

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Field Validation of Integrated Pest Management Package Against Leaf Eating Caterpillars of Cabbage in Bangladesh

S. N. Alam, D. Sarker, R. Srinivasan[1], M.A. Monim[2] And G. M. M. Bari[2]

Entomology Division, Bangladesh Agricultural Research Institute, Gazipur, Bangladesh. E-mail: snalambari@gmail.com

[1]Entomology Unit, AVRDC – The World Vegetable Center, Shanhua, Tainan, Taiwan, Republic of China

E-mail: srini.ramasamy@worldveg.org. [2]Regional Agricultural Research Station, Barishal, Bangladesh

Abstract

Studies were conducted in farmer's fields at Barishal and Jessore, situated in the southern part of Bangladesh during 2012-13 and 2013-14 winter season to validate a bio-rational management package against leaf eating prodenia caterpillar (*Spodoptera litura*) and diamond back moth (DBM) (*Plutella xylostella*). There were two treatments with six replications and the experiment was set following a dispersed RCB Design. The treatments were: I) IPM package: hand picking of infested leaves and caterpillars plus mass trapping with *S. litura* pheromone lures plus spraying of SNPV@ 0.2 g per liter of water at 10 day intervals after visible initial infestations plus application of Bt strain EG 7841 @ of 1g / litre of water at 10 day intervals after visible initial infestations, and II) Farmers' practice: spraying with different chemical insecticides every 3-7 days. The IPM package reduced leaf-eating caterpillars on cabbage during both study years. Pooled data showed a significantly lower head infestation of cabbage in IPM treated plots (3.2 %) compared to farmers' practice (31.7 %). Yields were 28-40 % higher in IPM plots compared to farmer's practice. A higher gross return and gross margin was also recorded from IPM than farmers practice in both years.

***Keywords*:** Farmers field, yield, DBM, *Spodoptera*, cost benefit.

Cabbage, *Brassica oleracea* L. is a popular vegetable in Bangladesh. Cabbage is attacked by a group of pests. Among them leaf eating caterpillar like diamond back moth (DBM), *Plutella xylostella* (L.) (Lepidoptera: Plutellidae), and prodenia caterpillar, *Spodoptera litura* (F.) (Lepidoptera: Noctuidae), are considered as the major pests in Bangladesh (Alam *et al.* 2010). Worldwide diamondback moth has for many years been considered to be the most important pest of cabbages and other brassica crops (Talekar and Shelton, 1993; Shelton, 2004; Furlong *et al.*, 2013), costing up to 4-5 billion US$ per year in damage and control costs (Zalucki *et al.* 2012). Newly hatched DBM caterpillars feed as leaf miners inside the leaf tissue. Older caterpillars feed on all plant parts. In cases of severe infestation, entire leaves can be damaged. Larvae of *Spodoptera* feed on both foliage and cabbage head. Young larvae feed gregariously and skeletonize. As they mature, larvae become solitary and their feeding causes large irregular holes in foliage. They also burrow into the crown or center of the cabbage head. Farmers frequently apply toxic pesticides to protect their crops from the attack of these pests without much success because of the fact that the pesticides hardly reach the target once the caterpillars enter the cabbage heads. Worldwide it has been reported that it is very difficult to control those two pests with synthetic chemical insecticides. Such pesticide use, besides being costly, detrimental to the environment and human health, is degrading the natural resource base by destroying potential predators and parasitoids of pest insects. So, it is important to develop integrated management packages to control those pests.

Materials and Methods

This study was undertaken on farmers' fields in Barishal and Jessore regions, situated in the southern part of Bangladesh during the winter season (October – March) of 2012-13 and 2013-14. At all locations the plot size was around 0.25 ha per treatment replicate. There were six dispersed replicates and the experiment was set in RCB design. There were two treatments: I) an IPM package that consisted of hand picking of infested leaves and caterpillars at weekly interval plus mass trapping with *S. litura* pheromone lures from two weeks of seedling transplanting plus spraying of SNPV@ 0.2 g per liter of water at 10 day intervals after visible initial infestations plus application of Bt strain EG 7841 @ 1g/litre of water at 10 day intervals

after visible initial infestations, and II) Farmers' practice: routine application of different insecticides every 3-7 d. At Jessore region, Voliam flaxi 300SC @ 0.5 ml/litre of water or Dimethoate 40 EC @ 2.5 ml/litre of water or Cypermethrin 10 EC @ 1 ml/liter of water or Quinalphos 25 EC @ 1 ml/ liter of water or Chlorpyriphos 20 EC @ 2 ml/ liter of water etc, were applied twice a week, while at Barishal region those insecticides were applied at 7-10 days interval. A minimum distance of 500 m was maintained between the IPM and Farmer's practice plots.

Numbers of healthy and infested heads were counted and recorded from randomly selected 2 m^2 area of each treatment replicate every 7 days. For each treatment the percent heads infested was calculated from the pooled data of twelve observations during 2012-13 and fourteen observations during 2013-14. Number of DBM and prodenia caterpillars were counted and recorded from ten randomly selected heads at 7 day intervals. For benefit cost analysis, records of the costs incurred for labour, insecticide, bio-pesticide and application costs in each treatment replicate were recorded. The prices of the harvested marketable healthy heads of each treatment were calculated at market price. The result of benefit-cost analysis was expressed in terms of Marginal benefit cost ratio (MBCR). Data were analyzed by using CropStat 7.2.3 software for analysis.

Results and Discussion

At all the locations cabbage head infestation by leaf eating caterpillars in the IPM plots were significantly less than that of farmers' practice. Percent head infestation in the IPM treated plots were 3.8 and 2.6 at Jessore and Barishal, respectively, compared to 36.7 and 26.7 % in the Farmer's practice plots. Lower head infestation in the IPM plots resulted in 1.27 and 1.39 times higher cabbage yield at Jessore and Barishal region that Farmer's practice fields (yield of cabbage was 58.6 and 54.8 t/ha, respectively at Jessore and Barishal, while that was 45.8 and 39.3 t/ha in the farmer's plots) (Table 1).

Table 1

Effect of different treatments on the management of leaf eating caterpillars (DBM and Spodoptera*) in cabbage as mean % heads infested and mean number of larvae per head (± SE) and their corresponding yield in Jessore and Barishal regions during winter 2012-13 & 2013-14 cropping season*

Treatment	Insect pest infestations			Yield (t/ha)
	Head infestation (%)	No. of DBM larvae/ head	No. of *S. litura* larvae/ head	
Jessore				
IPM	3.8±0.1 b	0.2±0.1 b	1.2±0.4 b	58.6 b
Farmer's practice	36.7±1.9 a	2.4±0.3 a	4.5±0.7 a	45.8 a
Barishal				
IPM	2.6±0.2 b	0.3±0.2 b	0.9±0.3 b	54.8 b
Farmer's practice	26.7±0.8 a	2.6±0.5 a	3.8±0.5 a	39.3 a

± SE, Means of 12-14 observations and 6 replications; Means followed by the same letter in a column did not differ significantly by paired t-test ($p<0.01$)

The benefit-cost ratio (BCR) based on the expenses incurred for total crop production cost and value of crops obtained from the IPM and Farmer's practice plots (Table 2) showed that at all the locations the highest benefit-cost ratios were calculated from the IPM trial plots (3.69 in IPM and 2.11 in Farmer's practice plots). The lower BCR in farmers practice plots could be attributed to the comparatively high prevalence of leaf eating caterpillars (even after repeated insecticide applications) leading to lower yield and the high cost of chemical insecticides.

Table 2

Overall benefit cost analysis of IPM and Farmer's practice in cabbage at Jessore and Barishal region combined

	Marketable yield (t/ha)	Gross return (Tk/ha)	Cost of production (Tk/ha)	Net return (Tk/ha)	B:C Ratio
IPM	56.7	554000	118000	436000	3.69
Farmer's practice	42.5	437000	140500	296500	2.11

Conclusions

The IPM package resulted in reduced leaf eating caterpillar damage on cabbage in the studied areas compared to standard local Farmers practice. The lower infestation status was reflected in the higher yield of healthy cabbage as well as higher gross return and gross margin. The developed IPM package can be utilized by the farmers for the sustainable management of leaf eating caterpillars in cabbage.

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Effect of Cabbage Plant Age on Parasitism of *Plutella xylostella* (L.) by *Cotesia vestalis* (Haliday): Implications for IPM

S. Amalina, Y. Mohd Hanifah[1], M.N. Mohamad Roff[2] and A.B. Idris[3]

School of Environmental and Natural Resource Sciences, Faculty Science and Technology, Universiti Kebangsaan Malaysia, 43600 Bangi, Selangor, Malaysia. E-mail: amalina767@gmail.com

[1]Pest and Disease Management Programme, Horticulture Research Centre, Malaysian Agricultural Research and Development Institute (MARDI). E-mail: hanifah58@yahoo.com

[2]Pest and Disease Management Programme, Horticulture Research Centre, Malaysian Agricultural Research And Development Institute (MARDI). E-mail: roff@mardi.gov.my

[3]School of Environmental and Natural Resource Sciences, Faculty Science and Technology, Universiti Kebangsaan Malaysia, 43600 Bangi, Selangor, Malaysia. E-mail: idrisgh@ukm.edu.my or idrisyatie@yahoo.com.myAbstract

Abstract

Cotesia vestalis (Hymenoptera: Braconidae) is one of the important larval parasitoids of the major insect pest of cruciferous crops, the diamondback moth (DBM), *Plutella xylostella* (Lepidoptera: Plutellidae) in many parts of the world, including Malaysia. We assessed the parasitism of DBM larvae by *C. vestalis* on three different ages of cabbage plant, *Brassica oleracea* L. var. *capitata* cv. K-K Cross under glasshouse conditions. Second instar DBM larvae infesting 4, 8 and 12-week-old cabbage plants were exposed to *C. vestalis* for one, three and six hours. There was a significant difference ($P < 0.05$) in the percentage parasitism of DBM larvae on cabbage of different plant ages in all exposure durations. Parasitism was significantly higher ($P < 0.05$) on 4-week-old plants after one hour of exposure than on the other two groups of older plants. For three hours of exposure, parasitism differed significantly ($P < 0.05$) only between 4 and 12-week-old plants. More ($P < 0.05$) DBM larvae on both 4 and 8-week-old plants than on 12-week-old plants were parasitized in six hours of exposure. Young cabbage plants infested by DBM larvae were more attractive to foraging *C. vestalis* and this information should be considered when utilising this biological control agent in integrated pest management of DBM. The application of biopesticides is likely to be a better control option for DBM during young crop stages as it will be less disruptive to *C. vestalis.*

Keywords: Biological control, glasshouse conditions, exposure duration, host searching

Diamondback moth (DBM), *Plutella xylostella* (L.) (Lepidoptera: Plutellidae) is one of the most damaging insect pests of cruciferous crops around the world (Furlong *et al.* 2013). In Malaysia, DBM was first recorded in 1925 (Ooi 1986). Its' larvae attack cabbage plants from seedlings to harvest stage, reducing yield quality and quantity (Mahr *et al.* 1993). Excessive use of insecticides in conventional control practices has decimated natural enemies (Idris and Grafius 1993a) and led to resistance to almost all insecticide products including *Bacillus thuringiensis* (Bt) (Idris and Grafius 1993b; Sarfraz and Keddie 2005). In order to tackle the problem, efforts have been made to include natural enemies in managing this pest (Furlong *et al.* 2013; Talekar and Shelton 2003).

Cotesia vestalis (Haliday) (Hymenoptera: Braconidae) is one of the key natural enemies of DBM in Malaysia, which was discovered in 1975 (Lim and Ko 1975; Shaw 2003). This endoparasitoid is known as one of the major biological control agents in Malaysia IPM program for DBM (Sivapragasam *et al.* 2011). During host searching, this parasitoid uses chemical cues such as monoterpenes and glucosinolates produced by wounds made by its herbivorous host (Potting *et al.* 1999; Vuorinen *et al.* 2004). Glucosinolate content varies among different host plants of DBM (Hopkins *et al.* 1998; Ulmer and Dosdall 2006) and changes over time according to plant developmental stage (Hopkins *et al.* 1998). Therefore, the objective of this study was to investigate the effect of plant age of cabbage on parasitism of DBM by its parasitoid, *C. vestalis*. The results may provide a useful understanding of the host plant-parasitoid interaction for effective control of the pest in IPM program for DBM, particularly in cabbage farming systems.

Materials and Methods

Plants

Cabbage plants (*Brassica oleracea* L. var. *capitata* cv. K-K Cross) were planted using a suspended pot non-circulating hydroponic method (Kratky 2004) in Malaysian Agricultural Research and

Development Institute (MARDI), Serdang, Selangor. Cabbage seeds were individually sown on a piece of wet sponge (2 cm × 2 cm) as a growing medium. After a week, the seedlings were transferred to net pots and the roots were immersed in nutrient solution. The solution was topped up throughout the plant growth. The plants were grown under glasshouse conditions at temperature 29 ± 4 °C, 78 ± 5 % relative humidity (RH) and a photoperiod of 12:12 (L:D) h. Three ages of plant were used in this study; 4-, 8- and 12-week-old plants.

Insects

The original cultures of *C. vestalis* and its host, DBM were initially collected from brassica vegetable farm in Kuala Lumpur. Both colonies were raised on *Brassica campestris* L. under laboratory conditions in MARDI at temperature 25 ± 2 °C, 63 ± 5 % RH and a 12:12 (L:D) h photoperiod. Adult insects of *C. vestalis* and DBM were fed with 10 % (v:v) honey solution. The two species had been reared over several generations before the test. Experimental *C. vestalis* were obtained by collecting cocoons from the stock culture and placed in mesh cage measuring 35 cm × 20 cm × 20 cm for adult emergence and mating. These adults were maintained without any contact with plant material or host larvae. Only 2 to 5-day-old mated naïve females were used in the test (Girling *et al.* 2011).

Effect of plant age on parasitism

The experiment was conducted in screen cage (1.5 m x 2.4 m x 1.5 m) under glasshouse conditions at 29 ± 4 °C and 78 ± 5 % RH. Three ages of cabbage plants were arranged in a completely randomized design (CRD) with 5 replicates. Each plant was infested with 30 second instar DBM larvae for 24 h prior to the experiment. Fifteen randomly selected naïve females of *C. vestalis* were released into the cage for three different exposure durations; 1 h, 3 h and 6 h. All exposed DBM larvae were collected and dissected to determine parasitism.

Data analysis

Parasitism data was transformed using arcsine square root transformation and data normality was confirmed with Anderson-Darling test. Data was then analyzed using two-way analysis of variance (ANOVA) and followed by Tukey's method when the analysis was significant ($P < 0.05$). All analyses were performed using Minitab Statistical Package Version 16 (Minitab, Inc., USA).

Results and Discussion

The mean percentage parasitism of DBM larvae was significantly different among cabbage plants of different ages ($F = 34.95$, $df = 2, 36$, $P < 0.05$) and among exposure durations of *C. vestalis* ($F = 24.90$, $df = 2, 36$, $P < 0.05$). However, there was no significant interaction effects ($F = 1.43$, $df = 4, 36$, $P > 0.05$) between plant age and exposure duration.

The mean percentage parasitism of DBM larvae by *C. vestalis* on 4-week-old plants was significantly higher ($P < 0.05$) than on 12-week-old plants in all exposure durations (Fig. 1). The longer the exposure durations, the more DBM larvae were parasitized by the parasitoid on 8-week-old plants compared to 12-week-old plants suggesting that host searching female *C. vestalis* are initially more attracted to younger plants that are infested by its host.

The lower responsiveness of *C. vestalis* to older cabbage plants in this study might be due to low content of glucosinolate in these maturing plants (Hopkins *et al.* 1998; Lambdon *et al.* 2003; Wallace and Eigenbrode 2002). This secondary plant product plays an important role in producing allelochemicals that attract parasitoids (Ahuja *et al.* 2010; Dicke and van Poecke 2002). This may explain the preference of *C. vestalis* to parasitize more DBM larvae on younger cabbage plants. However, the longer the exposure durations, more DBM larvae feeding on 8-week-old plants were parasitized. This may be because of the learning experience gained by the parasitoid. This was possibly explained by experiments by Karimzadeh (2005) showing that the preference of *C. vestalis* to parasitize DBM larvae feeding on a susceptible host plant compared to a partially-resistant host plant decreased and gradually disappeared when the exposure time was increased.

Talekar and Yang (1993) found that total parasitism by *C. vestalis* was greatest after cabbage transplanting and declined as the plant aged. Similar trend of parasitism was noticed by Sow *et al.* (2013) who reported significant relationship between the age of cabbage and total parasitism by natural enemies of DBM. However, parasitism by *Oomyzus sokolowksii* Kurdjumov, the eulophid that attacks older stage of

DBM larvae was not affected by host-plant age (Talekar 1997). The different effects of ages of host plants could be explained by various complex combinations of abiotic factors that may influence the parasitoid behavior under field conditions (Fournier *et al*. 2005; Heimpel and Casas 2008; Idris and Grafius 1998).

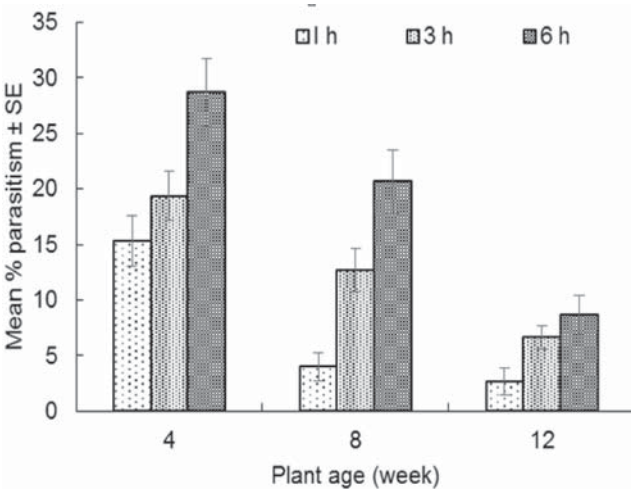

FIGURE 1

Parasitism of DBM larvae by C. vestalis on three plant ages of cabbage in three exposure durations

CONCLUSION

Cotesia vestalis parasitized more DBM larvae feeding on younger cabbage plants. The use of biopesticides should be encouraged to control the pest during young age of cabbage plants when implementing the IPM for DBM to avoid killing this natural enemy.

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Insecticidal Activity of Twelve Common Amazon Ecuadorian Plants Against *Plutella xylostella* (Lepidoptera : Plutellidae) : Laboratory Results

Cerda, H., Ledezma-Carrizalez, A.C.[1], Andino, M.[2], Chiurato, M.A.[3] and Carpio, C.[4]

Escuela Superior Politécnica De Chimborazo (ESPOCH) Riobamba, Chimborazo, Ecuador
E-mail: hugocerda04@gmail.com
1Escuela Superior Politécnica De Chimborazo (ESPOCH) Riobamba, Chimborazo, Ecuador
E-mail: cledezma974@gmail.com
[2]Universidad Estatal Amazónica (UEA), Puyo, Pastaza, Ecuador
E-mail: mandinoec@yahoo.com
[3]Universidad Estatal Amazónica (UEA), Puyo, Pastaza, Ecuador
E-mail: matteo.chiurato@gmail.com
[4]Escuela Superior Politécnica De Chimborazo (ESPOCH) Riobamba, Chimborazo, Ecuador
E-mail: fccarpio@yahoo.com

Abstract

Botanical insecticides are increasingly attracting research attention as an effective component for managing pests that have already developed resistance to conventional insecticides. Here, we report the insecticidal potential of twelve common Amazon Ecuadorian plants [*Lonchocarpus nicou* (Barbasco), *Urtica dioica* (Urticaria), *Xanthosoma undipes* (Camacho), *Brugmansia* sp. (Guanto), *Xanthosoma purpuratum* (Shungu panga), *Ocotea quixos* (Isupurunga), *Piper aduncum* (Matico), *Clibadium* sp. (Kakllambi), *Thesioides baccharis* (Ayaguachi), *Dieffenbachia costata* (Lalu), *Nicotiana tabacum* (Tabacco) and *Ficus insipida* Willd. (Oje)] against *Plutella xylostella*. Water extracts of *W. solanacea*, *D. costata*, *L. nicou* and *N. tabacum*s howed statistically higher *P. xylostella* larval mortality compared with water control.Water extracts of *Clibadium* sp., *X. purpuratum* and *W. solanacea* showed oviposition deterrence against *P. xylostella*. Based on the results presented in this paper, simply-prepared extracts from readily-available Amazonian Ecuadorian plants can be potentially used as botanical insecticides against *P. xylostella*.

***Keywords*:** *Plutella xylostella*, botanical insecticide, Ecuador, Amazon biodiversity

The diamondback moth (DBM), *Plutella xylostella* (L.) (Lepidoptera: Plutellidae), is the most important pest of crucifers worldwide (Shelton *et al.* 1993; Furlong *et al.* 2013). The estimated average control cost for DBM is greater than 4-5 billion dollars per year (Zalucki *et al.* 2012). Intensive insecticide use continues to be the primary method of control against *P. xylostella* (Grzywacz *et al.* 2010; Furlong *et al.* 2013), which represents a threat to the environment and human health (Devine *et al.*2008). Due to biological and behavioral traits of DBM (*e.g.*, great ability to disperse, high fecundity and short life cycle) and conditions offered by the environmental sites (*e.g.*, availability of various crucifers in neighbor fields and throughout the year), *P. xylostella* has developed a formidable resistance to chemical insecticides (Zalucki *et al.* 2012; Furlong *et al.* 2013). Therefore, research on alternative methods of control of this pest is essential. Botanical insecticide could play an important role as an alternative control method of this pest (Charleston *et al.* 2006; Boica Junior *et al*. 2013). Unlike their synthetic counterparts, botanical pesticides are made of many chemicals with complex and additive action mechanism such as lignans, neolignans, alkaloids, chalcones, kawapirones, flavones, essential oils, and amides (Miresmailli and Isman 2014). Worldwide four major types of botanical insecticides are being widely used for insect control, pyrethrum, rotenone, neem, and essential oils along with others in limited use (Isman 2006). Here, we report common Amazon Ecuadorian plants which potentially could have botanical insecticide properties against *P. xylostella*.

Materials and Methods

Sample collection

We collected leaves and roots of *Lonchocarpus nicou* (Barbasco), leaves of *Urtica dioica* (Urticaria), *Xanthosoma undipes* (Camacho), *Brugmansia* sp. (Guanto), *Xanthosoma purpuratum* (Shungu panga), *Ocotea quixos* (Isupurunga), *Piper aduncum* (Matico) and *Baccharis thesioides* (Ayaguachi), leaves and fruits of *Clibadium* sp. (Kakllambi), stem and leaves of *Dieffenbachia*. *costata* (Lalu), and bark of *Ficus insipida* willd. (Oje) within 2km of Centro de Investigación, Posgrado y Conservación Amazónica (CIPCA) of Universidad Estatal Amazónica (1°14'18.85" S, 77° 53' 4.30" W) Santa Clara, Pastaza, Ecuador in June-August 2014. The collected plants are traditionally used as food, medicine or natural products in the region.

Aqueous extracts

The collected materials from three or more plants of each species were thoroughly mixed, because secondary products chemistry can vary between individuals (Amoabeng *et al.* 2013). The mixed materials were dried at 60°C for 48 h in an oven, milled to fine powder and extracted with distilled water for 48 h to produce a plant aqueous extract.

Insect

Three hundred *Plutella xylostella* pupae were collected from a cabbage field near the city of Ambato (Ecuador) (1° 12' 16.65" S, 78°35'13.89" W) and cultured in cages containing potted plants of white heading Cabbage, *Brassica oleracea* convar. *capitata* L. var Alba OMBRIOS RS 91020 (Agripac CA, Ecuador). The population was maintained at room temperature of 20 to 28°C, 65-80% relative humidity (RH) and at natural photoperiod regime. Larvae or pupae were sometimes placed at 12ºC to reduce the rate of development.

Larvalmortality bioassay

The bioassay was conducted using the leaf dip method as described by Tabashnik *et al.* (1990). Leaf discs (4.8 cm diameter) were cut from middle leaves of six week-old cabbage plants using a sharpened metal hole punch. Each disc was immersed, with gentle agitation, for 10 s in the test solution of the plant aqueous extract. The discs were then drained for a further 10s to allow run-off of surplus solution and dried with the adaxial surface uppermost on corrugated sheets of aluminum foil for 1-2 h at room temperature. Control leaves were dipped in distilled water containing 0.1% Sunlight® detergent solution to give a 1-5% v/v final concentration. Once dry, each leaf disc was placed in an individual Petri dish (5 cm diameter) containing a single, moistened filter paper (Whatman® No. 1, 4.5 cm diameter). Five, 1 to 2 d-old 3rd instar larvae were placed on each leaf using a clean camel hairbrush. The test larvae were selected from lab culture in its third-sixth generation after field collection. Five petri dishes were set up per treatment, including the control. Petri dishes from each treatments group bound in stacks of 4 or 5 and kept at room environmental conditions. Larval mortality was determined after 5 d and the mortality percentage was calculated.

Anti-oviposition effect

Two cabbage leaf discs (4.8 cm diameter) were immersed in the test solution, drained and dried as briefed in the bioassay methodology. Two hours after treatment, the leaf cabbage discs were placed in plastic bottles with 40 *P. xylostella* adults that were 5–7 d old. After 2 d, the leaf discs were removed from the bottles and the number of eggs along the midrib of the leaf disc was counted.

Data analysis

Data of larval mortality was analyzed by Mann-Whitney test with $p < 0.05$ significance level. Data of oviposition deterrencewas analyzed using the t-test. The normality of the data was tested with the Shapiro-Wilk test. The complete statistical analysis was performed using the MINITAB 13.31 License WIN1331.05874 statistical PC program.

Results and Discussion

Table 1 showed that plant water extracts of *W. solanacea*, *Dieffenbachia*. costata, *L. nicou*, and *N. tabacum* resulted in statistically higher *P. xylostella* larval mortality, compared to water control. Table 2 showed that water extract of *Clibadium* sp., *X. purpuratum* and *W. solanacea* was the most effective deterrents against *P. xylostella* oviposition.

L. nicou and *N. tabacum* are already known to have in secticidal properties through rotenoids and nicotinoids, respectively (Lawson *et al.* 2010; Vandenborre *et al.* 2010). Hegazy *et al.* (1992) mentioned that *Dieffenbachia* sp. showed feeding deterrent effects on 2nd- and 4th-instar larvae of *Spodoptera littoralis* leading to mortality. They mentioned thiocyanates, tannins and alkaloids as possible active insecticidal compounds.

X. purpuratum has crystals of calcium oxalate (Finley 1999), which play a defensive role against herbivores (Konno *et al.* 2014). On the contrary in literature, *W. solanacea* did not have any insecticidal activity although Chinchilla-Carmona *et al.* (2014) mention its anti-leishmanial activity.

According to recent studies by Isman (2008 and2014), natural botanical insecticides have shown greater efficacy to control insects pests. These products are structurally very diverse. Unfortunately the mechanisms by which they exert their biological activity are not well known (Isman 2008 and 2014; Macias *et al.* 2008). Hence, additional research must be carried out to know the active compounds and mechanisms of action of the botanical insecticides screened in the current study.

Pest management using locally available botanicals offers producers the opportunity to reduce production costs substantially, because plants often grow wild around farms so that they can be obtained with little effort with zero or minimal cost. However, the selected botanical pesticides must be compatible with natural enemies in order to avoid resurgence of secondary pests. They should also possess low phyto and mammalian toxicities.

TABLE 1

Larval mortality effects of twenty common Amazon Ecuadorian plants against Plutella xylostella under laboratory conditions

Plant species	Mean (± SD) larval mortality (%) in plant extract	Mean (± SD) larval mortality (%) in control	P
Mr. Javier *Lonchocarpus nicou* (Barbasco)	100 ± 0.0	24 ± 16.7	0.01*
Lonchocarpus nicou (Barbasco) with *Urtica dioica* (Ortiga)	24 ± 21.9	24 ± 16.7	0.92
Xanthosoma undipes(Camacho)	32 ± 39.0	24 ± 16.7	0.92
Brugmansia sp. (Guanto)	12 ± 11	12 ± 26.8	0.53
Leaves of *Lonchocarpus nicou* (Barbasco)	12 ± 17.8	12 ± 26.8	0.83
Roots of *Lonchocarpus nicou* (Barbasco) tree	100 ± 0.0	12 ± 26.8	0.01*
Xanthosoma purpuratum (Shungu panga)	28 ± 17.8	12 ± 26.8	0.30
Ocotea quixos (Isupurunga)	32 ± 30.3	16 ± 16.7	0.46
Piper adencum (Matico)	48 ± 33.5	16 ± 16.7	0.12
Leaves of *Momordica* sp. (achonchilla)	44 ± 32.9	20 ± 24.5	0.21
Leaves of *Lycopersicon hirsutum* (Tomatillo)	44 ± 8.9	20 ± 24.5	0.12
Thesioides baccharis (Ayaguachi)	48 ± 39	24 ± 26.1	0.35
Witheringia solanacea (Tsimbio)	100±0.0	28 ± 33.5	0.02*
Leaves of (Lalu) *Dieffenbachia costata*	92.0±17.8	28 ± 33.5	0.02*
Clibadium sp. (Kakllambi)	44 ± 38.5	24 ± 26.1	0.47
Bark of *Ficus insipida* Willd (Oje)	40 ± 24.5	28 ± 33.5	0.47
Stem of *Dieffenbachia costata* (Lalu)	60 ± 28.3	28 ± 33.5	0.17
leaves of *Nicotiana tabacum* (Tabaco)	100 ± 0.0	34 ± 19.4	0.01*

n= 5 replicates. P to Mann-Whitney * P<0.05.
SD: Standard deviation of the mean

TABLE 2

Mean number of P. xylostella *eggs laid on leaves treated withextracts from twenty different Amazonian Ecuadorian aqueous plant versus water control under laboratory conditions*

Variable	N	No. of eggs	SD	P
Clibadium sp. (Kakllambi)	5	34.60	19.87	0.002**
Control	5	90.60	11.24	
Tabacum	5	18.40	19.91	0.43
Control	5	28.80	19.69	
Thesioides baccharis (Ayaguachi)	5	10.60	10.21	0.10
Control	5	39.80	29.10	
Xanthosoma purpuratum (Shungu panga)	5	1.60	0.89	0.007**
Control	5	37.60	15.96	
Witheringia solanacea (Tsimbio)	5	3.60	2.70	0.04*
Control	5	22.00	12.79	

N= 5;p of student t test * p <0.05; ** p <0.01

CONCLUSION

Aqueous extracts of *W. solanacea, D. costata, L. nicou*, *Clibadium* sp. and *X. purpuratum* have potential to be used as new botanical insecticides against *P. xylostella*. However their effectiveness under field conditions, the active compounds and their phyto and mammalian toxicity should be investigated in detail.

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Inundative Release of Egg and Larval Parasitoids as A Component of IPM of Leaf Eating Caterpillars of Cabbage

M.F. Khatun, S.N. Alam[1], N.K. Dutta[1] and D. Sarker[1]
Department of Entomology, Bangabandhu Sheikh Mujibur Rahman Agricultural University, Bangladesh
E-mail: k.fatemabsmrau@gmail.com
[1]Entomology Division, Bangladesh Agricultural Research Institute
Gazipur, Dhaka, Bangladesh

Abstract

We evaluated the efficacy of three *Trichogramma* species and a larval parasitoid, *Bracon hebetor* Say (Hymenoptera: Braconidae), against leaf eating caterpillars of cabbage; prodenia caterpillar, *Spodoptera litura* (F.) (Lepidoptera: Noctuidae) and diamond back moth (DBM), *Plutella xylostella* (L.) (Lepidoptera: PLutellidae); in microplots and fields during 2010-11 and 2011-12 winter season at BARI, Gazipur. In the micro plot study, two sets of experiments were done, in one set efficacy of only egg parasitoids was assessed and in other set both egg + larval parasitoids were evaluated against the pests in various treatment combinations. In the open field study there were two treatments, i) inundative release of the two parasitoids and ii) untreated control. Among the three species of *Trichogramma* in micro plots, highest parasitism of DBM were recorded from *T. chilonis* (84 % reduction in damage over untreated control) and lowest in *Trichogrammatoidea bactrae* (45 % reduction). Parasitism of *S. litura* was significantly less than that of DBM by *Trichogramma*. Together the egg and larval parasitoid significantly reduced leaf eating caterpillars in cabbage. In microplots, 85% DBM and 87 % *S. litura* caterpillars were reduced by the joint effort of *T. chilonis* and *B. habetor*. In the open field study, innundative release of *T. chilonis* and larval parasitoids reduced DBM and *S. litura* leaf eating caterpillar infestation by 45 and 56 % respectively over untreated control.

Keywords: natural enemy impact, parasitoids, field experiements, biologial control

Cabbage, a more or less compact leaf formed head of *Brassica oleracea* L., is a popular vegetable in Bangladesh. Two leaf eating caterpillars, prodenia caterpillar, *Spodoptera litura* (F.) (Lepidoptera: Noctuidae) and diamond back moth (DBM), *Plutella xylostella*, (L.) (Lepidoptera: PLutellidae) are the most destructive pests of cabbage in Bangladesh (Alam *et al.* 2010). Worldwide diamondback moth has for many years been considered to be the most important pest of cabbages and other brassica crops (Talekar and Shelton, 1993; Shelton, 2004; Furlong *et al.* 2013), costing up to 4-5 billion US$ per year in damage and control costs (Zalucki *et al*. 2012). In India, Krishnamoorthy (2003) reported a 52 % yield loss on cabbage due to diamond back moth. Worldwide it has been considered that biological control is the best option for effective management of these two pests (e.g. Furlong *et al.* 2004ab; Bopape *et al.* 2016) . However, in Bangladesh farmers continue to apply toxic chemical pesticides indiscriminately to control these two pests. Such pesticide use, besides being costly, detrimental to the environment and to human health, is also degrading the natural resource base by destroying predators and parasitoids. To better make use of natural enemies and reduce the pest population below economic injury level in a sustainable manner it is essential to assess their impact (MacFadyen *et al.* 2015; Zalucki *et al.* 2015). Here we assess the effect of different natural enemies using innundative releases of egg and larval parasitoids in small and larger scale field experiments..

Materials and Methods

Studies were undertaken in microplot and field scales during 2010-11 and 2011-12 winter seasons at experimental fields of Entomology Division, Bangladesh Agricultural Research Institute (BARI), Gazipur, Bangladesh. Twenty five day-old cabbage (var. Atlas 70) seedlings were transplanted to a 10 m^2 area with 45cm x 45cm spacing during last week of December in both years. Recommended management practices except plant protection measures were followed for raising the crop. For microplot studies ten cabbage heads (free from any insect infestation)

were covered with 70 meshes net (1.5 m x 1.5 m) 35 d after transplanting. Four pairs of newly hatched male and female DBM and two pairs of *S. litura* adults were released in the micro plots after 20 d of setting nets. After 2 d of insect pest release three species of egg parasitoid, *Trichogramma chilonis* Ishii, *T. evanescens* Westwood *and Trichogrammatoidea bactrae* Nagaraja were released separately in different microplots and other treatments established in combination with the larval parasitod *Bracon hebetor* Say (Hymenoptera: Braconidae). In microplots, two sets of experiments were done, in one set only egg parasitoids were evaluated and in other set both egg plus larval parasitiods were studied. There were four treatments/ set, i) release of pests + *T. chilonis,* ii) release of pests + *T. evanescens,* iii) release of pests + *Tr. bactrae,* and iv) only pests release. In another set joint efficacy of both the egg and larval parasitoids were determined, where the treatments were, i) release of pests + *T. chilonis*+ *B. hebetor,* ii) release of pests + *T. evanescens* + *B. hebetor,* iii) release of pests + *Tr. bactrae* + *B. hebetor,* and iv) only pests release. At 15 d after release of parasitoids, the nets were removed and the total number of *P. xylostella* and *S. litura* larvae per head counted and recorded. Microplot experiments were laid out in randomized complete block design (RCBD) with three replicates per treatment. In the open field study there were two treatments, i) innundative release of these two parasitoids and ii) untreated control. The field experiment was laid out in dispersed RCB design with six replications. Each field was atleast 200m away from each other. The egg and larval parasitoids for field release were collected from IPM laboratory of Entomology Division, BARI, Gazipur.

Results and Discussion

Egg parasitoid efficacy in microplots

The three species of trichogrammatids parasitised both the caterpillars' eggs but parasitism percentage was higher in *T. chilonis* treatments than other two, which was reflected in the per cent head infestation by the two pests (Table 1). Highest parasitism of DBM were recorded from *T. chilonis* (84 % reduction in damage over untreated control) and lowest in *Trichogramma-toidea bactrae* (45 % reduction in damage over untreated control) in micro plots (Table 1). Parasitism of *S. litura* was less than the diamond back moth. However like DBM, highest percent parasitism was also recorded in *T. chilonis* treated plots.

Egg and larval parasitoid efficacy in microplots

Together egg and larval parasitoids significantly reduced leaf eating caterpillars in cabbage (Table 2). The lowest head infestations were observed in the microplots where both the egg and larval parasitoids were released, while it was highest in the untreated control plots. Highest reduction of DBM (85 % reduction over untreated control) and *S. litura* (87 % reduction over untreated control) were recorded from *T. chilonis + B. hebetor* treated plots (Table 2).

Egg and larval parasitoid efficacy in open field

Innundative release of egg and larval parasitoids, *T. chilonis + B. hebetor* significantly reduced leaf eating caterpillar infestation (45 % DBM and 56 % *S. litura*) over untreated control in cabbage fields (Table 3).

Several studies have so far been done and reported on the biological control of different insect pests of cabbage (e.g. Furlong *et al.* 2004ab; Uelese *et al.* 2014). Inundative releases have been used especially on DBM. Release of adults of *Tr. bactrae* at 40,000 –50,000 adults per week ha^{-1}, from transplanting for 6-7 weeks, reduced DBM infestation by 30 % (Krishnamoorthy & Mani 1999). A total of six releases, at 50,000 adults per release $week^{-1}$ ha^{-1}. was recommended for effective control of DBM on cabbage (Krishnamoorthy 2003). Periodic releases of large numbers of egg parasitoids could also help in suppressing populations of *S. litura* (Singh & Jalali 1994). Result of the study here showed that there is considerable scope for increased use of natural enemies as components of IPM programmes for controlling different insect pests of cabbage, especially the most destructive ones, *P. xylostella* and *S. litura*, by combining egg and larval parasitoids.

TABLE 1

Efficacy of three species of trichogrammatids against infestations of leaf eating caterpillars on head cabbage during December - March 2010-11 & 2011-12 combined at Entomology Division experimental field, BARI, Gazipur, Bangladesh

Treatments	% head infestation (Mean of 2 seasons)	No. of DBM larvae/ head (Mean of 2 seasons)	% reduction of DBM over control (Mean of 2 seasons)	No. of *S. litura*/ head (Mean of 2 seasons)	% reduction of *S. litura* over control (Mean of 2 seasons)
T. chilonis	12.7±1.1 b	1.4±0.5	84.2	9.8±1.3	19.74
T. evanescens	16.8±0.9 b	2.8±0.8	67.81	10.3±1.7	15.65
Tr. bactrae	19.9±1.2 b	4.8±1.1	44.32	8.8±1.4	27.87
Control	64.4±3.2 a	8.7±2.2	-	12.2±1.9	-

Means followed by the same letter(s) in a column do not differ significantly by LSD ($p < 0.05$)

TABLE 2

Efficacy of joint effort of egg and larval parasitoid against leaf eating caterpillars infestation in cabbage in the micro plots during December - March 2010-11 & 2011-12 combined at Entomology Division experimental field, BARI, Gazipur, Bangladesh.

Treatments	% head infestation (Mean of 2 seasons)	No. of DBM larvae/ head (Mean of 2 seasons)	% reduction of DBM over control (Mean of 2 seasons)	No. of *S. litura*/ head (Mean of 2 seasons)	% reduction of *S. litura* over control (Mean of 2 seasons)
T. chilonis + *B. hebetor*	3.4±1.3 b	1.4±0.6	85.4	2.1±0.4	86.7
T. evanescens + *B. hebetor*	7.7±1.8 b	2.2±1.1	77.1	2.8±0.7	82.3
Tr. bactrae + *B. hebetor*	8.9±1.1 b	3.2±0.7	66.7	2.7±0.8	83.1
Control	61.1±2.5 a	9.6±2.1	-	15.9±1.7	-

Means followed by the same letter(s) in a column do not differ significantly by LSD ($p<0.05$)

TABLE 3

Efficacy of egg and larval parasitoid against leaf eating caterpillars infestation in cabbage field during December - March 2011-12 at Entomology Division experimental field, BARI, Gazipur, Bangladesh

Treatments	% head infestation	No. of DBM larvae/ head	% reduction of DBM over control	No. of *S. litura*/head	% reduction of *S. litura* over control
IPM plots *(T. chilonis* + *B. hebetor)*	7.4±0.4 b	4.7±0.6	44.7	4.1±0.5	56.3
Untreated control plots	42.8±1.2 a	8.5±1.1	-	9.4±1.1	-

Means of 12 observations and 3 replications; Means followed by the same letter did not differ significantly by paired t-test ($p < 0.01$)

CONCLUSION

Together, egg and larval parasitoids significantly reduced leaf eating caterpillars in cabbage. Inundative release of egg and larval parasitoids can be an effective component of IPM programmes for controlling different insect pests of cabbage, especially the most destructive *P. xylostella* and *S. litura*.

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Effect of *Nosema bombycis* on the Preference and Performance of *Plutella xylostella* (L.) (Lepidoptera: Plutellidae)

M.K. Nadia and A.B. Idris[1]

School of Environmental and Natural Resource Science, Faculty Science & Technology
Universiti Kebangsaan Malaysia, 43600 Bangi, Selangor, Malaysia. E-mail: nadia.khermani@yahoo.com
[1]idrisgh@ukm.edu.myor idrisyatie@yahoo.com.my

Abstract

Biological control, using pathogenic microsporidia, may provide a safer alternative to using pesticides against the diamondback moth (DBM), *Plutella xylostella* (L.) (Lepidoptera: Plutellidae). The microsporidium, *Nosema bombycis* (NB), is one of many disease agents that can be used in the Integrated Pest Management (IPM) of DBM. *Nosema bombycis* may affect DBM oviposition behaviour and their progeny performance on some host species. We infected second instar larvae of DBM and the emerged adults were used in preference and performance assays. Preferences and performance of DBM varied between plants. DBM performed best on *Brassica juncea* compared with the other plants used in this study (*B. chinese*, *B. rapa* and *B. oleracea*) with the greatest number of eggs laid, number of offspring emerged and the shortest developmental time. Interestingly, both normal and *Nosema*-infected DBM adults laid few eggs on cabbage. We suggest that *B. juncea* might be the best host plant to rear DBM. *Nosema* infection had negative impact on the fitness and performance of DBM but the infection has no effect on oviposition and performance of DBM on different plants. Knowledge of hierarchies of host plant oviposition preference by DBM females will be useful in understanding the population dynamics of this destructive pest, and for developing effective monitoring and management strategies.

Keywords: *Plutella* x*ylostella*, *Brassica*, microsporidia, preference, performance

The diamondback moth (DBM) *Plutella xylostella* (L.) (Lepidoptera: Plutellidae) causes considerable economic losses in cruciferous crops worldwide and occasionally other crops (Grzywacz *et al.* 2010). More than 90% of crop losses have been reported during outbreaks of *P*. *xylostella* (Verkerk and Wright 1997). Such outbreaks are in part due to the high population growth of *P. xylostella* with overlapping generations and its ability to migrate and disperse over long distances (Chapman *et al.* 2003; Li *et al.* 2012). The estimated cost for controlling this insect is US$ 4–5 billion annually (Zalucki *et al*. 2012). Several strategies are commonly used to control DBM: pest monitoring, various agricultural practices such as overhead watering, chemical control, biological control using *Bacillus thuringiensis* and other entomopathogens. Control of this pest is usually achieved through the application of synthetic insecticides, but DBM has become resistant to almost every insecticide applied in the field including spinosyns, avermectins, neonicotinoids, pyrazoles, and oxadiazines in many crucifer-producing regions (Zhao *et al.* 2006; Furlong *et al.* 2013). DBM was the first species to develop field resistance to *B. thuringiensis* (Bt) Cry toxins (Shelton *et al.* 2007). The developed resistance, high cost of insecticides, environmental contamination, and pest resurgence (Zhao *et al.* 2002) have encouraged the search for alternatives, which are more compatible with the environment.

Biological control, which includes the use of pathogens, parasitoids, and predators is one of the more favored and feasible approaches to deal with DBM control (Furlong *et al.* 2013). Microbial control using entomopathogen, like microsporidia could be developed as part of an integrated pest management (IPM) program. Microsporidia are intracellular pathogens, now thought to be closely related to fungi (Hibbett *et al.* 2007), that attack a broad range of eukaryote hosts including humans and *Drosophila*. Microsporidia are one of the natural controlling factors of some economically significant insect pest populations (Onstad and Carruthers 1990). The microsporidium, *Nosema locustae* Canning, has been developed to commercial use as a microbial insecticide in the biological control of grasshoppers in many countries (Lange and Cigliano 1999; Lomer *et al*. 2001). However, the possible use of microsporidia in biological control of different pest insects is still under debate.

Microbial control is an environmentally sound option to applying chemicals for controlling DBM. The microsporidia, *Nosema bombycis* Negali (Microsporidia: Nosematidae), is one of several important mortality factors of DBM (Idris *et al.* 2001). Although *Nosema* sp. can cause high mortality in the laboratory, especially at high doses (Kermani *et al.* 2013), it is unclear what effect this microsporidium has on host populations under field conditions. DBM despite being often exposed to microsporidia they seem to survive the infection in the field. The physiological and /or behavioral responses to the infection are thought to be responsible (Holt *et al.* 2013). Most studies focus on the effect of *Nosema* infection on development, mortality and fecundity (Idris *et al.* 2011; Kermani *et al.* 2013), yet little is known about its effect on adult oviposition behavior. The effects of microsporidia on adults may have a significant impact on DBM behavior and might modify their oviposition choice and larval performance. Thompson (1988) defined the oviposition preference as the number of eggs laid by a female on each of the plant species, in a choice trial, in which plants are presented in equal abundance and availability. Performance is used here as a composite term for survival and developmental period at all immature stages (egg, larval, pupal), pupal weight, and resultant adult fecundity and longevity.

DBM is an important pest of cruciferous plants. Cruciferous plants are well characterized by the presence of glucosinolates, which stimulate oviposition of the adults and feeding by the larvae (Marazzi *et al.* 2004). DBM prefers surfaces with reduced wax for oviposition (Justus *et al.* 2000; Shelton and Nault 2004) and larvae move more rapidly and spend more time searching on surfaces with reduced wax (Eigenbrode *et al.* 1991a; Eigenbrode *et al.* 1991b; Ulmer *et al.* 2002). Many studies have evaluated the effects of cruciferous plants on performance of *P. xylostella* (e.g. Golizadeh *et al.* 2009; Zhang *et al.* 2012), but here we investigate the effect of *Nosema* infection on the oviposition behavior of DBM and their larval performance on different plants. The objectives of this study were: (1) To determine whether or not some species of Brassica vegetables were more attractive to oviposition of both non-infected and *Nosema*-infected DBM than the other *Brassica* species, and (2) To compare the performance of DBM developing on those host plants.

Materials and Methods

Study site

Oviposition preference was conducted in a glasshouse while the larval performance was conducted in the parasitology laboratory at UKM, Bangi, Selangor, from August 2011 to December 2011. Experiments in the glasshouse were conducted in screen cages (1.50 m wide by 2.40 m long by 1.50 m high) covered with fine polyester mesh, at 25 ± 5 °C, 50-80 % RH

Experimental Plants and Insects

Four plant species were used in the study: sawi leaves (*Brassica juncea*), sawi bunga (*B. chinese*), pak choi (*B. rapa* var. *Chinensis*) and cabbage (*B. oleracea* var. *capitata* cv. K-K Cross). These plants were selected based on their importance as cultivated leafy green vegetables in Malaysia and they are commonly found in Malaysian food. Plants were grown in pots (11 cm diam.) filled with soil in screen cages. Plants at the 4–6 leaf stage were used in experiments. Experiments were conducted in the screen house at 25 ± 5 °C and a photoperiod of 12:12 (L: D) h. DBM larvae were obtained from a colony maintained at the Malaysian Agriculture Research and Development Institute (MARDI). They had been reared on Indian mustard to avoid possibly confounding effects of feeding history on host plant preference. After a few generations, 2nd instar larvae were treated by *Nosema* sp. spore concentration (103 spores/ìL) or sterile water as a control as described by Nadia *et al.* (2011).

Oviposition Preference Experiment in Screen house

To determine oviposition preference for different hosts by DBM females, a free-choice experiment was conducted in the glasshouse in August 2012. Oviposition preference was measured as the total number of eggs laid on the plant surface. Seven potted plants from each host plant species (i.e. a total of 28 plants) were randomly placed in wood-framed cages (1.50 m wide by 2.40 m long by 1.50 m high) with fine polyester mesh covers. The experiment was set up in completely randomized design. Plants were spaced at about 30 cm intervals. A total of 28 pairs in a 1:1 (male/female) sex ratio of newly emerged DBM adults were released in the cage and allowed to mate and oviposit. Adults of DBM were released in from a plastic container placed in the middle of the cage.

Placement of moths in the cages was done within 3 h of either sunrise or sunset to avoid the intense, midday sun. Adults were supplied with a cotton wick placed in a vial of 10 % honey solution, placed at the bottom of the cage served as a food source. After 48h (before the eggs would hatch) we assessed oviposition by removing all leaves and counting the number of eggs per plant in the laboratory. This experiment was repeated three times with new sets of host plants and insects. A similar protocol was used to quantify the eggs laid by *Nosema*-infected DBM.

P. xylostella *Performance on different hosts*

The parameters used to evaluate larval performance were development time and survival of the immature stages: All experiments in this study were performed in the parasitology laboratory maintained at 25 ± 1 °C, 65 ± 10 % RH, and a 12:12 L:D photoperiod.

Percent of egg hatch

Freshly laid eggs were counted and kept in batches of 50 and replicated 5 times for each host plant (i.e., a total of 250 eggs/plant) for *Nosema*-treated and untreated controls. The eggs laid on a particular plant species were placed on a new fresh leaf of the same plant in plastic containers and allowed to hatch. More than one leaf was used if necessary to have 50 eggs in each container. Eggs were monitored daily until all had hatched and numbers of eggs hatched were recorded. The egg hatching rate was calculated as: egg hatching rate = (number of larvae/ number of eggs) × 100. All laboratory bioassays were conducted at 25 °C, 65 % RH and a photoperiod of 12:12 (L:D) h.

Survivorship of immature DBM

A total of 50 newly hatched first instars (ten per replicate) were randomly selected per treatment (control or *Nosema*-treated) per each host plant to record the surviving pupae and adults. Ten larvae were placed on moist filter papers in plastic containers (5 ×15 × 15 cm) and reared on excised leaves from the same plant species. Freshness of leaf was maintained by wrapping moist cotton on to the leaf petiole, which was then covered with aluminum foil. Larvae were provided with fresh leaves every 24 h until pupation. Pupae were harvested, and kept individually in transparent plastic vials until adult emergence.

Development of immature DBM

To study the developmental periods (days) from hatching to pupation, 15 newly hatched first instars from both control and infected cohort were used (= 15 replicates, one larva per replicate per host plant). Larva were placed individually in similar type of containers (5 × 15 × 15 cm) as previously mentioned and kept as above. Larvae were fed with leaf of the respective host plants and leaves were replaced (as above) daily until pupation. The time (days) when pupae formed was recorded and larval duration was described as the number of days from hatching of the larvae to the pupal stage. Pupae within 24h of pupation were weighed using an electronic balance, kept separately in jars (5 × 15 cm) and monitored for adult emergence. The pupal durations were recorded.

Data Analysis

All data were tested for normality using Anderson- Darling test. Transformation of data was applied using Log10 (x+1) when data was not normally distributed to achieve normality before analysis. All measurements were analyzed by two-way analysis of variance (ANOVA) with infection status (infected or control) and host plant species as independent variables. When the independent variables or interactions between them were significant at $\alpha = 0.05$, the means were separated with Tukey's multiple range test. All the statistical analyses were conducted using MiniTab release 16 statistical software.

RESULTS

Oviposition preference

The mean number of eggs laid by DBM adults was affected by type of plant species ($F_{3,160} = 48.24$; $P < 0.05$), *N. bombycis* infection ($F_{1,160} = 15.02$; $P < 0.05$) and by their interaction ($F_{3,160} = 6.09$; $P < 0.05$). Mean numbers of eggs deposited by uninfected DBM were higher (76 ± 4.90) than infected DBM (43 ± 3.30). Oviposition preference of *P. xylostella* differed significantly among host plants (Fig. 1). There were no significant differences in mean total eggs laid by DBM (infected and control) on sawi, sawi bunga and pak choi ($P > 0.05$). *Nosema* infection did not deter oviposition by DBM, though *Nosema* did affect negatively the numbers of eggs laid ($P < 0.5$) on all plants except for cabbage.

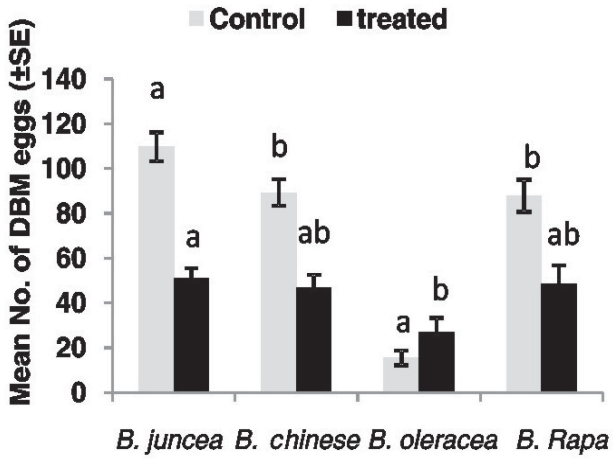

FIGURE 1

Mean ± SE number of eggs laid on different host plants species by control and infected-DBM; means with different lowercase letters are significantly different from each other (P< 0.05; Tukey's test)

P. xylostella *performance*

The mean percentage of eggs that hatched dramatically decreased ($F_{1,32}$ = 29.77; $P < 0.05$) in *Nosema*-infected DBM (40 ± 4.2 %) compared to untreated ones (71 ± 3.8 %) among the tested plants. However, there was no significant difference in egg hatch rate between host plants for the control DBM and infected treatments ($F_{3,32}$ = 1.57; P = 0.215) nor was there an interaction effect ($F_{3,32}$ = 0.18; P = 0.906).

The mean number of pupae developed from larvae ($F_{1,32}$= 91.62; $P < 0.5$) and adults emerged ($F_{1,32}$ = 109.51; $P < 0.5$) from these pupae were significantly decreased in *Nosema*-infected DBM compared to untreated ones among the tested plants. The effect of plant type on the number of pupae ($F_{3,32}$ = 0.98; P = 0.414) and adults ($F_{3,32}$ = 1.06; P = 0.377) of DBM produced was not significant.

The effect of interactions between type of plant and *N. bombycis* infection on larval ($F_{2,112}$= 2.31; P = 0.080; Table 1) and pupae ($F_{3,112}$ = 0.57; P = 0.633; Table 1) development was not significant. However, plant type and *Nosema*-infection significantly ($P <$ 0.05) impacted larval and pupal development time (Table 1). The offspring of DBM infected with *N. bombycis* developed more slowly (mean ± SE, 10.2 ± 0.11 d; 4.40 ± 0.11 d; for larval and pupal duration, respectively) than the offspring of non-diseased ones (mean ± SE, 9.7 ± 0.12 d; 4.0 ± 0.11 d; for larval and pupal duration, respectively), regardless of the plant they were fed.

TABLE 1

Two-way ANOVA results for mean developmental time (das) of DBM emerging from larvae (control or Nosema*-infected) fed on different host plants and the interactions between these factors. L.D. = Larval duration, P.D.= Pupal duration and D.P. = Developmental period*

	Source Source	***df df***	***Mean square***	***F-value***	***P-value***
L. D	Plant	3	0.056	20.52	< 0.05
	Infection status	1	0.010	10.96	< 0.05
	Plant × Infection status	3	0.006	2.31	0.080
	Error	112	0.102		
P.D	Plant	3	0.237	19.47	< 0.05
	Infection status	1	0.038	9.34	< 0.05
	Plant × Infection status	3	0.007	0.57	0.633
	Error	112	0.455		
D. P.	Plant	3	0.111	45.38	< 0.05
	Infection status	1	0.001	0.03	0.869
	Plant × Infection status	3	0.007	2.89	< 0.05
	Error	112	0.091		

The developmental time of DBM larvae ($F_{3,112}$ = 20.52; $P < 0.05$; Table 2) and pupae ($F_{3,112}$= 19.47; $P<$ 0.05; Table 2) were significantly longer for control and infected DBM fed on cabbage than with other plants except the pupal period for DBM fed on sawi bunga (Figure3). In contrast, the developmental time of DBM larvae and pupae fed on sawi, were significantly shorter than when fed on the other plants (Fig. 2 and 3). The developmental time from hatching until adult emergence (Fig. 4) were significantly ($F_{3,112}$ = 45.38; $P < 0.05$; Table 2) different among the plants. It was shorter when larvae were fed on sawi and longer on cabbage. However, the infection with *Nosema* did not affect the developmental time significantly for any type of plant ($P > 0.05$; Table 2).

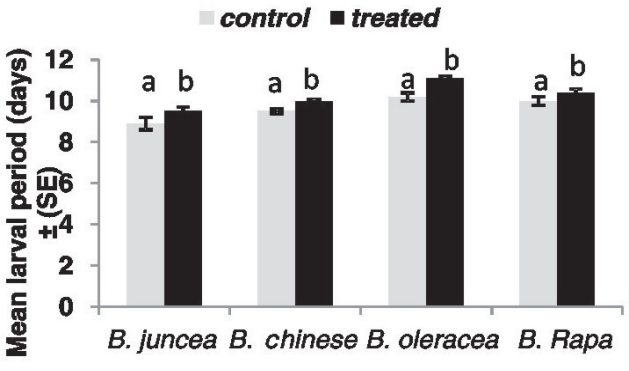

FIGURE 2

Mean development time (± SE) in days of DBM larvae (control and Infected) fed on each host plant. Means followed by the same letter are not significantly different from each other according to ANOVA, Tukey's test (P > 0.05)

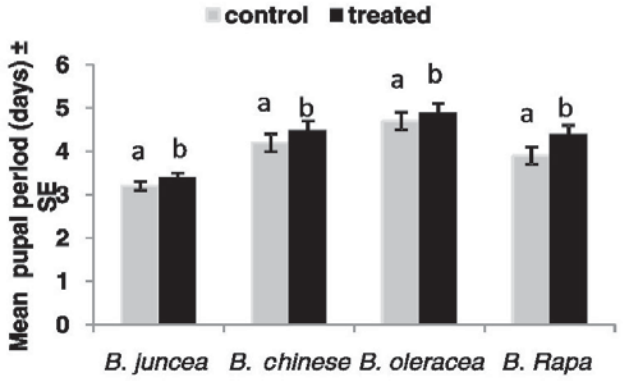

FIGURE 3

Mean (± SE) development time in days of DBM pupae (control and infected) developed from larvae fed on each host plant. Means followed by the same letter are not significantly different from each other according to ANOVA, Tukey's test (P > 0.05)

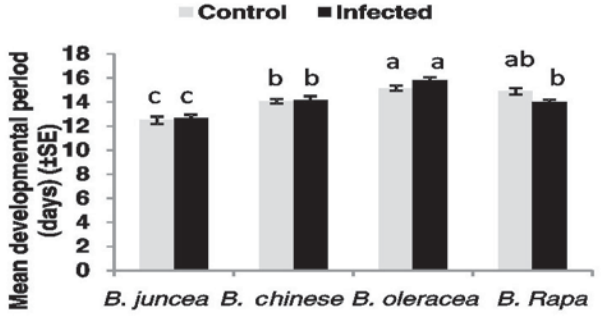

FIGURE 4

Mean (± SE) development time (days) from hatching to adult emergence in days of DBM (control and infected) developed from larvae fed on each host plant. Means followed by the same letter are not significantly different from each other according to ANOVA, Tukey's test (P> 0.05)

DISCUSSION

Oviposition preference and performance of DBM differed significantly among host plants. Most eggs were laid on *B. juncea* and the least on *B. oleracea* for both healthy and infected DBM. This study demonstrated that DBM oviposition choice varied among the tested plants, and *B. juncea* plants was the most preferred whereas *B. oleracea* was the least preferred plant in terms of oviposition. Badenes-Perez *et al*. (2004) reported similar observations and recorded more eggs, on the cruciferous *B. juncea* than on cabbage. However, *Nosema* infection did not affect host ranking but it did adversely impact the numbers of eggs laid on all plants except for cabbage. The reduction in the number of eggs laid may be related to several factors affecting the egg laying process, because *Nosema* infects certain tissues involved in egg production (Jolly and Sen 1972). The high spore concentration of *Nosema* in the gonads of *A. mylitta* Drury, *Antheraea assamensis* Helfer, and *Bombyx mori* L. was found to affect reproductive potential and fertility (Bansal *et al.* 1997). Reduction in the number of eggs deposited by infected females could be a response to the competition between the host and the microsporidia for nutrients (Goertz *et al.* 2008), as the microsporidia meets its nutritional requirements by taking resources directly from the host cytoplasm (Liu 1984). Infected females have been suggested to compensate for the loss of nutrients to the microsporidia by producing fewer eggs (Goertz *et al.* 2008). Our observations are consistent with our previous work on DBM (Kermani *et al.* 2013) and with the effects of other *Nosema* spp., such as *N. pyrausta* on the European corn borer, *Ostrinia nubilalis* (Hübner) (Bruck *et al.* 2001), and *Nosema* sp. on silkworm (Rath *et al.* 2003).

The mean percentage of egg hatching, pupae developed from larvae and adults emerged from pupae were dramatically decreased in *Nosema*-infected DBM compared to untreated ones among the tested plants. These results are similar with the sub-lethal effects of *Nosema* sp. on DBM (Kermani *et al.* 2013) and other microsporidia on insects (Becnel and Andreadis 1999). However, there were no significant differences in the egg hatching, pupae developed and adults between different host plants for the control and similarly for treated DBM. This means that the type of food plant did not affect the production of the DBM progeny. Also, *Nosema* infection did not alters DBM

performance on specific plant species. However, there were more progeny produced when DBM fed on *B. juncea* than on *B. oleracea* but this difference was not significant. Our results are similar to who found that *P. xylostella* survival on *B. oleracea* was significantly lower than other plants.

As expected, the development time for *Nosema*-infected DBM increased significantly compared to the control. An increase in developmental period due to microsporidia has been confirmed (Down *et al.* 2004; Blaser and Schmid-Hempel 2005). This delay could be a tactic from the microsporidia to spread the spores (Down *et al.* 2008)and to get as much as possible of the host resources (Hurd *et al.* 2001). This delay in adult emergence has been reported in the spruce budworm, *Choristoneura fumiferana* (Clem.) infected by *N. fumiferanae* (Campbell *et al.* 2007). Rath *et al.* (2003) suggested that the extended developmental period could be a tactic from the larvae to ingest more food to obtain best growth and accumulate enough nutrients for pupa and adult stages by extended larval period. We found that the development time of DBM from egg hatching to adult emergence varied greatly on different hosts. The difference also differed among the developmental stages of *P. xylostella*. The development period of control and *Nosema* infected was longest on cabbage (15.1 and 15.8 days respectively) and shortest (12.4 and 12.6 days respectively) on *B. juncea*. This means that the infection does not affect the DBM behavior on different plants in terms of development time. Variation in development time could be due to variation in nutritional quality (Syed and Abro 2003)in different hosts. In fact each host plant affects insect development rates differently (Soufbaf *et al.* 2012).

There are many studies on the effects of different species of cruciferous on the performance of DBM (Golizadeh *et al.* 2009; Sarfraz *et al.* 2007; Soufbaf *et al*. 2010; Zhang *et al*. 2012). Most studies demonstrated that each host plant affects the behavior and development of DBM differently (Sarfraz *et al.* 2007, 2008; Golizadeh *et al.* 2009). We found that *P. xylostella* performed best on *B. juncea* compared with the other plants used in this study, with the greatest number of eggs laid, number of offspring emerged and the shortest developmental time.

Based on our results, we suggest that *B. juncea* might be the best host plant to rear *P. xylostella*. This also might help to predict the pest's manifestation once it disperses to a new plant in adjacent fields. In general, *Nosema* infection had negative impact on the fitness and performance of *P. xylostella* in terms of life history parameters. However, infected DBM behaved very similarly to controls on different host plants. This means that the infection has no effect on oviposition and performance of DBM regarding to different plants. Females usually chose to lay egg on oviposition site which would be the best for their larvae (Jones 1991), and in our study this was true as the females preferred *B. juncea* for oviposition and their progeny performed better on it.

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Management of Diamondback Moth, *Plutella xylostella* L. Through Biorationals in Cabbage

R.H. Patil, H.M. Shaila and J.S. Awaknavar
Department of Agricultural Entomology, University of Agricultural Sciences, Dharwad – 580 005, Karnataka, India
E-mail: patilrh57@gmail.com

Abstract

In recent years there is a growing awareness and preference for organically produced crops. Governments of India and Karnataka have been earmarking significant funds for research and popularization of organic farming especially in consumable crops. This has led to development of organic packages of practices and modules to manage important insect pests of vegetables and particularly in cabbage. With this background, investigations on management of diamondback moth, *Plutella xylostella* L. through biorational pest control in cabbage were undertaken during 2011 and 2012 at University of Agricultural Sciences, Dharwad. The results of two year experimentation revealed that soil application of neem cake @ 500 kg/ha followed by sprays of vermiwash, garlic – chilli – kerosene extract + cow urine, NSKE 5 per cent and Neemazol at 15 d interval from 25 d after transplanting was found to be significantly superior in bio-efficacy and was comparable to the standard check, 100 per cent recommended dose of fertilizers (RDF) + recommended package of practices (RPP) (Malathion 50 EC @ 2.0 ml/l) in reducing the pest population and recording high yield. Among different eco-friendly insecticides tested, spinosad 48 SC @ 0.2 ml/l, emamectin benzoate 5 SG @ 0.25 g/l, indoxacarb 14.5 SC @ 0.3 ml/l proved significantly superior in reducing the larval population of *P. xylostella* throughout the crop period and recorded higher cabbage yield. Spinsoad proved to be cost effective and dipel, novaluron, spinosad and emamectin benzoate were in the mentioned order found to be safe to the parasitoids.

Keywords: Diamondback moth, Biorationals, Spinosad, IPM

India is the second largest producer of vegetables next to China with an annual production of 81.0 mt from 5.12 m. ha (Fageria *et al*. 2000). Among the vegetables, crucifers are among the most commonly grown vegetables. Cabbage is cultivated in 0.37 m ha with a production of 8.01 mt and average productivity of 21.5 t/ha in India (Indian horticulture data base 2014). In Karnataka it is grown in all the seasons over an area of 3,200 ha with annual production of 200,000 metric t having an average productivity of 24.6 t/ha.

Insect pests the major production constraint that hampers successful cultivation of crucifers. Among the insect pests, diamondback moth (*Plutella xylostella* L.) is a cosmopolitan defoliator pest causing 52 per cent crop loss in cabbage (Anuradha 1997). In spite of large scale and repeated application of insecticides, the pest has been found to occur in severe form in all cabbage growing areas mainly because of the fact that it has developed resistance to all major groups of pesticides. Presence of insecticide residues, management failure, adverse effects on natural enemies, environmental pollution and spiraling cost of synthetic insecticides have encouraged the present study on development of non-chemical and eco-friendly strategies for the management of diamondback moth in cabbage during 2011 and 2012.

Materials and Methods

Field experiments were carried out for two *rabi* seasons (October–January) of 2011 and 2012 at Main Agricultural Research Station, University of Agricultural Sciences, Dharwad (15°272303N), Karnataka. Application of soil amendments was done at the time of transplanting and spraying was taken up at 15 day intervals based on economic threshold levels (ETL), starting from 25 days after transplanting (DAT). Population count of DBM larvae was taken on 15, 30, 60 and 75 DAT.

Eco-friendly management trials were laid out with 10 treatments and each being replicated thrice using the randomized complete block design (RCBD) (Table 1). Cabbage variety Super Ball (F_1) was selected and 25 days old seedlings were transplanted during first week of December. Treatments were sprayed after 25 days of transplantation when the population reached ETL. Subsequent sprays were given at 15 days interval. Assessment of population density of DBM was taken on 10 randomly selected

plants in each treatment of each replication. Pre-treatment and post-treatment counts were made on one, three, seven and ten days after the treatment imposition. Cabbage yield was recorded from each treatment. Statistical analysis of data was done by using analysis of variance (ANOVA) after square root and angular transformation, followed by Duncan's Multiple Range Test (DMRT) for mean comparison.

TABLE 1

Details of organic amendments and spray sequence evaluated against P. xylostella

Tr. No.	Treatment details
T_1	50% RDF + Vermicompost (1250 kg/ha) superimposed with spraying of vermiwash 1:1 – NSKE (5 %) – GCK (1 %) + cow urine (5 %) – Neemazal (2 ml/l)
T_2	50% RDF + Vermicompost (2500 kg/ha) superimposed with spraying of vermiwash 1:1 – NSKE (5 %) – GCK (1 %) + cow urine (5 %) – Neemazal (2 ml/l)
T_3	50 % RDF + Neemcake (250 kg/ha) superimposed with spraying of vermiwash 1:1 – NSKE (5 %) – GCK (1 %) + cow urine (5 %) – Neemazal (2 ml/l)
T_4	50 % RDF + Neemcake (500 kg/ha) superimposed with spraying of vermiwash 1:1 – NSKE (5 %) – GCK (1 %) + cow urine (5 %) – Neemazal (2 ml/l)
T_5	50 % RDF + Pongamia cake (250 kg/ha) superimposed with spraying of vermiwash 1:1 – NSKE (5 %) – GCK (1 %) + cow urine (5 %) – Neemazal (2 ml/l)
T_6	50 % RDF + Pongamia cake (500 kg/ha) superimposed with spraying of vermiwash 1:1 – NSKE (5 %) – GCK (1 %) + cow urine (5 %) – Neemazal (2 ml/l)
T_7	50 % RDF + Poultry manure (1 t/ha) superimposed with spraying of vermiwash 1:1 – NSKE (5 %) – GCK (1 %) + cow urine (5 %) – Neemazal (2 ml/l)
T_8	50 % RDF + Poultry manure (2 t/ha) superimposed with spraying of vermiwash 1:1 – NSKE (5 %) – GCK (1 %) + cow urine (5 %) – Neemazal (2 ml/l)
T_9	100 % RDF + RPP
T_{10}	Untreated check (100 % RDF)

*Spraying at 15 days interval starting from 25 DAT (days after transplanting)

RESULTS AND DISCUSSION

Soil application of organic amendment with sequential sprays of botanicals revealed that treatments with neem cake (NC) were found to be the most effective, which recorded least larval population and were comparable with recommended package of practice (RPP). Among the other treatments, T2 [vermicompost (VC)-2500-SS)] was significantly superior at all the intervals followed by T1 (VC-1250-SS). However, at 75 DAT, all the treatments with neem cake and vermicompost were found to be on par. Treatments including Pongamia cake were next in the order of efficacy (Table 2).

Treatment effect was manifested on yield in both the years of study (2011-2012). The pooled data of yield indicated significantly higher yield of cabbage in T4 (NC-500-SS), which was as good as RPP. This was followed by T3 (NC-250-SS), while significantly lower yield was recorded in untreated check. Among the treatments, maximum benefit cost (B:C) ratio was recorded in RPP followed by T3 (NC-250-SS) and T4 (NC-500-SS). The lowest BCR was recorded for poultry manure (PM-1000) treatment. The highest net return was also recorded for RPP, followed by neem cake treatments (Table 2).

Eco-friendly molecules spinosad and emamectin benzoate recorded significantly maximum percent larval reduction at all observations. Pooled analysis of the data on larval population indicated that spinosad and emamectin benzoate were on par in efficacy. The next best molecules in bio-efficacy were indoxacarb, novaluron and dipel, whereas *N. rileyi* was found to be the least effective in reducing *P. xylostella* larval population (Table 3). Pest reduction in various treatments clearly translated in yield increase. Pooled yield of cabbage over two years did not vary significantly among plots treated with spinosad and emamectin benzoate. By virtue of good protection offered by these insecticides the yield increased significantly in spinosad and emamectin benzoate. They were followed by indoxacarb, novaluron, and dipel. The benefit to cost ratio (B:C ratio) was maximum in spinosad followed by indoxacarb.

Proportion of investment to benefit was so close between novaluron and dipel.

Insecticidal control of DBM has posed problems of resistance development and pesticide residues in cabbage and hence switching over to organic agriculture with development of a non-chemical package or a program with least pesticide usage is the need of the hour in cabbage production. In this context, use of organic soil amendments and botanical pesticides in crop production assume greater significance. With this idea, an attempt has been made in the present study to know the effect of various organic amendments along with a non-chemical spray sequence on pest activity and management on cabbage in two field trials during 2011 and 2012. The literature pertaining to effect of organic soil amendments in DBM management appears scanty except few reports. Krishnamoorthy *et al*. (2001) reported that soil application of neem cake @ 250 kg/ha led to significant reduction in DBM larval population compared to untreated check. Similarly, Chakraborti (2001) reported the superiority of pre-sowing application of neem cake @ 300 kg/ha, followed by foliar application of NSKE and 1-2 applications with phosphamidon to be effective against DBM. However, in the present study RPP was superior over other treatments with neem cake application. Perhaps the absence of any chemical in the spray sequence in the present study might be the reason for variation of these results. Further Krishnamoorthy *et al.* (2001) reported that three applications of NSKE enabled harmonious blending of effective components of pest control methods in the light of the importance of conserving natural enemies, while pesticides are being used as a tool for the control of DBM.

In the present investigation, spinosad, a fermentation product of soil actinomycete, *Saccharopolyspora spinosa* with novel mode of action and emamectin benzoate were found to have strong larvicidal property against DBM. Several earlier researchers have also reported the effectiveness of spinosad and emamectin benzoate against DBM (Arora *et al.* 2003; Pramanik and Chatterjee 2004; Kumar and Devappa 2006; Kanna *et al.* 2005). The superiority of indoxacarb against DBM is in accordance with the works of many of the earlier authors (Babu *et al.* 2002; Liu *et al.* 2003). Among the treatments, spinosad registered highest yield and proved highly productive treatment. This is in agreement with earlier report by Dhanraj (2000). This was found to be on par with emamectin benzoate treated plots, which corroborate with the results of Kumar and Devappa (2006) and Kanna *et al.* (2005).

Conclusion

Soil application of neem cake @ 500 kg/ha followed by sprays of vermiwash, garlic - chilli – kerosene extract + cow urine, NSKE @ 5 per cent and Neemazol at 15 days interval was found as effective as standard check, 100 per cent RDF + RPP in reducing the pest population and recorded higher yield. Among different insecticides tested, spinosad 45 SC @ 0.2 ml/l, emamecgin benzoate 5 SG @ 0.25 g/l, and indoxacarb 14.5 SC @ 0.3 ml/l proved significantly superior in reducing the larval population of *P. xylostella* throughout the crop period. Spinosad was also proved to be highly cost effective. Among the tested chemicals, dipel, novaluron, spinosad and emamectin benzoate in the mentioned order were found to be safe to the parasitoids.

TABLE 2

Effect of organic amendments with spray sequence on P. xylostella *population and influence on yield in cabbage (pooled data of two years)*

Treatments	Larvae / plant					Yield (t/ha) (INR)	Net returns (INR)	Incremental returns	Treatment cost (INR)	IBCR
	15 DAT	30 DAT	60 DAT	75 DAT	Mean					
T_1- Vermicompost 1.25 t/ha+SS	1.38^{de} (1.37)	2.72^{d} (1.79)	2.65^{d} (1.77)	2.55^{abc} (1.74)	2.33	14.54^{e}	24350	19100	4200	4.54
T2- Vermicompost 2.5t/ha+SS	0.92^{cd} (1.19)	2.42^{cd} (1.71)	2.32^{cd} (1.68)	2.22^{abc} (1.65)	1.97	16.26^{cd}	28650	23400	5450	4.29
T_3- Neem cake 250 kg/ha+SS	0.62^{bc} (1.05)	2.15^{ab} (1.63)	1.98^{bc} (1.58)	1.83^{ab} (1.53)	1.65	17.34^{bc}	31350	26100	3950	6.60
T_4- Neem cake 500 kg/ha+SS	0.42^{ab} (0.95)	1.95^{ab} (1.56)	1.80^{b} (1.52)	1.62^{ab} (1.46)	1.45	18.90^{ab}	35250	30000	4950	6.06
T_5- Pongamia cake 250 kg/ha+ SS	2.22^{f} (1.65)	3.58^{e} (2.02)	3.47^{e} (1.99)	3.35^{bc} (1.96)	3.16	13.76^{ef}	22400	17150	5200	3.30
T_6- Pongamia cake 500 kg/ha+ SS	1.68^{ef} (1.47)	3.33^{e} (1.96)	3.25^{e} (1.94)	3.12^{bc} (1.90)	2.85	15.17^{de}	25925	20675	7450	2.78
T_7- Poultry manure 1 t/ha+ SS	3.70^{g} (2.05)	5.20^{g} (2.39)	5.32^{g} (2.41)	5.00^{d} (2.34)	4.80	11.56^{fg}	16900	11650	3950	2.35
T_8- Poultry manure 2 t/ha+ SS	3.02^{g} (1.87)	4.37^{f} (2.20)	4.25^{f} (2.18)	4.07^{cd} (2.14)	3.93	12.33^{g}	18825	13575	4950	2.74
T_8- Recommended package of practice	0.10^{a} (0.77)	1.60^{a} (1.45)	1.27^{a} (1.33)	1.13a (1.27)	1.03	19.41^{a}	36525	31275	4523	6.91
T_{10}- Untreated control	5.60^{h} (2.46)	6.63^{h} (2.67)	11.00^{h} (3.93)	12.35e (3.58)	8.89	6.90^{h}	5250	–	–	–

Figures in parentheses are square root transformed values (“x+05)

In a column means followed by the same alphabet do not differ significantly by DMRT (0.05%)

DAT – Days after transplanting, SS – Spray sequence

IBCR— – Insecticide benefit cost ratio

TABLE 3

Bioefficacy of eco-friendly insecticides against P. xylostella *and their influence on yield of cabbage*

Treatments	Dosage (g ai/ha)	Per cent larval reduction over pre treatment						Yield (t/ha)	Net returns (INR)	Incre mental returns (INR)	Treat ment cost (INR)	IBC
		First spray			Second spray							
		1 DAS	3 DAS	7 DAS	1 DAS	3 DAS	7 DAS					
T_1- Spinosad 2.5 SC	15	59.75^{a} (0.63)	94.11^{a} (76.72)	87.55^{a} (69.63)	49.84 (44.92)	96.06^{a} (80.93)	93.37^{a} (77.06)	35.28^{a}	72700	51025	3720	13.71
T_2 Emamectin-benzoate 5 SG	7.5	53.45^{a} (47.99)	84.97^{a} (71.98)	80.77^{a} (64.72)	42.76^{a} (41.01)	87.35^{a} (74.64)	88.01^{a} (71.71)	33.17^{a}	67425	48250	6038	7.99
T_3- Novaluron 10 EC	50	30.11^{c} (33.25)	40.29^{cd} (30.37)	64.74^{b} (53.73)	31.50^{c} (34.05)	59.09^{c} (50.32)	68.94^{b} (56.29)	25.69^{bc}	48725	29500	5600	5.28
T_4- Indoxacarb 14.5 SC	35	45.03^{b} (42.11)	71.26^{b} (57.96)	69.95^{b} (56.96)	39.75^{b} (39.03)	75.62^{b} (61.09)	72.99^{b} (58.98)	28.76^{b}	56400	37225	3600	10.34
T_5- Bt (Dipel 8L)	60	23.91^{d} (29.20)	32.28^{c} (34.58)	37.00^{c} (37.43)	26.60^{c} (30.94)	52.27^{cd} (46.33)	46.85^{c} (43.19)	23.71^{c}	43775	24600	4795	5.13
T_6- *Nomuraea rileyi*	2 g/l	11.69^{f} (19.53)	13.80^{e} (21.61)	18.24^{d} (25.26)	8.53^{ef} (16.85)	15.83^{g} (23.03)	25.66^{f} (30.33)	10.79^{e}	11475	-	1400	-
T_7- *Beauveria bassiana*	5 g/l	14.45^{f} (22.31)	20.22^{d} (26.67)	22.15^{d} (27.85)	11.30^{ef} (19.34)	27.04^{f} (30.91)	30.52^{ef} (33.50)	14.51^{d}	20775	1600	3100	1.45
T_8- NSKE	5%	20.97^{de} (27.23)	26.34^{cd} (30.85)	25.32^{d} (30.06)	20.46^{d} (26.58)	45.28^{dc} (42.28)	40.59^{cd} (39.55)	15.10^{d}	22250	3075	1200	2.50
T_8- Malathion 50 EC	250	18.54^{e} (27.48)	23.43^{d} (28.92)	22.17^{d} (28.07)	12.30^{e} (20.33)	36.04^{ef} (36.88)	35.24^{de} (36.38)	16.50^{d}	25750	6575	1690	3.89
T_{10}- Untreated control	-	-3.93^{g} (11.36)	-3.87^{f} (11.31)	-5.46^{e} (13.35)	-7.87^{f} (-15.97)	-3.09^{h} (-9.80)	-6.35^{g} (-14.35)	7.67e	-	-	-	-

In a column means followed by the same alphabet do not differ significantly by DMRT (0.05%)

DAS – Days after spray, Figures in parenthesis are angular transformed values ("x+05)

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Session 6

Biotechnological tools and novel approaches to manage diamondback moth and other crucifer pests

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Toxicity and Sublethal Effects of *Bacillus thuringiensis* B. δ-Endotoxin Cry1Ab on the Biological Parameters of Diamondback Moth, *Plutella xylostella* L.

M. KANNAN, S. LOKESH, J.N. PRITHIVA AND M. MOHAN[1]

Department of Nanoscience and Technology, Tamil Nadu Agricultural University, Coimbatore - 641 003, India
E-mail: kanento@gmail.com

[1]Division of Molecular Entomology, National Bureau of Agricultural Insect Resources (NBAIR), Bengaluru - 560 024
E-mail: Mohan_iari@yahoo.com

ABSTRACT

The toxicity and sublethal effects of *Bacillus thuringiensis* (*Bt*) endotoxin Cry1Ab on development and reproductive behaviour of the diamond back moth, *Plutella xylostella* L. was assessed by leaf dip method. The results of probit regression analysis of dose response mortality data for the bioassay of Cry1Ab to *P. xylostella* was recorded and the LC_{25}, LC_{50} and LC_{75} were 0.014, 0.115, 0.916 ìg/ml, respectively. The results of the present study indicated that prolonged developmental durations (egg, larval, pupal and adult periods), retardation in larval growth and development, reduction in adult emergence, increase in malformation, reduced fecundity, oviposition periods and fertility together with significant decrease in late larval and pupal weights with increased toxin concentrations. In the sublethally exposed insect the percentage of larvae that survived and pupated increased with a decrease in the toxin concentrations. Furthermore, delayed larval development with slow death may expose the pest to natural enemies and also reduce the risk associated with resistance development by decreasing the dominance and inheritance of resistance.

***Keywords*:** *Bacillus thuringiensis*, toxicity, sublethal effects, Cy1Ab, *P. xylostella*

DIAMONDBACK moth (DBM), *Plutella xylostella* (Linnaeus) (Lepidoptera: Plutellidae), is one of the most damaging cosmopolitan insect pests of cruciferous vegetables in many parts of the world. The crop loss is estimated to vary from 52 to 100 per cent (Calderson and Hare 1986). It is one of the major constrains in the profitable cultivation of cole crops. In 1993, the estimated annual cost for its control on cruciferous crops worldwide was $ 1 billion USD, primarily with insecticides (Talekar and Shelton 1993). In 2012 the estimated annual total costs associated with damage and management of DBM worldwide was 4-5 billion USD (Zalucki *et al.* 2012) and in India, it is about 168 million USD (Sandur 2004). The main method of control has been the use of insecticides and the pest has become very serious in many regions because of its ability to establish in newer areas, coupled with high reproductive potential and shorter life cycle and year-round availability of host plants. Its rapid generation time, high proliferation and particularly extensive selection pressure in the field, resulted in resistance to various types of traditional insecticides. The extensive use of many commercial insecticides aggravated the development of resistance across various countries (Shelton *et al.* 1993).

Bt is a rod shaped, gram positive soil bacterium that, produces insecticidal crystal proteins during sporulation. The crystals comprising of one or more δ-endotoxins referred to as Cry proteins, vary among different *Bt* strains. The mode of action of *Bt* in the insect midgut includes the following steps: solubilization and enzymatic activation of crystal inclusions, binding of activated toxin to receptors on midgut epithelium and pore formation leading to osmotic imbalance and subsequent septicemia, toxemia and eventual death (Knowles and Dow 1993).

The bio-pesticide market currently accounts for 2 per cent of the worldwide crop protection market or about 600 million USD. Products based on *Bt* are the most successful microbial pesticides used in agriculture, forestry and public health accounting for more than 90 per cent of bio-pesticide sales (Sanchis and Bourguet 2008). Many commercial formulations containing high levels of δ-endotoxins have been proven to be as effective as chemical insecticides. Besides, they exhibit high specificity to target pests without detrimental effects on beneficial insects and animals. Furthermore, δ-endotoxins are primary translation products of bacterial genes and are amenable to genetic engineering techniques.

It was once believed that resistance development in insects would be unlikely for bio-pesticides like *Bt* because they have co-evolved naturally over millions of years. Subsequently, with laboratory and field evolved resistance to *Bt* documented in the diamondback moth demonstrated adaptation by pests is now considered the biggest threat to the long term success of *Bt* in *P. xylostella* management. Considering the importance of crucifer crops in India, it is essential to study the toxicity and effect of sublethal doses of *Bacillus thuringiensis* endotoxin Cry1Ab on the biological performance of *P. xylostella* to devise an appropriate resistance management strategy. Since, fitness costs can be a key factor influencing the evolution of resistance.

Materials and Methods

Insects

About 250 larvae were collected from Thondamuthur village of Coimbatore district, Tamil Nadu, India and mass cultured under laboratory condition on mustard and cauliflower at the Insectary, Department of Agricultural Entomology, TNAU, Coimbatore to establish the stock culture of *P. xylostella* (with a mean temperature of 27^{0}C, 60% RH and with a photoperiod of 14:10 (L: D)). Larvae were examined regularly to ensure that they remained pathogen-free for rearing and maintained.

Purification of recombinant B. thuringinsis *Cry1Ab endotoxin*

Recombinant *E. coli* (JM 103) expressing the *Cry1Ab* gene which was cloned in pKK 223-3 was kindly provided by Dr. D.R. Zeigler, BGSC, Columbus, USA. Culturing, isolation, purification and quantification of Cry1Abprotein followed the protocol of Lee *et al.* (1992). Cells were cultured on Luria agar plates containing 50 µgml^{-1} ampicillin for 48h at 37 °C. The cells were harvested by scraping, content was centrifuged at 8,000 rpm for 10 min and the pellet was suspended in 50 ml of lysis buffer (50 mM Tris-hydrochloride, 50 mM ethylene diamine tetra-acetic acid, 15% sucrose, pH 8.0, 2 mgml^{-1} lysozyme) and incubated for 12 h at 37 °C. After sonicating the suspension, it was given extensive washing with ice cold 0.5 NaCl containing 2% Triton X-100 followed by 0.5M sodium chloride and sterile distilled water. In all the steps the protease inhibitor, phenyl methyl sulfonyl fluoride was added at 1mM concentration. The final pellet containing inclusion bodies was solubilized in 50 mM sodium carbonate buffer, pH 10.5, containing 10 mM dithiotheritol at 37 °C for 6 h. Then it was centrifuged at 10,000 rpm for 10 min, and the supernatant containing the solubilized protoxin was activated by Bovine trypsin (0.1 mg stock) at 10:1 ratio for 12 h and then stored at -20 °C. Toxin protein was quantified by Bradford dye binding method (Bradford, 1976) and was estimated to be 0.33 mg/ml.

Bioassay method

Leaf-dip bioassay method was used (Tabashnik *et al.* 1991). The cabbage leaves were first washed with distilled water containing 0.1% Triton X-100 thoroughly and air-dried. Leaf discs of 6-8 cm diameter were cut and dipped in different concentrations of the toxin. Each disc was dipped for 5-10 seconds and allowed to air-dry under shade for 1 hour. After complete evaporation, the leaves were transferred to clean bioassay containers over a moistened filter paper. The leaf discs were placed on a slant to rest on the side of the container so that larvae could move on either side. Eight to ten 2rd instars were released in each dish and four to five replicates were maintained per treatment. A treatment without *Bt* protein served as control. Larval mortality was recorded every 24 h, consecutively for seven days. All the experiments were carried out in a room with a photoperiod of 12:12 (L: D) and experiments with control mortality more than 20% were discarded and repeated. The mortality of neonates was observed up to 7 days for $LC_{25,}$ $LC_{50,}$ LC_{75} and until adult emergence for growth and development studies. Probit regression analysis and ANOVA were carried out Finney (1971) and Gomez and Gomez (1984), respectively.

Exposure of P. xylostella *to sublethal doses* following

The development and reproductive behaviours of *P. xylostella* were studied by continuous exposure to sublethal doses of Cry1Ab. Dose levels were chosen to represent an LC_{25} (0.014 ìg/ml), LC_{50} (0.115 ìg/ml) and LC_{75} (0.916 ìg/ml), respectively as determined in the bioassays. The leaf discs coated with different doses of Cry1Ab toxin was used in the study. Hundreds of neonate larvae were released in each treatment and leaf discs were changed at every 3 days until pupation in respective doses. Data on the development *viz*., egg, larval and pupal periods, adult

longevity, adult emergence, larval, and pupal weights were recorded. The reproductive behaviours of *P. xylostella viz.*, fecundity, fertility and fecundity period were studied by rearing the adults emerging from respective treatments. Adult emergence was recorded daily and the percentages for adult emergence, healthy and malformed, were calculated. The number and percentage hatch of eggs laid were recorded for each female (Salama *et al.* 1981).

Results and Discussion

Toxicity of Cry1Ab toxin to P. xylostella

The results of studies on the toxicity of Cry1Ab to *P. xylostella* by the leaf-dip bioassay method and the result of probit regression analysis are shown in Table 1. The LC_{25}, LC_{50} and LC_{75} were 0.014, 0.115 and 0.916 ìg/ml, respectively. The mean slope of the probit regression for Cry1Ab was 0.738. The results of the present study are in agreement with findings of Mohan and Gujar (2002), who reported that the populations from Iruttupallam and Ottanchathiram in the southern state of Tamil Nadu had significantly higher LC_{50} values (ranged from 0.007–1.25 ìgml^{-1}) than the populations collected from the northern part of India. Similarly, Phani on deshire and Gujar (2005) also reported that the populations of *P. xylostella* collected from seven states in India were susceptible to Cry1Ab (LC_{50} values ranged from 0.002 to 0.386 ìg/ml).

Table 1

Probit regression analysis of mortality data to Cry1Ab for P. xylostella

Sub lethal dose (µg/ml) (seven days)	95% fiducial limits		Slope ± SE	χ^{2*}	Degree of Freedom (df)
	Lower	Upper			
LC_{25} (0.014)	0.0068	0.0305	0.738 ± 0.22	0.389	4
LC_{50} (0.115)	0.0538	0.2467			
LC_{75} (0.916)	0.2018	4.1573			

* In each case χ^2 value from the goodness-of-fit test was less than the tabular value, (p = 0.05), indicating that the data fit the probit model.

Sublethal effects of Cry1Ab on the developmental parameters of P. xylostella

The result of the present study indicated that prolonged developmental durations occurred: egg (4.00, 3.00, 3.00 and 4.00 days in LC_{25}, $LC_{50,}$ LC_{75} and control, respectively); larvae (11.00, 11.00, 10.00 and 12.00 days in LC_{25}, $LC_{50,}$ LC_{75} and control, respectively); pupae (4.00, 3.00, 3.00 and 4.00 days in LC_{25}, $LC_{50,}$ LC_{75} and control, respectively); adults (male: 8.00, 9.00, 9.00 and 8.00; female: 9.00, 10.00, 10.00 and 9.00 00 days in LC_{25}, $LC_{50,}$ LC_{75} and control, respectively). There were also significant decreases in late larval (2.11, 2.02, 1.71 and 2.21 mg/larva in LC_{25}, $LC_{50,}$ LC_{75} and control, respectively) and pupal weights (3.55, 3.53, 3.48 and 3.61 mg/pupa in LC_{25}, $LC_{50,}$ LC_{75} and control, respectively) (Table 2). In sublethally exposed insects the percentage of larvae that survived and pupated decreased with an increase in the toxin concentrations.

The results on sub lethal doses of Cry1Ab on *P. xylostella* prolonged the total developmental duration *viz.*, egg, larvae, pupae and adults and, reduced the larval and pupal weights. Similar results were observed in *Heliothis armigera* (Dulmage *et al.* 1978; Salama *et al.* 1981). In the present study, the larvae exposed to sublethal doses of ä-endotoxin Cry1Ab during 12 days showed severe reduction in larval, pupal weights and more of growth inhibition. These findings are in agreement with the results of Gujar and Mohan (2000). Kannan and Uthamasamy (2006) reported that exposure of *H. armigera* to low doses of *Bt* toxin delayed the larval and pupal periods, inhibited the larval growth and reduced the larval and pupal weights.

TABLE 2

Sublethal effects of Bacillus thuringiensis *B. endotoxin Cry1Ab on the developmental parameters of Diamondback moth.*

Sub lethal dose (µg/ml)	Egg period (days)*	Larval period (days)*	Pupal period (days)*	12th day larval weight (mg/larva)	Pupation* (%)	Pupal weight (mg/pupa)*	Oviposition Period (days)*
LC_{25} (0.014)	4.00a	11.00b	4.00a	2.11[ab]	71.66b	3.55[b]	5.00[a]
LC_{50} (0.115)	3.00b	11.00b	3.00b	2.02[b]	45.71c	3.53[b]	5.00[a]
LC_{75} (0.916)	3.00b	10.00c	3.00b	1.71[c]	32.00d	3.48[c]	4.00[b]
Control	4.00a	12.00a	4.00a	2.21[a]	86.66a	3.61[a]	5.00[a]
Grand Mean	3.50	11.00	3.50	2.01	59.00	3.54	4.75
SED	0.121	0.389	0.121	0.073	2.041	0.12	0.164
CD (0.05)	0.252	0.825	0.253	0.154	4.259	0.2621	0.342
CV (%)	5.75	5.71	5.75	5.83	6.19	5.70	5.75

*Mean of 30 observations; means followed by different letters within a row indicate significant differences (P<0.05; LSD)

Sublethal effects of Cry1Ab on the reproductive parameters of P. xylostella

Sub-lethal exposure of *P. xylostella* larvae to the Cry1Ab toxin significantly influenced the reproductive capacity of moths. The present results show the existence of physiological infection of Cry1Ab on *P. xylostella* during developmental stages. There was a significant reduction in adult emergence (90.00, 86.66, 80.00 and 96.66 per cent in LC_{25}, $LC_{50,}$ LC_{75} and control, respectively), increase in malformation (7.69, 15.38, 26.08 and 4.00% in LC_{25}, $LC_{50,}$ LC_{75} and control, respectively) and a significant decrease in fecundity (59.00, 55.00, 41.00 and 67.00 eggs/ 2 pairs % in LC_{25}, $LC_{50,}$ LC_{75} and control, respectively), oviposition period (5.00, 5.00, 4.00 and 5.00 days in LC_{25}, $LC_{50,}$ LC_{75} and control, respectively) and fertility (88.13, 80.45, 80.36 and 93.52 per cent in LC_{25}, $LC_{50,}$ LC_{75} and control, respectively) (Table 3). The present results thus, agree with the earlier studies of Salama *et al.* (1981). We believe that the low emergence, less fertility, less fecundity and malformation as a result of poor physiological condition of the survivors are the direct effects of the toxin on the sexual process.

Furthermore, the delayed growth and development are in turn significant from the concept of Integrated Pest Management, where insects die a slow death, exposing them to attack of parasitoids and predators (Gujar and Mohan 2000). The increased level of natural enemies interaction would aid in reducing the overall insect population. It may also reduce the risk associated with resistance development by decreasing the dominance and inheritance of resistance and such interactions are gaining importance as IPM is increasingly practiced in crucifer production systems.

CONCLUSION

The results of the above studies calculated LC_{25}, LC_{50} and LC_{75} values of 0.014, 0.115 and 0.916 ìg/ ml, respectively. Similarly, sub lethal doses of Cry1Ab on *P. xylostella* prolonged the total developmental duration *viz*., egg, larvae, pupae and adults. Cry1Ab also reduced larval and pupal weights, reduced adult emergence, increased malformation and reduced fecundity, oviposition periods and fertility. These results on *P. xylostella* may favour natural enemies interaction with the host insect and thereby reduce insecticide applications which may benefit ecological based sustainable IPM in cruciferous crops.

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TABLE 3

Sublethal effects of Bacillus thuringiensis *B.* δ*-endotoxin Cry1Ab on the reproductive parameters of Diamondback moth,* Plutella xylostella

Sub lethal dose (µg/ml)	Adult emergence * (%)	Healthy adult emergence * (%)	Malformed adult emergence * (%)	Adult longevity male (days)	Adult Longevity female (days)	Fecundity (No. of eggs laid) **	Fertility ** (%)
LC_{25} (0.014)	90.00[b]	92.30[b]	7.69[c]	8.00[b]	9.00[b]	59.00[b]	88.13[b]
LC_{50} (0.115)	86.66[c]	84.61[c]	15.38[b]	9.00[a]	10.00[a]	55.00[c]	80.45[c]
LC_{75} (0.916)	80.00[d]	73.91[d]	26.08[a]	9.00[a]	10.00[a]	41.00[d]	80.36[c]
Control	96.66[a]	96.00[a]	4.00[d]	8.00[b]	9.00[b]	67.00[a]	93.52[a]
Grand Mean	88.33	80.11	17.88	8.50	9.5	55.5	85.6[2]
SED	3.145	2.926	0.780	0.361	0.346	1.865	2.96[2]
CD (0.05)	6.560	6.103	1.628	0.753	0.722	3.889	6.18[0]
CV (%)	5.73	5.77	6.90	5.71	5.70	5.85	5.77

*Mean of 30 observations; means followed by different letters within a row indicate significant differences (P<0.05; LSD), ** No. of observation = 2 pairs

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Progress in The Development of Transgenic Cabbage, Cauliflower and Canola Expressing Stacked Bts for Caterpillar Control and RNAi for Aphid Suppression

D.A. Russell, D. Huang[1], P. Bhalla[2], M. Singh[3], C. Robin[4], J. Golz[5], A. Williams[6], R. Good[7], O. Edwards[8], B. Carroll[9], R.K. Bhatnagar[10], R.C. Bhattacharya[11], P. Kalia[12], G.T. Gujar[13] and V. Kalia[14]

Faculty of Veterinary and Agricultural Sciences, University of Melbourne, Parkville, Australia 3010
E-mail: derek.russell@unimelb.edu.au

[1 & 2] Faculty of Veterinary and Agricultural Sciences, University of Melbourne, Parkville, Australia 3010
E-mail: premlba@unimelb.edu.au

[3] Faculty of Veterinary and Agricultural Sciences, University of Melbourne, Parkville, Australia 3010
E-mail: mohan@unimelb.edu.au

[4]School of Biosciences, University of Melbourne, Parkville, Australia 3010. E-mail: c.robin@unimelb.edu.au

[5]School of Biosciences, University of Melbourne, Parkville, Australia 3010. E-mail: j.golz@unimelb.edu.au

[6]School of Biosciences, University of Melbourne. Parkville, Australia 3010. E-mail: a.williams@unimelb.edu.au

[7]School of Biosciences, University of Melbourne, Parkville, Australia 3010

[8]CSIRO Land and Water Flagship, Floreat, Western Australia. E-mail: o.edwards@csiro.edu.au

[9]School of Chemistry and Biological Sciences, University of Queensland, St Lucia, Australia
E-mail: b.carroll@uq.edu.au

[10]International Centre for Genetic Engineering and Biotechnology, Aruna Asif Ali Marg, New Delhi 110067
E-mail: raj@icgeb.res.in

[11]National Research Centre for Plant Biotechnology, Pusa Institute, New Delhi, India
E-mail: bhattacharyarc@rediffmail.com

[12]Division of Vegetable Science, Indian Agriculture Research Institute, New Delhi, India. E-mail: pritam.kalia@gmail.com

[13]Division of Entomology, IARI, New Delhi, India. E-mail: gtgujar@yahoo.com

[14]Division of Entomology, IARI, New Delhi, India. E-mail: vkalia_1998@yahoo.com

Abstract

Building on the earlier technical success of a Public Private Partnership in India using pyramided Bt genes *cry1B* and *cry1C* for the control of *Plutella xylostella, Crocidolomia pavonana, Hellula undalis and Pieris spp.* in cabbage and cauliflower, a consortium of Australian and Indian public institutions has been extending that work to include caterpillar control in canola in Australia and aphid control with RNAi in the three crops. A re-engineered Bt construct has proved effective in trials with *Arabidopsi*s and canola in Australia and is currently under test in transformed cabbage and cauliflower in India. The efficacy of constitutive and phloem specific promoters for aphid RNAi expression have been determined. *Myzus persicae* have been challenged with >20 RNAi sequences designed against genes involved in critical functions, without however identifying clearly effective, taxonomically unique, sequences. A targeted gene insertion system is under development to enable the insertion of aphid RNAi sequences next to existing Bt cassettes in the crop plants when effective sequences are demonstrated.

***Keywords*:** Bt brassicas, aphid RNAi, *Myzus persicae*, *Plutella xylostella*

The very considerable success of insect resistant transgenic crops, and in particular Bt crops, since 1996 has encouraged their deployment for the control of caterpillar and other insect pests in brassicas. Grzywacz *et al*. (2010) reviewed the potential contribution of caterpillar resistant Bt brassicas to farmers in Asia and Africa. Shelton *et al*. (2008) reviewed the progress in the development and use of Bts in brassica crops. Diamondback moth (DBM) was the first insect to develop resistance to sprayed Cry Bt proteins in the field (Tabashnik *et al*. 2003) resulting in work with stacked Bts with differing binding sites within diamondback moth which are better able to withstand resistance selection (Cao *et al.* 2002).

From 2002 to 2010 a public/private initiative, CIMBAA 'the *Collaboration for Insect management in Brassicas in Africa and Asia*' developed and tested stacked *cry1B/cry1C* Bt genes in cabbage and cauliflower for India with a view to commercialization there (Russell *et al.* 2008, 2011). Aphids are a major set of vectors of brassica viral diseases in addition to the damage caused directly by their feeding activity, but have not yet been shown to be sufficiently susceptible to Bts (even Bts modified with gut binding peptides (Chougule *et al.* 2013)) for Bts to provide a realistic prospect of control. A successor to the CIMBAA programme, the CARiB (Caterpillar and Aphid Resistance in Brassicas) (2012-16) project is attempting to use RNA interference for the control of key brassica aphid pests and to stack this with the *cry1B/cry1C* Bt combination.

The CIMBAA project (2002-2010)

A description of the structure of this public/ private partnership between Nunhems Seeds BV and the University of Greenwich, Cornell University, Melbourne University and AVRDC, the World Vegetable Center with support from a number of technical partners, notably TERI University and IARI in New Delhi, can be found in Russell *et al.* (2008). A discussion of the scientific advances made and the subsequent obstacles to commercialization resulting in the closing of this very technically promising programme can be found in Russell *et al.* (2008 and 2011) and Shelton *et al.* (2009). Here we briefly summarise the earlier technical advances on which the present work is predicated

After consideration of cross resistance patterns in diamondback moth between various Bt proteins (Zhao *et al.* 2001, 2003), the Bt gene combination *cry1B/cry1C* was chosen on the basis of efficacy, the polygenic resistance to Cry1C and having separate binding sites in the brush border membrane of diamondback moth (Ballister *et al.* 1999). Work using leaf-dip assays on 26 field collected populations in India, USA, China, Indonesia and Australia (Russell *et al.* 2008, Shelton *et al.* 2009) confirmed the efficacy of this protein combination against diamondback moth (with LC50s for Cry1B less than twice that of the standard susceptible (Geneva88) population and less than 3 times the value for the susceptible population for Cry1C). *Crocidolomia pavonana* and *Hellula undalis* also proved to be very susceptible to both of these proteins. *Pieris rapae* and *B. brassicae* were susceptible to Cry1B but *P. rapae* (from the USA) was rather less susceptible to Cry1C and *Spodoptera litura* and *Helicoverpa armigera* were considerably less susceptible to either protein.

The Bt *cry1B/cry1C* combination was transformed into cabbage and cauliflower varieties by the private sector partner and extensively tested and selected in greenhouse/ screenhouse trials in India from 2006 to 2010. As reported in Kaliaperumal *et al.* (2011) trials in India near Delhi and Bengaluru in 2008-2009 showed outstanding (100%) control of first instar *P. xylostella*, *C. pavonana* and *H. undalis* on selected elite lines of both cabbage and cauliflower. *P. brassicae* had no survival on Bt cauliflower but some larval survival (no pupation) on cabbage. *S. litura* mortality was > 90 % on cabbage and up to 80 % on cauliflower with no pupation on either crop. *H. armigera* was not well controlled but larval weights were strongly reduced with <4 % eventual pupation.

Attempts at Melbourne University to select for resistance in diamondback moth with Cry1B and Cry1C separately did not produce a resistance ratio over 20 generations of more than 20-fold. Resistance was autosomal, functionally recessive (dominance co-efficient (h) 0.24-0.29 for Cry1B in a range of field strains and 0.4 for Cry1B) and highly unstable, implying considerable fitness costs. Neither 'resistant' strain proved to have heightened tolerance to a Cry1B/ Cry1C protein combination and efforts to select with that combination achieved an unstable resistance of less than 4 fold (Behere, Nair, Russell and Gujar unpublished). Further selection for resistance was undertaken in India, reaching >400 fold for Cry1B compared with Indian field strains and 20 fold for Cry1C. Neither 'resistant' strain showed any improved survival on the selected *cry1B/cry1C Bt* plants although some insects fed for longer than did the susceptible controls.

Work on the IPM context for Bt brassicas in India (Kaliaperumal *et al.* 2011) showed that in sprayed Bt fields (regulatory restrictions prevented Bt crop planting in open fields) predators and parasites were more active than in fields under conventional management. Control of aphids in those fields could be managed for up to 45 days post planting using imidacloprid (as Confidor® 600 FS) in experimental soil drenching and seed pelleting treatments, with one to two applications of *Verticillium lecanii* thereafter. The < 10 % of *S. litura* and *H. armigera* experimentally inoculated into the plots which survived the Bt sprays, could be controlled with their species-specific NPVs at $1.04x10^9$ PIB.

Despite a number of setbacks – most notably the decisions by AVRDC and the University partners not to play a role in the commercialization phase of the project outputs - by 2010 the selected Elite Event cabbage and cauliflower were ready to enter the Indian biosafety approvals process. However, the combination of pressure to provide transformed germplasm to other public and private bodies before it was approved for commercialization, the problems of asynchronous de-regulation in other regional growing or import countries and the very unfortunate decision by the Indian Minister of Environment and Forests in early 2010 not to proceed with the commercialization approval of Cry1Ac Bt brinjal (egg plant) resistant to brinjal stem borer (despite the recommendation of the regulatory body (Shelton 2010), resulted in a decision of the CIMBAA Board to terminate this very technically promising programme in March 2010. Details can be found in Russell *et al.* (2011).

The CARiB project (2012-16)

The Australia-India Strategic Research Fund, which had been a funder for some of the CIMBAA technical work, agreed to fund a fully public sector programme building on the basis of CIMBAA and adding the dimension of stacking along with the Bts, RNAi control of the key aphid pests of cabbage, cauliflower (*Brassica oleracea*) and mustard (*B. juncea*) in India and canola (*B. napus*) in Australia. Indian partners are the International Centre for Genetic Engineering and Biotechnology, the National Research Centre for Plant Biotechnology and the Indian Agricultural Research Centre's divisions of Vegetable Science and Entomology. Australian partners are Melbourne University, CSIRO's Land and Water Flagship and the University of Queensland. The project commenced in May 2012.

Bt construct

The CIMBAA Bt construct patent was granted to the private partner in 2010 (just after the closure of the project). This construct was offered to the Indian Council of Agricultural Research (ICAR) for continued development of the Bt crops wholly in the public sector within the new project. Unfortunately, ICAR had no mechanism to accept the offer, necessitating a re-design of the DNA sequences at the University of Melbourne to avoid patent infringement and potential liability risks for the private partner in the earlier project.

RNA interference for aphid control

RNA interference technology (RNAi) aims to block the translation of mRNA into proteins. Success in the agricultural arena has been achieved in plant viral suppression eg. papaya ring spot virus (Gonsalves 2014), plum pox virus (Scorza *et al.* 2013) and, bean golden mosaic virus (Bonfirm *et al.* 2007) (see Good *et al.* proceedings of this meeting and Good et al. 2015 for details). Christiaens and Smagghe (2014) and Smagghe and Swevers (2014) have recently reviewed this field. RNAi can be highly specific, down to the level of species within a genus (Whyard 2015). A maize cultivar using RNAi designed against corn rootworm beetle (*Diabrotica virgifera*) is progressing through regulatory approval in the USA (Bolognesi *et al.* 2012, Bachman *et al.* 2013).

RNAi control of pest aphids has proved challenging. Mutti *et al.* (2006, 2008) were able to affect a salivary gland transcript in the pea aphid (*Acyrthosiphon pisum*) and Pitino *et al.* (2011) have shown RNAi induced gene silencing in *Myzus persicae* feeding on transgenic *Arabidopsis*. Coleman *et al.* (2015) have gone on to show fecundity/population growth reductions and transgenerational effects on gene expression using RNAi designed against *Rack1* (shuttling and anchoring proteins and interaction with ribosomal machinery and cell surface receptors), *MP2* (salivary gland effector protein) and especially *C002* (aphid-plant interaction modulator). However, this effect has not yet come close to being adequate for deployment as a field control strategy.

Three aphid species are significant pests of leafy and oilseed brassicas in India and Australia: *Myzus persicae* (green peach aphid), *Brevicoryne brassicae* (cabbage aphid) and *Lipaphis erysimi* (mustard or turnip aphid). The project is seeking to stack RNAi mediated control of these species with the Bt control for caterpillars. Use could be made of RNAi sequences specific to each species of aphid and/or of sequences common to these (and possibly other) aphids but absent in non-target organisms. In principle effective RNAi target genes should be expressed in tissues likely to be exposed to a high dose of dietary RNAi; be stably expressed and vital regardless of environmental variables; have sequences confined to target pest species and be absent in other species likely to be exposed to the RNAi in the particular agroecosystem; and have a minimal chance of resistance development.

The technical challenges here are to identify taxonomically circumscribed vital genes susceptible to RNAi effects (see Good *et al.* (2015 and proceedings of this meeting) for methods of identifying such sequences), to achieve high enough levels of phloem expression to produce deleterious effects on the aphids and then to successfully stack them with the Bts, initially in *Arabidopsis* and then in crop plants.

Materials and Methods

Genetic constructs

Bt constructs

Sequences containing *cry1Ba2* and c*ry1Ca4* driven by single or double subterranean clover stunt virus promotors and incorporating the *Bar* herbicide tolerance gene or the *NPTII* kanamycin gene as a selection marker were designed at the Univ. of

Melbourne, avoiding the earlier CIMBAA patent. These 'Bt-only' constructs were provided to IARI Vegetable Division for transformation into cabbage (cv Golden Acre) and cauliflower (cvs PBSK1 and Pusa Meghna) and to NRCPB for Indian mustard. These constructs were introduced into *Arabidopsis* in Melbourne by floral dipping with *Agrobacterium* and successful transformants selected using Basta.

RNAi sequences

RNAi sequences were designed against *M. persicae* sequences homologous to sequences from the *B. brassicae* partial transcriptome (the only available transcriptome from the three aphid species of interest). 20 genes in Australia and further candidates in India were targeted, drawn from genes involved in:

a. Phloem sucking – salivary proteins, phenol oxidase, other detoxifiers, aphid effectors, calcium binding proteins, osmotic pressure associated, C002
b. Buchnera associated – *Buchnera* encoded transporters, aphid encoded transporters, LOCs
c. Life cycle, parthenogenesis, telescoping of generations – ecdysone and juvenile hormone mechanisms
d. Dosage sensitive housekeepers – ribosomal proteins, elongation factors, other minutes, haplolethals, haplo/triplo lethals
e. Membranes – vacuolar ATPase, Alpha tubulin, ESCRT

dsRNAi was designed from candidate sequences and produced synthetically using Megascript® kits.

Bt+RNAi constructs in plants

An RNAi hairpin designed against four 'Best bet' candidate *M. persicae* genes (*Snf7, vATPase, ACE* and *C002*) which had shown transcript knockdown in published studies in a variety of organisms, were incorporated into a cassette with the *cry1B/cry1C* sequence. This construct was introduced using *Agrobacterium* into *Arabidopsis*, tobacco and canola and provided to the Indian team for possible use in cabbage, cauliflower and Indian mustard**.**

BIOASSAYS

Bt bioassays

Leaves from the selected Bt *Arabidopsis* and from the Bt+RNAi canola were placed on moist filter paper in 40 mm diam x 50 mm high plastic cups and bioassayed against first instar diamondback moth larvae (10 larvae on each of two leaves per plant). Mortality and leaf feeding damage were assessed at 48 hrs (and 72 hrs if there was any survival in the treatments at 48 hrs).

RNAi diet and microinjection bioassays

RNAi designed against candidate genes and synthetically produced were bioassayed against *M. persicae* (CSIRO clone 61) < 48hrs old in artificial diet feeding assays (7.5 ng/ul of diet) using 5 neonates/ replicate and 10 replicates per treatment. Diet was replaced after 3 days. Impacts were assessed by weighing the aphids in each replicate after 3, 4 and 5 days as *M. persicae* commence asexual reproduction from 6-7 days onwards. Leather *et al*. (1984) demonstrated a direct relationship between mean relative growth rates of aphids and fecundity/ population growth rate.

Using a photometric technique at the University of Melbourne to measure the plan area of test aphids, a group of 8 candidates RNAs was assayed over 12 days on artificial diet, with and without the addition of chitosan, a cationic RNAi stabilizer and protectant (10 aphids per treatment).

Microinjection of candidate RNAi into *M. persicase* was undertaken at ICGEB/IARI in New Delhi using a Nanoject II to deliver 46 nl of RNA to aphids in groups of 25, replicated three times. Control aphids were injected with nuclease free water. Injected aphids were placed on excised young leaves to feed and target RNA expression levels measured after 24 hrs.

RNAi + Bt bioassays with transformed plants

Efficacy against DBM was assayed as previously described. *M. persicae* bioassays utilized clip cages *(see Figure 6*) with 10 adult females per cage. The number of resulting aphids was measured 8 days later (the aprox. maximum time for good leaf quality in excised leaves)

RESULTS AND DISCUSSION

RNA expression in phloem

For interfering RNA to be present in useful quantities in the phloem, suitable promotors need to be found (perhaps with a focus on companion cell expression). Vascular tissue-specific expression would

be more energetically efficient than constitutive expression. Work at the Univ. of Queensland using yellow fluorescent protein mRNA in a x3YFP:NLS incapable of passing through plasodesmata, showed cell localization of RNA for *Dem2* (meristem and vascular tissue), *RolC* and *Suc2* (phloem expression). However, phloem expression of RNA using the constitutive promotor *35S* was > 10X higher than with the other promotors. Although SUC2 proved to be silenced less often than *35S*, *35S* has been the promotor of choice for subsequent construct design.

Bt expression in Arabidopsis

Cry1C expression in transformed *Arabidopsis* was 500-600 ppb (Figure 1), comparable to the levels in CIMBAA *B. oleracea* which had been shown to be effective in the control of a range of lepidopteran pests.

The use of single or double SCSV promotors to drive each Bt gene did not significantly affect Bt expression and the project has continued to use the double promotor version (as in CIMBAA).

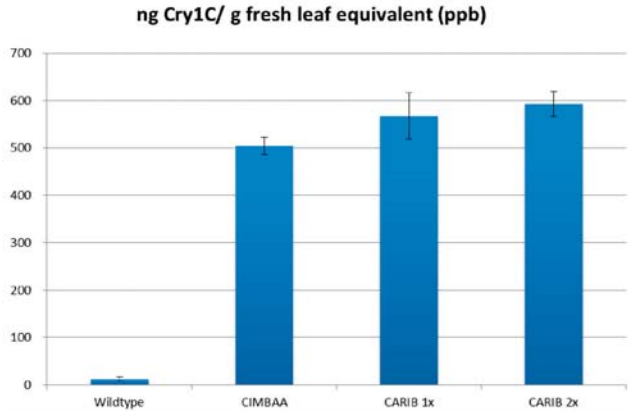

FIGURE 1

Bt Cry1C expression in CARiB Arabidopsis *(single and double SCSV promotors), CIMBAA* B. oleracea *(double SCSV promotor) and untransformed control. ELISA values ngCry1C/g fresh weight equivalent*

Efficacy of Cry1B/1C plants against DBM

The bioassay system using excised leaves in plastic cups is shown in Fig 2. Bioassay of Cry1B/1C expressing *Arabidopsis* with first instar DBM using 10 insects/ cup resulted in 99 % larval death by 48 hrs and 100 % in 72 hrs with no leaf damage (Figure 3 B), against 1.5 % deaths in the controls which showed extensive plant feeding damage at 48 hrs (Figure 3A).

Initial cabbage and cauliflower transformations at IARI using kanamycin as the selection agent resulted in plants on which all 1st instar DBM died within 24 hrs. The Basta selection on the *Bar* version saw some survival to 3 days.

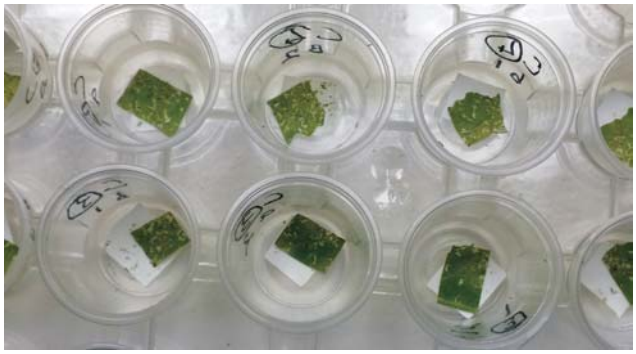

FIGURE 2

Canola leaf squares on moist filter paper in 5cm pots – controls damaged by DBM

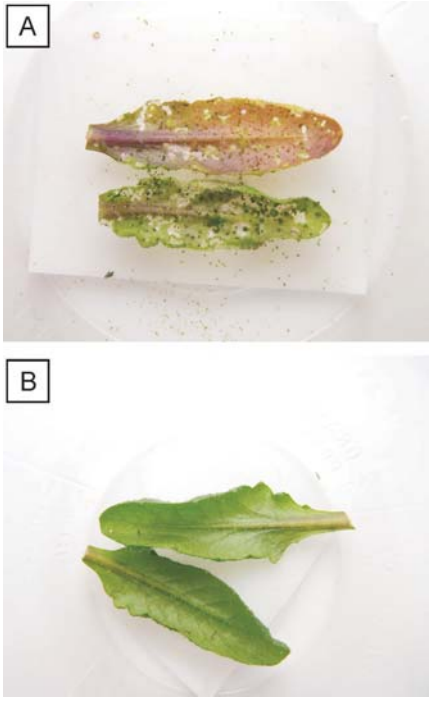

FIGURE 3

DBM bioassay Arabidopsis *(10 1st instar larvae per leaf). Leaf damage 3 days post inoculation. A. untransformed control; B. Cry1B/1C transformed*

RNAi diet bioassays with Myzus persicae

Artificial diet bioassay with *M. persicae* at CSIRO (10 replicates per treatment, 5 aphids per replicate) showed no significant differences in mean aphid weights after 5 days when compared to the controls in any of the 20 RNAi candidates tested.

Using average aphid size (plan area) as a measure of growth, artificial diet assays at the University of Melbourne showed no effect of 8 RNAi candidates (SHRUB, VPS24, VPS2, KAT 60, ATPase 69, ATP SYN B, RpS19a, RpS 13) delivered together, with or without chitosan (Figure 4).

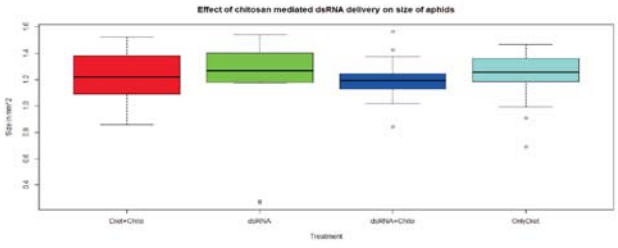

FIGURE 4

Effect on average size (plan area) after 12 days of M. persicae *fed 8 RNAi candidates, with or without chitosan as a stabilising agent, compared to a diet only control. (Line in box is the median, box boundaries include 50% of measurements, bars are the max. and min. observations and circles are individual outliers)*

The high osmotic pressure of their sugar rich diet is challenge for phloem feeding insects. Aphid osmoregulation requires a functional gut sucrase. IARI tested three siRNA sequences designed against a pea aphid *Acyrthosiphon pisum*) sucrase gene at 0.5µg siRNA/ gm in artificial diet assays (Table 1).

TABLE 1

Percentage mortality in B. brassicae *and* M. persicae *using three siRNAs designed from* A. pisum *sucrase gene*

Treatment 0.5µg siRNA/gm of diet	**% Mortality**		
	24hrs	**48hrs**	**72hrs**
Brevicoryne brassicae			
Control	0	6.6	13.3
APSUC1	16.6	23.3	53.3
APSUC2	10	43.3	73.3
APSUC3	3.3	33.3	60
Myzus persicae			
Control	0	10	30
APSUC1	10	20	43.3
APSUC2	10	40	50
APSUC3	6.6	30	43.3

Results for APSUC2 in particular are promising and further work is being undertaken.

RNA injection assays

Survival of M. persicae adults injected at ICGEB with nuclease free water (NFW) was c. 80 % (Table 2).

TABLE 2

Survival of M. persicae *adults in nuclease free water microinjection assays*

Sample	**Volume injected**	**No of aphids**	**Survival (Day)**			
			1	**2**	**3**	**% 3**
Injected	46 nl	25(x3)	23	22	20	80%
Control	46 nl	25(x3)	25	25	24	96%

Only one candidate gene target showed strong RNA knockdown as measured by RT-PCR following 24hrs of feeding on an excised leaf post injection (Figure 4) – a sucrase gene (A1). Further work is continuing with this target.

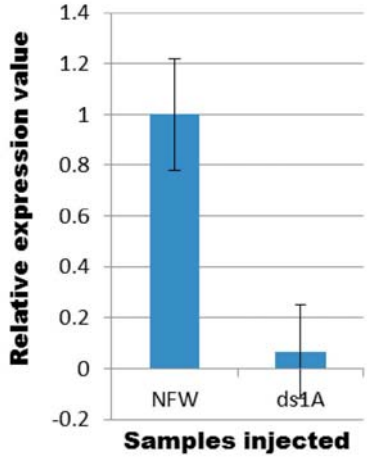

FIGURE 5

Relative RNA expression (+/- s.d.) as measured by RT-PCR of aphids injected with dsRNAi to a sucrose gene (1A) and nuclease free water (control)

Bt+RNAi 'Best-bet' construct assays in plants

Despite considerable efforts, canola transformation with the large construct proved challenging. Early assays with putatively transformed plants using 2 clip cages containing 10 adult *M. persicae* excised per leaf (Figure 6) did not result in significant reductions in fecundity compared with the controls. Elisa assay confirmed the presence of the Bt Cry1C sequence and PCR confirmed Cry1B and the RNAi construct only in one plant (transformant 45 of cv Bonanza). This plant was subsequently cloned and used for DBM and aphid bioassays. The DBM

bioassay used leaf squares (c. 2 cm^2) from 10 of the cloned plants, with 2 leaves per plant and two pieces of each leaf in separate cups and with 10 1st instar DBM per cup (400 larvae in all) (Figure 2). This resulted in 100% mortality of DBM in 48 hrs on the Bt transformed leaves as opposed to 2 % mortality of the 200 DBM on leaves from 5 non-transformed control plants.

However, despite confirming the presence of the RNAi constructs in the canola by PCR, bioassay using 2 clip cages on individual leaves of 10 plant clones with 10 adult *M. persicae* adults per clip cage, did not result in any reduction in aphid fecundity over 7 days (mean production 1.6 nymphs per adult) when compared with results from non-transformed control canola (mean 1.7 nymphs per adult).

FIGURE 6

Clip cages with 10 adult aphids/ cage on individual leaves from putatively RNAi transformed canola

Tobacco proved to be more readily transformed with the Bt + RNAi construct (as confirmed by Elisa and PCR). However, clip cage tests with individual leaves in vials of water, over 7 days, also did not show any difference in aphid fecundity between transformed and untransformed plants.

The Bonanza 45 plants using in these assays were close to seeding with leaves in early senescence and this work is being repeated using whole young plants from the next generation.

CONCLUSION

The redesigned *cry1B/1C* gene sequence expressed in *Arabidopsis* and canola is entirely effective in killing 1st instar DBM (efficacy in transformed cabbage and cauliflower is being tested now). The proteins express in *Arabidopsis* at a level close to that of the earlier CIMBAA cabbage and cauliflower plants.

The designed RNAi candidates did not result in slower *M. persicae* growth rates in diet bioassays individually or as a group of 8 (presented with or without chitosan). The four 'Best-bet' RNAi candidates incorporated as a single construct along with the Bts into tobacco and canola did not result in reductions in *M. persicae* growth rates using clip cages on leaves of mature plants. However, microinjection and diet bioassays of RNA candidates at ICGEB/IARI shows some initial prospects of knockdown of transcription using sucrase gene sequences.

However, the recent work of Coleman *et al.* (2015) suggests that population growth assays over a longer period (three generations – c. one month) may be necessary to show clear RNAi effects and this is being trialed now.

The project is working on a targeted gene insertion technology using a novel homologous recombination system. It is hoped that this will allow stacking, next to the Bt sequences in Bt transformed and characterized lines, of effective aphid RNAi sequences when these are proven

ACKNOWLEDGEMENT

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Evaluation of Insecticidal Activity of Insecticidal Crystal Related Protein (ICRP) Toxin from *Photorhabdus luminescens* for the Control of Diamondback Moth

M. Kshirsagar, S. Madankar[1], K. Babar[2], S. Parimi[3] and L. Alamalakala[4]
Maharashtra Hybrid Seeds Co. Ltd.. At: Dawalwadi, Tq. Badnapur, P.O. Box-76, Jalna-431203, India
E-mail: mangesh.kshirsagar@mahyco.com; [1]E-mail: santosh.madankar@mahyco.com
[2] E-mail: kiran.babar@mahyco.com; [3]E-mail: srinivas.parimi@mahyco.com; [4]E-mail: leela.alamalakala@mahyco.com

Abstract

The diamondback moth (DBM), *Plutella xylostella* (L.) (Lepidoptera: Plutellidae) is the most important pest of cruciferous vegetables worldwide. DBM has developed resistance to all classes of insecticides including *Bacillus thuringiensis* (Bt) spray formulations. Consequently it is becoming increasingly difficult to control this pest. One approach for effective pest control and resistance management is the deployment of proteins with novel modes of action. Here, we report the cloning of a gene of 411 bp from *Photorhabdus. luminescens,* designated as *Pl-ICRP* (*P. luminescens*- insecticidal crystal related protein) and the expression of histidine-tagged-ICRP recombinant protein (17 kDa) in *E. coli* BL21 (DE3) strain. The Pl-ICRP protein shares 29 % predicted amino acid sequence similarity to a 13.6 kDa crystal protein gene of *B. thuringiensis.* Protein expression was analyzed by SDS-PAGE and protein identity was confirmed by peptide mass fingerprinting. The insecticidal activity of His-tagged-ICRP protein was assessed against DBM larvae by cabbage leaf-dip bioassay. Larval mortality was observed in DBM at different Pl-ICRP protein concentrations (10 ppm-3000 ppm). Pl-ICRP could be a potential candidate for the development of transgenic crops resistant to the diamondback moth.

***Keywords*:** *Photorhabdus*, *Plutella xylostella*, ICRP, Leaf-dip bioassay

The diamondback moth, *Plutella xylostella* (L.) (Lepidoptera: Plutellidae) is considered to be the most destructive pest of cruciferous crops throughout the world (Talekar and Shelton 1993; Furlong *et al.* 2013). Due to its ability to rapidly develop resistance to insecticides (Zhao *et al.* 2006), chemical control of this pest remains difficult (Huang and Wu 2003; Li *et al*. 2012). Development of transgenic crops expressing insecticidal proteins is one of the most preferred strategies for pest control in agricultural systems, since it can lead to reduced insecticide applications and significant environmental benefits (Li *et al.* 2009). The success and benefits of transgenic technology can be compromised due to resistance evolution in target pests (Morin *et al.* 2004). One approach to effectively control DBM and manage resistance evolution is the deployment of novel insecticidal proteins or proteins with novel modes of action. Entomopathogenic soil bacteria produce a plethora of insecticidal proteins. Testing the activity of these bacterial proteins will facilitate the development of novel strategies for crop protection.

Photorhabdus luminescens, a gram-negative, entomopathogenic bacterium, produces a wide range of proteins with entomotoxic properties. These bacteria are associated with entomopathogenic nematodes of the family Heterorhabditidae (ffrench-Constant *et al.* 2003; Joyce *et al.* 2006), colonizing the intestine. *P. luminescens* produces a number of toxins that have been tested against different lepidopteran insect pests. The toxin complex (Tc) proteins (Waterfield *et al.* 2001) are insecticidal proteins that have been shown to be orally toxic to the Colorado potato beetle, *Leptinotarsa decemlineata* (Say) and sweet potato whitefly, *Bemisia tabaci* (Gennadius) (Blackburn *et al.* 2005). The *Photorhabdus* insecticidal toxin (Pit) protein demonstrated insecticidal activity against *Galleria mellonella* (Linnaeus) and *Spodoptera litura* (Fabricius) via hemocoel injection and inhibited the growth of *S. litura* and *Helicoverpa armigera* (Hübner) upon oral delivery (Li *et al.* 2009). Here we analyzed the insecticidal effects of a 411 bp gene designated as ICRP from *P. luminescens* subsp. *luminescens* strain Hb on laboratory-reared diamondback moth larvae. ICRP gene from *P. luminescens* was cloned into *E. coli* BL21 (DE3) cells for protein expression. Cabbage leaf-dip bioassay (Shelton *et al.* 1993) was conducted to evaluate the insecticidal activity of the partially purified Pl-ICRP protein against DBM larvae.

Materials and Methods

Selection of Pl-ICRP candidate gene

The *ICRP* gene from *P. luminescens* was shortlisted as a probable candidate gene for testing

insect toxicity. The *Pl-ICRP* nucleotide sequence is available in the GenBank database under accession number DQ305347.1.

Total genomic DNA isolation

Photorhabdus luminescens subsp. *luminescens* strain Hb obtained from the American Type Culture Collection was grown from a single colony in Luria-Bertani (LB) medium (1 % tryptone, 0.5 % yeast extract and 1 % NaCl, pH 7.0) at 28 °C for 18 h with shaking at 180 rpm. High-molecular weight genomic DNA was isolated using CTAB method (Daborn *et al.* 2003)

Amplification, cloning and sequence analysis of* Pl-ICRP *gene

Pl-ICRP gene-specific forward and reverse primers containing *NdeI* and *HindIII* restriction sites, respectively, were designed. The desired gene was amplified using *Taq* DNA polymerase (*Merck Genei*, India) with the following primer set: forward primer: 5'-ATAGCATATGTCAGAGAT CGAAGC-3' and reverse primer 5'-CAGAAG CTTGCTTACAATCATA-3' in a 50-µl reaction volume in a Veriti 96-Well Thermal Cycler (Applied Biosystems). PCR conditions were as follows: 3 min initial denaturation at 95 °C, followed by 35 cycles of denaturation at 95 °C for 30 sec, annealing at 44 °C for 30 sec, and extension at 72 °C for 2 min and final extension for 7 min at 72 °C. The 411 bp PCR amplicon was purified using QIAquick gel extraction kit (Qiagen), and cloned into the pGEM-T Easy vector (Promega, USA). Competent *Escherichia coli* DH5á cells were used as a host strain for transformation, and the transformed *E.coli* DH5á were grown on LB agar plates with ampicillin (100 µg/ml) for 16 h at 37 °C. Broth cultures were grown from a single colony in LB containing ampicillin (100µg/ml) for 16 h with constant agitation (37 °C, 180 rpm). The recombinant plasmid was isolated using alkaline lysis method (Birnboim and Doly 1979). Gene cloning was confirmed by restriction digestion followed by sequencing (Applied Biosystems 3130xl Genetic Analyzer).

Expression of Pl-ICRP recombinant protein in E. coli BL21 (DE3) cells

The pGEM-T Easy recombinant plasmid containing *Pl-ICRP* gene was digested with *Nde* I/ *Hind* III restriction enzymes and resulting gene product was cloned into *pET-20b* (+) expression vector. Recombinant *pET-20b* (+) plasmid with *Pl-ICRP* gene was used for transformation of *E. coli* BL21 (DE3) cells. An isolated colony harboring *Pl-ICRP* gene was pre-cultured in 5 ml LB medium containing ampicillin (100 µg/ml) for 16 h with constant agitation (37 °C, 180 rpm). This pre-culture was added into 500 ml LB broth with ampicillin (100 µg/ml) and incubated for 8 h with constant agitation (37 °C, 180 rpm). Expression of ICRP was induced after 8 hours with Isopropyl 1-thio-â-D-galactopyranoside (0.5 mM IPTG final concentration) and the culture was incubated further at same conditions for 16 hours for the induction of protein expression. The bacterial culture was centrifuged for 10 min at 10,000 rpm at 4 °C and the resulting pellet was suspended in lysis buffer (10 mM Tris buffer pH 8.0, 100 mM NaCl, 10 mM Imidazole, 100 µM Phenyl methylsulfonyl fluoride (PMSF) and 1 % Triton X100). The cell suspension was vortexed and sonicated by applying twenty 15 sec pulses separated by 15 sec cooling (0.250, TRANS-O-SONIC, Mumbai). Sonicated cell lysate was centrifuged at 10,000 rpm for 30 min at 4 °C. Soluble cellular supernatant was collected. Protein purification was performed at room temperature with gravity flow using Protino® Columns (35 ml) containing 1ml of Ni-NTA Agarose following the protocol described in The QIAexpressionist™ (Qiagen). After eluting the 6X His-tagged Pl-ICRP protein, dialysis was performed for desalting to prevent the potential toxic effects of imidazole in insect bioassays. The purified protein was quantified on Nanodrop 8000 spectrophotometer (ThermoFisher Scientific). The affinity column purified protein band (15 kDa Pl-ICRP with 2 kDa 6X-His tag) was analyzed by SDS-PAGE and Coomassie brilliant blue R-250 (Sigma-Aldrich) staining. The protein was confirmed by MALDI-PMF analysis.

Insect bioassay

Toxicity of ICRP was assessed using a leaf-dip bioassay. The DBM population used in this study was a laboratory colony maintained in the insectary (Mahyco, India). Leaf tissue from the cabbage plants (*Brassica oleracea* var. Dynasty) raised in the greenhouse was used for leaf-dip bioassays. The cabbage leaf was cut into squares (2 x 2 cm) covering either side of midrib, and individual leaf squares were immersed in the 6X His-tagged-Pl-ICRP protein

solutions of different concentrations (i.e. 10 ppm, 100 ppm, 1000 ppm, and 3000 ppm) for 30 sec and allowed to air-dry for 1 h. For the controls, buffer (5 mM Tris, 100 mM NaCl) was used for dipping the leaf squares (for buffer control) and untreated cabbage leaves were used as untreated control. Moist filter paper (Whatman No. 1, 90 mm diameter) was placed inside an 8.75 cm diameter plastic Petri dish and treated cabbage leaf tissue was placed on top of the filter paper. Two first instar DBM larvae were released on a single cabbage leaf square in each Petri dish and 10 replications were prepared, resulting in a total of 20 larvae per treatment. The assay was maintained at suitable growth conditions (26 - 28 °C temperature and 65 - 70 % humidity) in a dark room. Observations on the larval mortality were recorded 72 h after the release of larvae.

Results and Discussion

Cloning and bioinformatics analysis of Pl-ICRP *gene*

The Pl-ICRP gene cloned in pGEM-TEasy vector and *pET-20b* (+) expression vector showed 100 % nucleotide sequence similarity with sequence available in the NCBI database.

Expression of Pl-ICRP recombinant protein in E. coli *BL21 (DE3) cells*

SDS-PAGE analysis of his-tagged-Pl-ICRP protein purified by Ni-NTA chromatography showed good expression of the recombinant protein at the expected size (17 kDa) confirming the expression of Pl-ICRP in *E.coli* BL21 (DE3) cells (Figure 1).

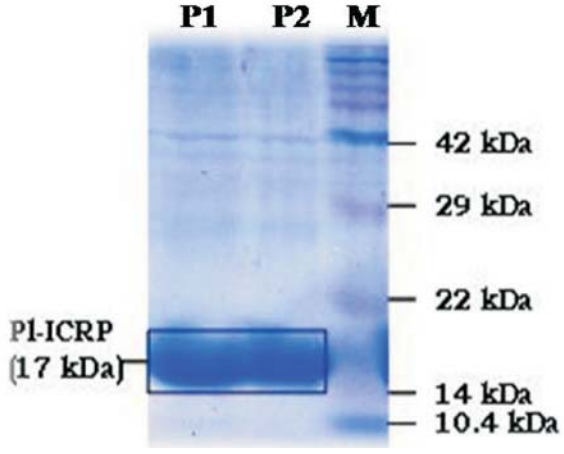

Figure 1

SDS-PAGE analysis (Coomassie blue stained) of 6X-His-tagged-Pl-ICRP protein (17 kDa), Lane P1 and P2- His-tagged-Pl-ICRP protein; M-molecular weight marker

Pl-ICRP protein identification

The Pl-ICRP protein band (17 kDa) analyzed by MALDI-TOF **(Figure 2 and Figure 3)** (Sandor Proteomics Pvt. Ltd., Hyderabad, India) further confirmed the presence of the desired protein.

Mascot Search Results

Search title	:
Database	: NCBInr 20141019 (51471198 sequences; 18460256539 residues)
Taxonomy	: Bacteria (Eubacteria) (34995986 sequences)
Timestamp	: 27 Oct 2014 at 06:24:54 GMT
Top Score	: 91 for gi|83629109, insecticidal crystal-related protein [Photorhabdus luminescens subsp.

Figure 2. MALDI-TOF analysis of Pl-ICRP protein

gi|83629109 **Mass:** 15042 **Score:** 91 **Expect:** 0.026 **Matches:** 8

insecticidal crystal-related protein [Photorhabdus luminescens subsp. luminescens]

	Observed	**Mr(expt)**	**Mr(calc)**	**ppm**	**Start**	**End**	**Miss**	**Peptide**
1.	832.2715	831.2642	831.3723	-129.93	52	59	0	R.SNDNTPGK.A
	844.3723	843.3650	843.4814	-137.96	29	35	0	K.LIQDSIR.L
	1085.5848	1084.5775	1084.6604	-76.41	27	35	1	K.LKLIQDSIR.L
	1248.5358	1247.5285	1247.6106	-65.77	64	76	0	R.SDSNSLLGGVSGR.C
	1358.5948	1357.5876	1357.6738	-63.56	52	63	1	R.SNDNTPGKAIWR.S
	1866.8599	1865.8526	1865.9636	-59.44	36	51	0	R.LDQGEWTTLPPQVINR.S
	2692.3269	2691.3196	2691.4344	-42.65	29	51	1	K.LIQDSIRLDQGEWTTLPPQVINR.S
	2933.4813	2932.4740	2932.6134	-47.52	27	51	2	K.LKLIQDSIRLDQGEWTTLPPQVINR.S

Figure 3

MALDI-TOF MS analysis of tryptic digests of Pl-ICRP protein fragments

Cabbage leaf-dip bioassay

Leaf-dip bioassays were conducted to determine the efficacy of Pl-ICRP protein against diamondback moth larvae**.** Growth inhibition was observed in *P. xylostella* larvae fed with cabbage leaves dipped in different concentrations (i.e. 10 ppm, 100 ppm, 1000 ppm, and 3000 ppm) of 6X-His tagged Pl-ICRP after 72 h (Table 1). The dead and moribund *larvae were recorded* after 72 h as *larval mortality*. Larval mortality greater than 75% was observed when Pl-ICRP protein concentrations of 1000 ppm or more were used to treat leaf squares. In leaf squares treated with 100 ppm of protein around 65-75 % larval mortality was observed. The lowest protein concentration used in this study was 10 ppm in which 25-40 % larval mortality was observed. No mortality was observed in DBM larvae feeding on untreated cabbage leaves. Less than 5 %mortality was observed in larvae feeding on leaves used as buffer control, which confirmed that the Pl-ICRP protein is responsible for mortality and growth inhibition of DBM larvae.

TABLE 1

Toxicity effect of His-tagged-Pl-ICRP protein on DBM larvae

Treatments	Percent mortality
Untreated Diet	0%
Buffer	<5%
10 ppm protein	25-40%
100 ppm protein	65-75%
>1000 ppm protein	> 75%

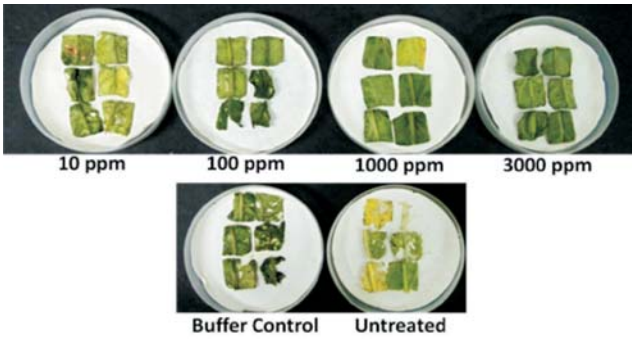

FIGURE 4

Leaf feeding assay-feeding damage by DBM larvae in Pl-ICRP treated (10 ppm, 100 ppm, 1000 ppm and 3000 ppm) and control (buffer treated and untreated) leaves

Leaf tissue damage due to insect feeding was minimal or not observed in leaves treated with ICRP protein (Fig. 4). At lower protein concentrations larval growth inhibition was observed whereas mortality was observed in insects feeding on leaves treated with protein concentrations > 1000 ppm. The bioassay clearly demonstrated the toxicity of Pl-ICRP protein to *P. xylostella* larvae when introduced orally and the observed biological effects *viz.*, larval mortality or growth inhibition was dependent on the protein concentration used.

CONCLUSION

We investigated the insecticidal activity of Pl-ICRP protein and established the efficacy of ICRP on diamondback moth larvae. The Pl-ICRP gene was amplified, cloned and sequenced. Sequence analysis showed that Pl-ICRP protein shares 29 % predicted amino acid sequence similarity to a 13.6 kDa crystal protein gene of *B. thuringiensis*. A recombinant protein of 17 kDa was successfully expressed in *E.coli* (BL21) cells. Cabbage leaf-dip bioassays showed that the Pl-ICRP possesses oral insecticidal activity towards *P. xylostella* larvae. The mortality of DBM larvae exposed to cabbage leaves treated with different concentrations of protein was significantly higher than that of DBM larvae exposed to leaves treated with buffer establishing the insecticidal potential of Pl-ICRP protein. It is hypothesized that the feeding inhibition probably caused the mortality of the DBM larvae. Gut histology studies will be carried out to decipher the mode of action of the protein. The present study thus opens up an avenue for developing transgenic crops resistant to diamondback moth based on the Pl-ICRP gene.

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OffTargetFinder – A Web Tool for RNAi Target Design for *Brassica* Aphids and Other Pests

R.T. Good, T. Varghese[1], O. Edwards[2], C. Robin[3] and D.A. Russell[4]

Genetics Department, Univeristy of Melbourne, Parkville, Australia 3010. E-mail: rtgood@unimelb.edu.au
[1]Atlas of Living Australia, Facilities & Collections, CSIRO, Canberra, Australia 2601. E-mail: temi.varghese@csiro.au
[2]CSIRO Land and Water Flagship, Canberra, Act 2601, Australia. E-mail: o.edwards@csiro.edu.au
[3]Genetics Department, Univeristy of Melbourne, Parkville, Australia 3010. E-mail: c.robin@unimelb.edu.au
[4]Faculty of Veterinary and Agricultural Sciences, University of Melbourne, Parkville, Australia 3010
E-mail: derek.russell@unimelb.edu.au

Abstract

Increasingly, pesticide resistance is emerging to a plethora of chemicals and alternative methods of control are urgently needed. The use of RNA interference (RNAi) to study gene function has become relatively commonplace in experimental animal models (*C. elegans, Drosophila, Danio, Mus*, etc) but it is increasingly becoming used as a tool in crop pest management. A major advantage of RNAi is the exquisite specificity of the process which means that it is possible to differentially inhibit the function of alleles that vary by only one SNP and so to target the specific gene sequence in particular insect species.

One delivery method for RNAi involves the molecule being genetically incorporated into plants being fed upon by the target pest(s). Potential concerns of the regulatory authorities as to the effects of RNAi on non-target species (especially beneficial ones) has prompted us to develop a bioinformatic tool to search the known transcriptional space for possible RNAi interactions that could potentially harm species other than the intended targets.

'*OffTargetFinder*' is a web-based tool for searching for suitable RNAi targets for introduction into potential transgenic organisms (including crops). We have used it to design RNAi sequences for *M. persicae* and other brassica aphids in the context of a larger project for aphid control in cabbage and cauliflower in India and canola in Australia.

'*OffTargetFinder*' uses the 'bowtie' algorithm to rapidly search transcriptomes (currently 102, predominantly insects, plus some model organisms) for matching 21mers (with or without mismatches) for small interfering RNAi (siRNA) sequences of interest, which are then visualised in the '*canvasxpress*' genome browser package. The various outputs are then used to refine the appropriate region of a gene of interest to eliminate potential off-target effects in other arthropod species. Here we present a case study of the *Myzus Rdl* gene showing the capacity of OffTarget Finder to show the level of specificity to the target species.

Keywords: Myzus persicae, RNAi, off-targets, Off Target Finder

RNA interference technology (RNAi), which blocks the translation of mRNA into proteins, has been used for a number of years in transgenic plants capable of defending themselves from viral attack (Papaya Ring Spot Virus (Gonsalves 2014), Plum Pox Virus (Scorza *et al.* 2013), Bean golden Mosaic Virus (Bonfirm *et al.* 2007). The use of RNAi for insect pest control is now an advancing field (Smagghe and Swevers 2014). RNAi formulations have been proposed as species-specific (or species assemblage specific) insecticidal sprays and a genetically modified maize product using RNAi designed against a corn rootworm beetle (*Diabrotica virgifera*) Snf7 gene has been shown to be lethal and is in the advanced stages of regulatory approval by Monsanto (Bolognesi *et al*. 2012; Bachman *et al.* 2013). The extremely high level of species specificity attainable with RNAi (down to species specificity within a genus (Whyard 2015)) is a great advantage of the method, minimizing unintended effects on non-target organisms both within and beyond the crop. However, specificity can only be assured if the targeted gene sequence is unique to the particular target organism(s). The active mechanism of RNAi for insect control involves the ingestion of double stranded RNA (dsRNA) applied topically on the plant by spraying or as a plant produced molecule. Within the target insect the dsRNA is then cut by Dicer enzymes into short double stranded fragments of c. 21 nucleotides – small interfering RNA (siRNA). Each siRNA strand unwinds into two single RNA molecules, a passenger strand which degrades and a guide strand which is incorporated into an RNA-induced silencing complex (RISC). When the guide strand binds to a

complimentary mRNA sequence in the cell cytoplasm this induces cleavage of the mRNA by the RISC enzyme Argonaute. In the nematode *Caenorhabditis elegans* and some other organisms this process is able to spread systemically, allowing major suppression of a gene product from an initially very limited quantity of RNAi ingested. In the corn rootworm example, Bachman *et al.* (2013) showed that, of the 10 insect families surveyed, only a few closely related beetles were affected and that these shared at least three of the 21-mers within the Snf7 sequence. In general, it would seem likely that only fairly conserved gene sequences in quite closely related organisms would share identical, multiple, 21 nucleotide sequences. However, given the enormous number of potential off-target organisms in the world and the possibility that some may share sequences, there is concern within the scientific regulatory community that evidence be provided of the level of specificity of siRNAs resulting from specific dsRNA sequences. Making detailed searches of the specificity of all possible 21mers across the genomes available would be a very time consuming process if not automated, and the chance of missing matching sequences would be very high.

The authors are partners in a project aimed at adding aphid control by RNAi to the existing Bt control of caterpillars in brassica crops (cabbage and cauliflower in India and canola in Australia) (Russell *et al.* 2011; Russell *et al.* Proceedings of this meeting,) although this is expected to be challenging (Christaens and Smagghe 2014). Of the three target aphid species, *Myzus persicae (*green peach aphid), *Bervicoryne brassicae* (cabbage aphid*)* and *Lipaphis erysimi* (the mustard or turnip aphid), a partial transcriptome is available only for *B. brassicae*. As part of this programme we have developed software – OffTargetFinder (Good *et al.* 2015) which very greatly simplifies the process of checking sequence uniqueness and identifying taxonomic groups which might need to be screened for off-target effects if they occur in the agro-ecosystem of interest (Romeis *et al.* 2013).

Materials and Methods

OffTargetFinder searches specific transcriptomes for close nucleotide sequence matches against all the possible 21 nucleotide siRNA molecules which may be produced from a provided DNA target sequence. Bowtie software (Langmead *et al.* 2008) using a custom PEARL script is used for the search which is then visualized in the CanvasXpress genome browser (http://canvasxpress.org). The database of transciptomes searched includes sequences from the 1000 Insect Transcriptome Evolution (1KITE) data set (http://www.1kite.org) which is still in the process of development by an international consortium (Misof *et al.* 2014). At the time of writing, the searchable database includes 102 transcriptomes (mostly insects) from the Arthropoda (Hexapoda, Crustacea, Myriapoda and Chelicerata). Using Exonerate (Slater and Birney 2005) we have used the pea aphid transcriptome to annotate the *M. persicae* genome and it is included in the data set as are human, chicken, *Arabidopsis* and some other representative transcriptomes.

OffTargetFinder can be accessed free at http://146.118.96.106.rnai/.

Using the Program

Entering a DNA sequence

The user pastes into the definition line a chosen DNA sequence (one which is potentially a good target in terms of its vital importance to insect survival) in FASTA format (coding for 24 amino acids plus 3 special codes), leaving no blank spaces. The system allows a user to specify the allowed number of mismatches (0,1,2,3) for which they wish the programme to show returns. At present the programme runs best in Chrome or Firefox browsers and less well in Explorer.

Submitting a query

Pressing the 'FindOffTargets' button submits the query. Processing takes c. 15 mins depending on the number of mismatches allowed. The job status appears in the 'History' table (Figure 1). When the job is complete the green text shows that hits were found and red where none are found. Clicking the 1Kite motif for a search shows the full results.

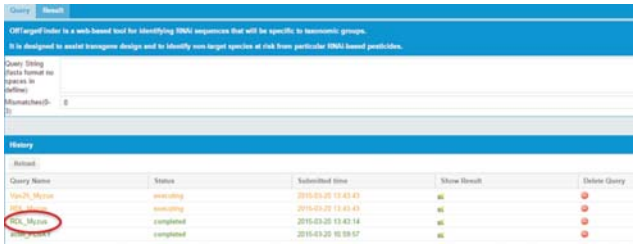

Figure 1

List of records on 'OffTargetFinder', 1 Myzus *and one* Plutella *search complete, 2* Myzus *queries still running*

Results and Discussion

Results are shown here for the *Myzus persicae* Rdl gene query sequence to demonstrate the tool's functionality.

Results panels

The results page shows the taxonomic position of each organism which has returned sequence alignments within the target sequence (allowing 0,1,2 or 3 mismatches as selected by the user). Several views of the data are possible by selecting from the dropdown menu under the 'View' button.

Browser View

This view shows the position of the matching sequences for each species returning a hit (in yellow) against the target sequence (in red). With no mismatches (Figure 2) 10 species are shown to have at least one 21-mer of sequence identity within the target sequence.

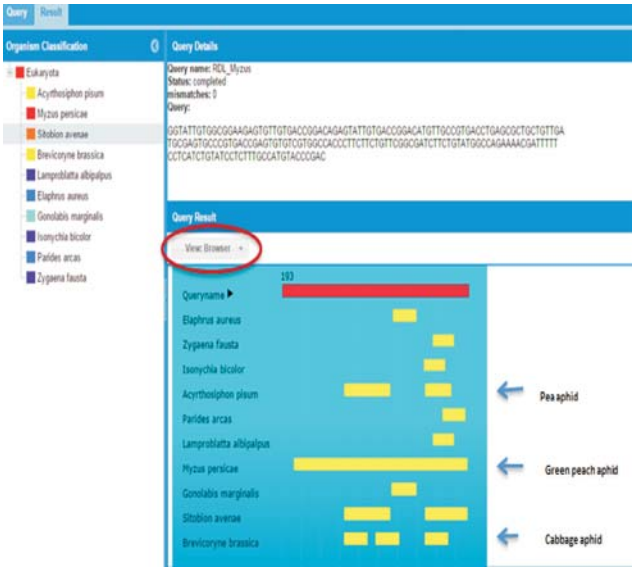

Figure 2

Myzus persicae *RDL gene – Browser view of sequence identity hits for other species (0 mismatches in 21-mer allowed)*

Raw Data View

This view lists the species returning hits and the number of those hits within the sequence. In Figure 3, three other aphids in the list of organisms showed hits with >20 21-mer matches to the *M. persicae* sequence, while the other organisms had <5.

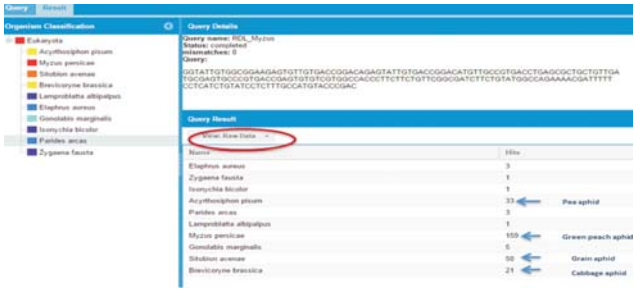

Figure 3

Myzus persicae *Rdl Gene – Raw data view showing the number of 21mer hits on the target sequence per Off-Target species. (0 Mismatches per 21-mer allowed)*

Bar Graph view

This view (Figure 4) has the x-axis as the species for which hits are returned and the y-axis shows the number of hits within the sequence (i.e. graphs the Raw Data view).

Figure 4

Myzus persicae *Rdl gene – Bar Graph view showing the number of 21mer hits on the target sequence per off-target species. (0 mismatches per 21-mer allowed)*

Query Hit Regions View

This view shows the exact nucleotide sequence hits within the supplied target sequence, which can be selected in the left panel for any taxon level which has returned hits. The matching sequences are shown in yellow for single hits in and in red for double hits. Figure 5 shows the result specifically for *Brevicoryne brassicae*.

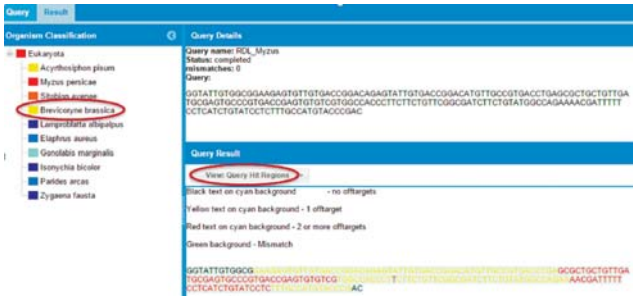

Figure 5

Myzus persicae *Rdl gene – Query Hit view showing the number of 21mer hits on the target sequence per off-target species. (0 mismatches per 21-mer allowed)*

Conclusion

The example given in this paper shows how OffTargetFinder may be used to refine the target sequences for experimental testing of RNAi. Within a longer gene sequence query, there may be stretches of nucleotides which are not shared with the other organisms in the data base. These may prove to be better targets in terms of species specificity and avoidance of accidental non-targets, though those non-target species would need to be present in the agro-ecosystem in which the RNAi strategy was to be used for any potential adverse impacts to be seen in practice. On the other hand, where multiple species are legitimate targets for pest management, sequence identity which is limited to that pest group may provide appropriate RNAi targets (*e.g*., In Figure 5, *B. brassicae* is a target aphid pest for the authors' current RNAi project along with *M. persicase* and there are shared sequences available).

OffTargetFinder (http://146118.96.106/rnai/) is a freely available web-based tool which allows checking for 21 –mer matches within proposed dsRNA molecules for unintended sequence identity with non-target organisms, and consequent sequence refinement for improved specificity. Currently this search facility is limited to just over 100 insect sequences (most from the 1Kite verified data base) but it is intended to add insect and other genomes as they become publically available.

Acknowledgement

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Selection and Inheritance of Cry1Ac Resistance in Diamondback Moth

S.R. Sandhya, S.N. Mandlik[1], D.K. Shirale[2], M.G. Patil[3] and S. Parimi[4]
Maharashtra Hybrid Seeds Co. Ltd., Jalna. 431203, India
E-mail: sandhya.sr@mahyco.com; [1]E-mail: suneetamandlik@gmail.com; [2]E-mail: dattatray.shirale@mahyco.com;
[3]E-mail: mangesh.patil@mahyco.com; [4]E-mail: srinivas.parimi@mahyco.com

Abstract

Diamondback moth (DBM) (*Plutella xylostella*), is a major pest on all the vegetables belonging to the family Brassicaceae. It was the first insect reported to have evolved resistance to Bt formulations in the field. There are several studies in DBM that demonstrate the laboratory-selected resistance to Bt proteins. In this study, a laboratory colony, DB-RM, was selected with Cry1Ac protein for several generations. DB-RM colony demonstrated 900-fold resistance compared to a laboratory susceptible colony. With an objective of understanding the inheritance of the selected Cry1Ac resistance in DB-RM colony, male and female larvae of resistant and susceptible colonies were separated at third instar stage and reared individually. The adults that emerged from resistant (DB-RM) and susceptible colonies were used as mating pairs for the crosses *viz*., susceptible (♀) x DB-RM (♂) and DB-RM (♀)) x susceptible (♂). The F1 neonates of these crosses were subjected to bioassay using Cry1Ac protein and observations were recorded after 72 hrs. The LC_{50} values were calculated and were used to estimate the dominance ratio (*D*). The calculated LC_{50} values of the crosses DB-RM (♀) X S (♂) and DB-RM (♀) x S (♂) were 0.015 and 0.0061 ppm, respectively. The degree of dominance (*D*) as determined by Stone's formula indicated that the resistance to Cry1Ac protein in DBM larvae is an autosomal trait that is inherited in an incompletely recessive or partially recessive manner.

Keywords: *Plutella xylostella*, Cry 1Ac, inheritance

Crucifers are among the most important vegetables used for consumption. Diamondback moth (DBM), *Plutella xylostella* (L.) (Lepidoptera: Plutellidae) is a destructive insect pest of cruciferous vegetables. The larval infestations of the crowns or growing points of young plants causes contamination and substantial economic losses in cabbage and cauliflower. In India the losses can be up to 90% when no insecticides are used and around 35% when used (Sandur 2004). The wide spread use of synthetic insecticides resulted in resistance of the pest to many insecticides (Sandur 2004). Several Bt proteins have been documented to be effective against DBM and are being used in developing cabbage/cauliflower tolerant to DBM. Consequently, understanding the risks involved in resistance development to these proteins and deciphering the mechanisms of resistance inheritance are important for designing sustainable resistance management plans. Here we present results of a study conducted to evaluate the inheritance of Cry1Ac resistance in DBM.

Materials and Methods

Two colonies of the insect were used in our study *viz*, laboratory susceptible colony (S) and a Cry1Ac-resistant colony (DB-RM). The Cry1Ac-resistant DB-RM colony was developed through selection of DBM larvae using MVP II as the source of Cry1Ac protein. The selection of DBM to Cry1Ac was done at a concentration of 0.010 ppm initially and was later increased in successive generations.

A resistance inheritance study using a susceptible and resistant colony of DBM was carried out in laboratory conditions. Initially the third instars of each colony were identified and separated for rearing on cabbage leaves in separate containers. When the adults emerged from these containers, the adults of Cry1Ac-resistant and susceptible colonies were allowed to mate individually, resulting in development of two pairs of crosses *viz.*, Susceptible Female *X* Resistant Male and Resistant Female *X* Susceptible Male. The F1 progeny produced by single pair matings in separate containers were used in the bioassays.

The F1 neonates of these crosses were subjected to cabbage leaf dip bioassays with different concentrations of the Cry1Ac protein. The leaf discs were dipped in protein dilution and allowed to air dry for 30 min. Then four 48hr-old larvae were placed on the leaves. The leaves were dipped in 0.1 % Triton solution before dipping in the protein dilutions. Observations were recorded after 72 hrs of incubation for mortality and survival of larva. A temperature of 27 $\pm$ 1°C and relative humidity of 65 % was maintained in the incubation chamber.

Mortality data were subjected to statistical analysis by using probit analysis (POLO Software) and LC_{50} values and the estimate of dominance (D_{LC}) were calculated. The degree of dominance (*d*) was determined using the formula of Stone (1968) [$d = (2x2 - x1 - x3)/x1 - x3$, where, $x1$, $x2$, and $x3$ are the logarithms of the LC_{50} (concentration for 50 % lethality) values for resistant, F1 hybrid, and susceptible strains, respectively. The estimate of dominance (D_{LC}) = (d+1)/2.

Results and Discussion

The susceptibility value of the resistant colony increased 900-fold compared to the susceptible laboratory colony after 64 generations of exposure to Cry1Ac protein (Fig 1).

The LC_{50} value of resistant parent (DB-RM) after 64 generations of exposure (at 65th generation) was 0.728 ppm where as susceptible laboratory colony exhibited LC_{50} value of 0.0028 ppm. The LC_{50} values were 0.015 and 0.0061 ppm for the crosses DB-RM (♀) X S (♂) and DB-RM (♀) X S (♂), respectively (Table 1). The D_{LC} value varies from *0* to 1; 0-0.2 indicates recessive, 0.2-0.4 indicates incompletely recessive or partially recessive, 0.4-0.6 indicates semi-dominant, 0.6-0.8 indicates incompletely dominant or partially dominant, and 0.8-1.0 indicates dominance of resistance inheritance. The dominance ratios of the crosses were estimated at 0.30 and 0.14, for DB-RM (♀) X S (♂) and DB-RM (♀) x S (♂), respectively, which indicated that F1 is partially or completely recessive (Table 1).

Generally, DBM resistance to the Cry1A toxins has been found to be completely or partially recessive (Hama *et al.* 1992; Tabashnik *et al.* 1992; Martínez-Ramírez *et al.* 1995; Tabashnik *et al.* 1997; Tang *et al.* 1997 a & b; Sayyed *et al.* 2000).

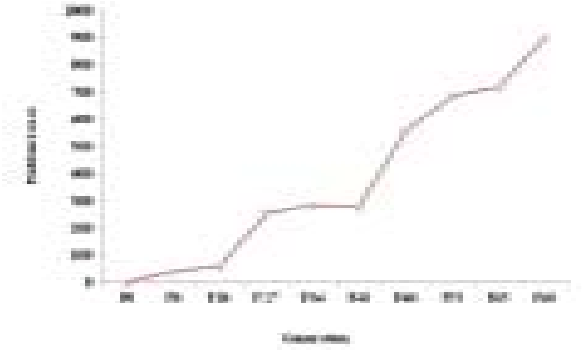

Figure 1

Fold increase in susceptibility value of resistant colony in comparison with the susceptible laboratory colony

Table 1

Summary of assay data and the heritability values estimated through Cry 1Ac leaf-dip bioassay

Population	n	χ^a	LC_{50} (95% CI) ^{b}	Dominance Ratio
DB-RM (Resistant)	120	0.18	0.728 (0.900-0.99)	-
S (Susceptible)	120	1.44	0.0028 (0.00018-0.0078)	-
DB-RM(@&) x S(B&)	140	1.12	0.015 (0.004-0.029)	0.30
DB-RM (B&) x S(@&)	140	2.21	0.006 (0.002-0.012)	0.14

Cry1Ac resistance in DBM has never been found to be dominant, although a Thai strain appeared to have polyfactorial control of resistance (Imai and Mori 1999). Insect bioassays and assessment of resistance inheritance results preliminarily suggested the inheritance of resistance to a low level of Bt broccoli in a Cry 1Ac resistant strain to be recessive (Dengxia *et al.* 2015). Reciprocal genetic crosses between Cry1Ac-reselected and susceptible population (ROTH insects) indicated that resistance was autosomal and showed incomplete dominance. At the highest dose of Cry1Ac tested, resistance was recessive while at the lowest dose it was almost completely dominant (Sayyed *et al.* 2000).

A strain from South Carolina selected to > 60,000-fold resistance to Cry1C displayed autosomal, incompletely recessive inheritance when evaluated by a leaf-dip bioassay and completely recessive behaviour on Cry1C-expressing transgenic broccoli (Zhao *et al.* 2000). The inheritance of resistance evaluated in the progeny derived from the backcross of the offspring of the Cry1B resistant strain (University of Melbourne) crossed with the

susceptible Australian (Waite and Queensland) strain, to the Cry1B resistant strain was found to be recessive with dominance co-efficient (h) values of 0.24-0.29 (Kaliaperumal *et al.* 2011).

Inheritance of resistance to Cry toxins Cry1Aa, Cry 1Ab, Cry1Ac and Cry1F was recessive in Pennsylvania and Newton populations (Tabashnik *et al.* 1997a). The results presented here suggest that resistance of the NO-Q strain of diamondback moth to a commercial formulation of *B. thuringiensis* was recessive, autosomally inherited, and controlled by one or a few loci (Tabashnik *et al.* 1992).

Conclusion

This study indicates that laboratory selected *P. xylostella* developed 900-fold resistance when compared with laboratory susceptible colony. It was observed that resistance of the DB-RM to Cry1Ac is inherited as recessive or incompletely / partially recessive. The recessive form of inheritance fits into the basic assumptions of insect resistance management strategy for Bt crops. When the resistance inheritance is recessive in nature, the progeny from matings of resistant and susceptible insects will die on Bt crops, substantially slowing the evolution of resistance (Tabashnik *et al.* 2009).

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Session 7

At the farm level; Barriers to and innovations for management of diamondback moth and other crucifer pests

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Landscape-level IPM for Brassica Pests: Patterns, Problems and Prospects

N.A. Schellhorn
CSIRO, Brisbane, Australia. E-mail: Nancy.schellhorn@csiro.au

Abstract

Landscape ecology studies show a general trend for a positive relationship between natural enemy abundance and habitat diversity; greater resources are provided to natural enemies in complex landscapes. A similar pattern is seen for the sub-set of studies on brassica insect pest abundance and suppression by natural enemies. However, increasing evidence shows that intensive farming practices such as habitat disturbance and frequency of insecticide application can mediate the effect of brassica pest-natural enemy interactions more than availability of resources. With the demands for agricultural products restricting habitat management interventions, such as increased areas of non-crop habitat for the capture of biological pest control in production landscapes, we must look for opportunities that can align with production goals and constraints. I show that fundamental to brassica insect pest suppression is identifying the interaction between species movement, disturbance, and the farm-landscape context. Yet, more has to be done to demonstrate the impact of such integrated pest management to increase adoption by farmers.

Keywords: landscape structure, biological control, disturbance, non-crop habitat, natural enemies, area-wide management, research impact

Over the past 20 years brassica vegetable production has increased by 39 %, and oil-seed rape by 59 % (see review Furlong *et al.* 2013). Undoubtedly, our knowledge of the biology and ecology of diamondback moth (DBM), *Plutella xylostella* and other cruciferous pests has also increased, as well as solutions to pest management. However, what is the impact of this knowledge? This question is in line with that posed by Prof. Shelton in his opening key note address of this Workshop asking the audience whether we've met the Talekar challenge. The question of impact of our collective research is increasingly important as agriculture strives to meet the demand of food increases, while reducing farm inputs such as pesticides and fertilizers, minimizing the degradation of agricultural land, and reducing crop loss. This is nowhere more important than with the production of Brassica vegetables and crops. DBM continues to develop resistance to all chemical groups that it encounters, and insecticide use continues to rise. Although much is now known about the best way to manage DBM and other brassica pests, the demonstration of the uptake and impact of these management options is limited. Linked to the management objective, we need to provide evidence of lesser problem, greater savings, and adoption of science-based solutions for the management of Brassica pests. Here I describe the current state of play of landscape-level IPM for Brassica pests and evaluate the patterns, problems and prospects for the future. Two main messages are brought to bear in this proceedings, firstly if Brassica pest management is going to meet the demands and challenges of increasing food production than we must think beyond the scale of the crop, and secondly we must demonstrate the impact of our work starting with a quantification of the problem prior to working towards solutions.

Patterns, Problems, Prospects

Patterns

The seminal paper by Thies and Tscharnkte (1999) on landscape structure and biological control in agricultural systems moved the thinking of pest management beyond the scale of the crop. Using the oil-seed rape, pollen beetle (*Meligethes aeneau*), and Ichneumonid parasitoid system, they showed an increase in parasitism of pollen beetle with an increase in non-crop area, and a decrease in crop damage. This finding spurred many to ask similar questions across different production systems, landscapes and countries. The conclusion from now more than 65 studies is that the trend is positive for natural enemy (NE) abundance and diversity with increasing amount of non-crop area in agricultural landscapes (Chaplin-Kramer *et al.* 2011; Veres *et al.* 2013); few studies focus on pests, and even fewer have demonstrated pest suppression. Of these 65 studies nine are on brassica systems (Table 1). Four studies focus on the brassica vegetable-pest-natural enemy systems; two from the Netherlands using sentinel egg cards and sentinel

larvae in Brussels sprout and cabbage systems, one from Germany on Brussel sprout-pests-parasitoid system, and one from California, USA on broccoli-cabbage aphid-predator system; four studies are from Europe on oil-seed rape-pollen beetle-parasitoids; one from New Zealand using forage brassica-*Plutella* sentinel larvae-predation/parasitism. Most of these studies show increased natural enemy abundance and / or increased pest suppression with increased non-crop habitat (Table 1). One shows increased colonization of pests with increased land use intensification (Ludwig *et al.* 2014), while one shows that parasitism of *P. xylostella* by *Diadegma semiclausum* is more strongly affected by disturbance rather than the availability of complex habitats (Jonsson *et al.* 2012).

Problems

While studies on landscape scale ecology of natural enemies and pests has clearly demonstrated the link between multiple spatial scales, crop, farm, landscape and region, there are gaps that need filling. Most of these studies focus on habitat complexity and natural enemy abundance and diversity; we need more that focus on pests. Most are conducted in Europe and the USA; we need more in Asia, Africa and the southern hemisphere. Several are demonstrating pest suppression, for example predation or parasitism as a function of landscape complexity and percentage non-crop habitat, however pest suppression does not equal pest control, which informs about economic injury and impact (Schellhorn *et al.* 2015). A few studies are incorporating disturbance as an explanatory variable. Given the often high insecticide use with brassica production, we need more studies that include this factor. Reducing disturbance from broad-spectrum insecticides is a far more likely option than re-vegetating large areas of land. Therefore, understanding the strength of these various components is likely to point us to management actions.

Finally, numerous local in-field studies have shown natural enemies to significantly reduce Brassica pests, especially studies on parasitoids (Furlong *et al.* 2013). We must next go beyond the scale of the crop to ask about sub-populations of natural enemies that are available in habitats on-farm or in the local area to colonize new plantings of brassica crops. Where are the natural enemies found? Are they using other crops and non-crop habitat? When are they there?

Prospects

A way forward for landscape-scale IPM for brassica pests is to connect the multiple spatial and temporal scales, and to demonstrate impact of the science-based solutions. There are several ways to achieve this. Firstly, a tremendous amount of knowledge has been demonstrated on various aspects of brassica pest biology and ecology; plant insect interactions, host plant resistance, pest-natural enemy interactions, host finding, physiology and chemical ecology to name a few. However, more work is needed to connect these disciplines across scales, and integrate the knowledge into 'know-how' for pest management. For example, cover crops such as rolled cereal rye were shown to reduce host finding and colonization of DBM and *Brevicoryne brassicae* (Broad *et al.* 2008a, 2008b). A greater mechanistic understanding of these findings could be achieved by scaling down to understand the chemical ecology of the cover cropping, and links to foraging behavior, as well as by scaling up to understand whether the effect of reduced host finding will hold in different landscape contexts. For example, is host finding reduced on Brassica vegetable embedded in rolled cereal when present in landscapes dominated by brassica production compared to landscapes that are dominated by non-crop or non-host crop habitat?

Secondly, much evidence points to the need to think beyond the scale of the crop to capture the services of natural enemies, and to coordinate pest management practices. In general, arthropod pests are highly mobile, and Brassica pests are as well. Learnings from pests of grain and vegetable landscapes in Australia, including canola, show us that weeds harbor pests (especially weedy pasture), whereas native plants rarely harbor these pests, but are hosting many of the natural enemies (Parry *et al.* 2015). These natural enemies move from these native remnants into the crops (Macfadyen *et al.* 2015, Bianchi *et al.* in review). In some cases Lucerne provided the best source habitat for natural enemies, and aphid suppression was positively related to increasing area of Lucerne at 1.5 km (Costamagna *et al.* 2015). Given the high demands for agricultural products and lack of arable land, Lucerne provides a reasonable option as a habitat to support beneficial insects, not host pests of Brassica crops, and provide an economic return. On-farm habitat management and area-wide approaches are needed for hosting natural

enemies of Brassica systems. However, this will require highly coordinated groups of neighboring farmers working together to achieve shared outcomes.

Finally, prior to researching a solution, we must generate a baseline assessment of the pest problem. As standard practice, researchers should conduct surveys of growers prior to starting the research in order to benchmark the problem. We need to ask questions like 'when was the worst pest year, and why?', 'when was the lightest year, and why?', 'on a scale of 1-10, what year was most severe, and how bad was it (with 10 being the most severe)?'; 'what year did you spend the most money controlling the pest, and how much?'; 'what year did you spend the least money, how much?'; 'why was the pest problem light in those years?'; 'how much does it currently cost you to control brassica pests?'. These types of questions can be linked to management objectives and an assessment of the impact of the research program in terms of a lesser problem or greater savings on pest control. Towards the end of the research program, and beyond, follow-up surveys can assess the changes in awareness, and practices by the growers, and ultimately document any changes in the severity of the pest problem.

TABLE 1

Landscape Studies for Brassica pests & natural enemies

Crop and Country	Study System	Effect
[1]Broccoli, CA, USA	*Brevicoryne brassicae*, syrphid -predation	Reduction of pests greater with increased complexity at local and landscape scale.
[2]Brussels sprout, NL	*Mamestra brassicae*; egg parasitism/ predation	Predation and parasitism related to landscape variables at 0.3, 1, 2 and 10 km; positive relationship with predation and woody habitats, and parasitism and pasture.
[3]Cabbage, NL	*Plutella xylostella*, parasitism/ predation	Parasitism positively related with the area of forests at scales of 1, 2 and 10 km.
[4]Brussels sprout, Germany	*Brevicoryne brassicae*, *Aleyrodes proletella*	Pest colonization higher on Brussels sprouts in landscapes with greater area of oil-seed rape.
[5]Oil-seed rape, Germany	*Meligethes aeneau*, parasitism	Oil-seed rape damage was lower and parasitism higher in structurally complex landscapes compared to simple landscapes with high areas of agriculture.
[6]Oil-seed rape, Germany	*Meligethes aeneau*, parasitism	% non-crop area was negatively related to herbivory and positively related to parasitism of larvae.
[7]Oil-seed rape, France	*Meligethes aeneau*, parasitism	Positive relationship with proportion of grassland and forest (small and large scales) and previous year OSR field.
[8]Oil-seed rape, Austria	*Meligethes aeneau*, parasitism	Stem weevil parasitism negatively related to grassy fallow; pollen beetle parasitism positively related to crop density and roadside strips
[9]Forage Brassica; New Zealand	*Plutella xylostella*, parasitism	Parasitism declined with increasing annual crop cover; decreasing habitat diversity in the landscape had little effect.

[1]Chaplin-Dramer *et al.* 2012; [2]Bianchi *et al.* 2005; [3]Bianchi *et al.* 2008; [4]Ludwig *et al.* 2014; [5]Thies and Tscharntke 1999; [6]Thies *et al.* 2003; [7]Rusch *et al.* 2011; [8]Zaller *et al.* 2009; [9]Jonsson *et al.* 2012.

Conclusion

The discipline of Brassica pest management has generated considerable knowledge, but many challenges lie ahead. Managing pests beyond the scale of the crop is technically and socially challenging, and will require integration across disciplines and new knowledge on the role of surrounding habitats in supporting pests and natural enemies. In addition, it will require well- coordinated groups of farmers working towards shared goals. However, most important is the need for researchers to demonstrate impact of their programs in solving pest management problems. Such an approach can be highly impactful, and either highlights the gaps and the failings or shine the spot light on the successes.

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Towards Developing an Integrated Pest Management Strategy for Cabbage Production Systems in Lowlands of Taiwan, Republic of China

R. Srinivasan, M.-Y. Lin[1], F.-C. Su[2] and C.-C. Huang[3]
Entomology, AVRDC – World Vegetable Center, Shanhua, Tainan 74151, Taiwan, Republic of China
E-mail : srini.ramasamy@worldveg.org; [1]E-mail : mei-ying.lin@worldveg.org
[2]E-mail : fu-cheng.su@worldveg.org; [3]E-mail : amy.huang@worldveg.org

Abstract

No single method of pest management can achieve a level of control of cabbage pests acceptable to producers in the lowlands of Taiwan, Republic of China. Therefore, an effective integrated pest management (IPM) strategy has been developed and validated. Since Indian mustard (*Brassica juncea*) has been reported to be an effective trap crop, we screened 89 Indian mustard accessions from the Genetic Resources and Seed Unit (GRSU) of AVRDC – The World Vegetable Center. We identified three very attractive accessions to diamondback moth *viz.,* VI33061, VI33129 and VI36395. When these accessions were subsequently tested as trap crops with cabbage, the yield and pest damage did not show significant differences compared to the cabbage crop without trap crops. Improved sex pheromone lures of *Plutella xylostella* attracted significantly higher numbers of moths. However, the yield and pest damage did not differ significantly among the treatments. Seventeen chemical and bio-pesticides were tested against *P. xylostella*, *Crocidolomia pavonana* and *Spodoptera litura*. Spinetoram, spinosad, chlorfenapyr, chlorantraniliprole, emamectin benzoate, fipronil, cartap, indoxacarb and *Bacillus thuringiensis* were effective against *P. xylostella* at the recommended concentrations. *C. pavonana* was susceptible to almost all these insecticides, particularly to spinetoram, spinosad, indoxacarb, chlorfenapyr and chlorantraniliprole and *B. thuringiensis*. However, *B. thuringiensis* was not effective against *S. litura*. Based on these results, a pesticide window strategy was developed and evaluated in 2013. Significantly low pest damage and higher yields were recorded using the insecticide window strategy, compared to the farmers' practice. Trap cropping did not have any effects in managing the cabbage pests in the lowlands. Although the insecticide window strategy alone could provide sufficient control of major lepidopterans on cabbage in lowland production systems in Taiwan, Republic of China, it would be improved if combined with the pheromone lures which serve as the monitoring tool for timing applications.

***Keywords*:** Cabbage, lowland, trap crop, sex pheromones, pesticide window strategy

Introduction

Cabbage (including Chinese cabbage) is an important vegetable crop in Taiwan, Republic of China. They are cultivated on ca 10,000 ha with an annual production of over 457,000 t (Council of Agriculture, 2014). Diamondback moth (DBM), *Plutella xylostella,* is one of the most serious pests of cabbages in Taiwan, Republic of China as well as other parts of the world. Though *P. xylostella* is the predominant pest in cabbages, it can be brought under reasonable control in the highlands of Taiwan, Republic of China by introduced parasitoids such as *Diadegma semiclausum* and *Diadromus collaris*. Although the lowlands of Taiwan, Republic of China and Southeast Asia have another parasitoid, *Cotesia plutellae* (Talekar and Shelton 1993), it alone cannot keep the *P. xylostella* populations below levels causing economic damage. In addition, pests of secondary importance including cabbage head caterpillar (CHC, *Crocidolomia pavonana*), cabbage web worm (CWW, *Hellula undalis*), common armyworm (CAW, *Spodoptera litura*), and imported cabbageworm (ICW, *Pieris rapae*) often lack parasitoids or other integrated pest management (IPM) strategies. Thus vegetable growers predominantly rely on chemical insecticides to control these secondary pests. However, this practice inadvertently kills the natural enemies of *P. xylostella*, resulting in the resurgence of *P. xylostella* populations in the lowlands of Taiwan, Republic of China as well as other parts of Southeast Asia (Talekar 2004). Hence, alternative pest management strategies are warranted for lowland brassica producers in Taiwan, Republic of China.

Indian mustard (*Brassica juncea*) has been reported to be an effective trap crop. It was proven to be effective in managing *P. xylostella* in India (Srinivasan and Krishna Moorthy 1992) and South Africa (Charleston and Kfir 2000). However, it was not attractive for the population in the Pacific (Muniappan *et al.* 2001). Badenes-Perez *et al.* (2004) reported that Yellow rocket (*Barbarea vulgaris*) may be a better trap crop than Indian mustard. Variable

attractiveness of Indian mustard as a trap crop can be attributed to its genotypic variations. Hence, we screened about 89 Indian mustard accessions that are available with the Genetic Resources and Seed Unit of AVRDC – The World Vegetable Center, to identify the most attractive accession(s) to be used a trap crop(s).

Synthetic sex pheromone and new attractant formulations are highly effective in forecasting the occurrence of *P. xylostella* in cabbage and Chinese cabbage fields in Shanghai County, People's Republic of China (Dai *et al*. 2011). Besides using sex pheromone traps as an effective forecasting tool, they can also be employed as a control method. For instance, *P. xylostella* populations were reduced about 60 % by continuously using sex pheromone traps during a planting season in Yunnan, People's Republic of China (Chen *et al*. 2011). Hence, improved formulations of sex pheromone lures were evaluated in the current study.

Bio-pesticides have an important role in managing brassica insect pests. *Bacillus thuringiensis* toxins and formulations have been evaluated against the larvae of *P. xylostella, C. pavonana, H. undalis* and *Pi. rapae*. The toxins Cry1Ac, Cry1Aa and Cry1Ca were equally toxic to *P. xylostella*. *C. pavonana* was highly susceptible to all Cry1A toxins and it was least sensitive to Cry1Ca (Srinivasan and Hsu 2008). Cry 1A toxins are commonly found in *B. thuringiensis* subsp. *kurstaki* based formulations. Conversely, *H. undalis* was highly sensitive to Cry1Ca, but less susceptible to Cry1A toxins. Hence, the susceptibility of *H. undalis* to Xentari®, a formulation containing Cry1C as a major toxin, was significantly higher (Srinivasan and Hsu 2008). This was also confirmed in earlier studies which showed that application of *B. thuringiensis* subsp. *kurstaki* based formulations was ineffective against *H. undalis* in Taiwan, Republic of China (AVRDC 1987). Hence, different formulations of *B. thuringiensis* can be deployed in managing various lepidopteran pests on cabbages. However, it will be more sustainable if the bio-pesticides are combined with chemical insecticides in a proper insecticide window strategy. In the window strategy, insecticides are rotated with each one used only during a specific 'window.' The insecticide window strategy has been shown to be effective in maintaining the susceptibility of *P. xylostella* in other countries. Therefore, we attempted to develop and validate an effective IPM strategy based on trap crops, sex pheromone lures and insecticide window strategy in lowland cabbage production in the current study.

Materials and Methods

(1) Screening of Indian mustard germplasm against major lepidopteran insects

Seeds of 89 Indian mustard accessions were obtained from the GRSU of AVRDC – The World Vegetable Center, and sown in seedling trays. Three week-old seedlings were planted during the spring season of 2012. For each accession, two replications were maintained, and each replication had 10 plants. The plants were not sprayed with any insecticide.. Beginning three weeks after transplanting, the number of insects present on each plant was counted at weekly intervals for three weeks. The observations included *P. xylostella, C. pavonana, H. undalis, Pi. rapae* and *Phyllotreta striolata*. Based on the mean insect population, the most attractive accessions were selected for advanced screening.

The nine most attractive Indian mustard accessions from the spring trial were screened to confirm their attraction during the autumn season of 2012. Each accession was planted in three replications following a randomized complete block design (RCBD), and each replication had 10 plants. The plants were not sprayed with any insecticide. Beginning three weeks after transplanting, the number of insects present on each plant was counted at weekly intervals for three weeks. The observations included *P. xylostella, C. pavonana, H. undalis, Pi. rapae* and striped flea beetle (SFB, *Phyllotreta striolata*). Data were subjected to analysis of variance (ANOVA) and Tukey's Honest Significant Difference (HSD) test for mean comparisons.

(2) Evaluating Indian mustard as a trap crop against major lepidopteran pests of brassicas

The three most attractive accessions (VI33061, VI33129 and VI36395) were evaluated as trap crops. The trial conducted during spring 2013 included seven treatments *viz*., three selected accessions in two modes of deployment each, and the untreated check containing only cabbage as the main crop. Each treatment was replicated for three times, following RCBD.

(i) VI33061 planted twice (15 days before- and 25 days after planting of cabbage)

(ii) VI33061 planted thrice (15 days before-, 25 days after- and 50 days after planting of cabbage)

(iii) VI33129 planted twice (15 days before- and 25 days after planting of cabbage)

(iv) VI33129 planted thrice (15 days before-, 25 days after- and 50 days after planting of cabbage)

(v) VI36395 planted twice (15 days before- and 25 days after planting of cabbage)

(vi) VI36395 planted thrice (15 days before-, 25 days after- and 50 days after planting of cabbage)

(vii) Untreated check (cabbage only)

Five plants were randomly selected in each replication, and the total number of *P. xylostella, C. pavonana* and *Pi. rapae* larvae on each plant was counted at weekly intervals until the harvest starting from two weeks after transplanting. The mean number of larvae per plant for each pest species was compared between the treatments using ANOVA and Tukey's HSD for mean comparisons.

(3) Effectiveness of improved sex pheromone lures against* P. xylostella *for their potential in monitoring and/or mass-trapping

A field trial was conducted at AVRDC – The World Vegetable Center - during the autumn season of 2012 to evaluate the efficiency of an improved sex pheromone lure for *P. xylostella*, which was obtained from Bio-Control Research Laboratories (BCRL), Bangalore, India. There were two treatments: (i) sex pheromone lure and (ii) untreated check. Each treatment was replicated seven times, and the design was RCBD. The number of *P. xylostella* adults attracted to each trap was counted at weekly intervals. The total number of insects attracted in each treatment was subjected to ANOVA and Tukey's HSD for mean comparisons.

A second field trial was conducted in farmer's field in Shanhua during the autumn season of 2012 to evaluate the efficiency of improved sex pheromone lures for *P. xylostella*. There were three treatments: (i) sex pheromone lure – India; (i) sex pheromone lure – Taiwan; and (iii) check. Each treatment was replicated six times following the RCBD. The number of *P. xylostella* adults attracted to each trap was counted at weekly intervals. The total number of insects attracted in each treatment was subjected to ANOVA and Tukey's HSD for mean comparisons.

According to the field trials conducted during autumn 2012, the pheromone lures could be used as a monitoring tool in an IPM strategy for *P. xylostella*. Since we received newer pheromone lures from Taiwan Agricultural Chemicals and Toxic Substances Research Institute (TACTRI), additional trials were conducted during 2013. The third trial had three treatments: (i) AVRDC pheromone lure, (ii) TACTRI pheromone lure, and (iii) check. The number of *P. xylostella* adults attracted to each trap was counted at weekly intervals. In addition, the number of *P. xylostella* and *P. rapae* larvae on ten plants in each replication was also counted randomly. The marketable yield of cabbage during harvest was recorded. The data were subjected to ANOVA and Tukey's HSD for mean comparisons.

(4) Development and evaluation of an insecticide window strategy against key lepidopteran pests

Commercially available bio-pesticide formulations based on *B. thuringiensis* such as Crymax® and strain E-911® (*B. thuringiensis* subsp. *kurstaki*), Xentari® (*B. thuringiensis* subsp. *aizawai*), and selected chemical pesticides were bio-assayed in the lab against *P. xylostella, C. pavonana* and *S. litura*. A few log-based concentrations (i.e. 10, 100, 1000 and 10000 ppm) for each bio-pesticide or chemical pesticide were used in the preliminary range-finding tests. At least five concentrations for each compound that caused 10–90% mortality, according to the preliminary tests, plus an untreated control were included in further bioassays. Insect bioassays were performed by treating the cabbage leaf discs with selected concentrations of each compound. Ten early second-instar larvae were introduced into each cup containing treated leaf disc that served as an experimental unit. The cups were covered with lids. The experiment was replicated five times. The cups were incubated at 27±1 °C, and 70±10 % RH, L12:D12. Larval mortality were recorded after 3-4 days; at each assessment, larvae were classed as either alive or dead. The lethal concentrations causing 50 % mortality (LC_{50}), 90 % mortality (LC_{90}), their 95 % fiducial limits (FL) and the slope value of probit line were assessed according to probit analysis methodology using the statistical program LdP line (Ehab Mostofa Bakr, Cairo, Egypt), and the LC_{50} values and relevant statistics were calculated.

Differences in toxicity were considered significant when 95% fiducial limits (FL) of LC_{50} values did not overlap.

An insecticide window strategy was developed using the chemical and bio-pesticides based on the bio-assay results, and a field trial was conducted in spring 2013.

Window 1 (spring): spinetoram, chlorfenapyr, indoxacarb and *B. thuringiensis* subsp. *kurstaki*

There were three treatments: (i) window strategy, (ii) farmers' strategy which was mainly based on chemical insecticides without regard to frequency of use, and (iii) untreated check plots. Each treatment was replicated four times, and the design was RCBD. Ten plants were randomly selected in each replication, and the total number of *P. xylostella* and *Pi. rapae* larvae on each plant were counted at weekly intervals for six weeks starting from four weeks after transplanting. The marketable yield of cabbage during harvest was also recorded. The data were subjected to ANOVA and Tukey's HSD for mean comparisons.

A second field trial was conducted during autumn 2013.

Window 2 (autumn): emamectin benzoate, fipronil, chlorantraniliprole and *B. thuringiensis* subsp. *aizawai*

There were five treatments: (i) window strategy, (ii) window strategy + *P. xylostella* sex pheromone lures, (iii) window strategy + *P. xylostella* sex pheromone lures + Indian mustard trap crop, (iv) farmers' strategy based on chemical insecticides alone, and (v) untreated check plots. Each treatment was replicated four times, and the design was RCBD. Ten plants were randomly selected in each replication, and the total numbers of *P. xylostella*, *Pi. rapae*, *C. pavonana* and *S. litura* larvae on each plant were counted at weekly intervals for six weeks starting from four weeks after transplanting. The marketable yield of cabbage during harvest was also recorded. The data were subjected to ANOVA and Tukey's HSD for mean comparisons.

Results

(1) Screening of Indian mustard germplasm against major lepidopteran insects

Of the 89 accessions screened during spring 2012, the nine most attractive accessions, *viz.*, V1010245, V1033061, V1033129, V1033134, V1036395, V1037401, V1041182, V1048139 and V1048315 were selected for advanced screening (data not shown). From the advanced screening trial, VI36395 was identified as the most attractive accession to. *striolata* (F=2.58; p=0.05), whereas VI33061 was found to harbor more *P. xylostella* and *C. pavonana*, although there was no significant difference among the accessions for *P. xylostella* (F=1.72; p=0.18), *C. pavonana* (F=2.30; p=0.07), *H. undalis* (F=1.63; p=0.19) and *P. rapae* (F=1.01; p=0.47) (Table 1). VI33129 was found to have more *Pi. rapae* larvae and VI37401 hosted more *H. undalis* larvae. Hence, three accessions (VI33061, VI33129 and VI36395) were evaluated as trap crops with cabbage as the main crop in the following trials to manage the major lepidopteran pests. Since *H. undalis* mostly appears only sporadically, VI37401 was not included in the subsequent trials.

(2) Evaluating Indian mustard as a trap crop against major lepidopteran pests of brassicas

None of the selected Indian mustard accession was able to reduce the number of larvae of *P. xylostella*, *Pi. rapae* and *C. pavonana* in either mode of deployment (Table 2). Hence, the larval population of *P. xylostella* (F=0.73; p=0.63), *Pi. rapae* (F=1.08; p=0.43) and *C. pavonana* (F=2.03; p=0.10) did not differ significantly on cabbage plants with or without the trap crop

(3) Effectiveness of improved sex pheromone lures against P. xylostella *for their potential in monitoring and/or mass-trapping*

The improved pheromone lure from BCRL, India attracted significantly higher numbers of *P. xylostella* adults (154/week) compared to the untreated check (51/week) (F=17.83; p=0.008) in the trial conducted at AVRDC during autumn 2012 (Table 3). Similarly, the improved pheromone lures from India and Taiwan attracted almost an equal number of insects recording 344 and 331 insects/week, respectively, which were significantly higher than the untreated check (145/week) (F=16.21; p=0.0007) (Table 4). Hence, we chose the Taiwan lures for subsequent trials.

TABLE 1

Attraction of selected Indian mustard accessions to major lepidopterans (autumn 2012)

Accession	Mean no. of DBM / plant	Mean no. of CWW / plant	Mean no. of CHC / plant	Mean no. of ICW . plant	Mean no. of SFB / plant
VI10245	0.08 a	0.09 a	2.17 a	1.22 a	1.72 ab
VI33061	0.13 a	0.03 a	3.51 a	1.39 a	1.76 ab
VI33129	0.01 a	0.08 a	1.59 a	3.41 a	1.91 ab
VI33134	0.03 a	0.05 a	2.76 a	1.27 a	1.63 ab
VI36395	0.03 a	0.09 a	0.00 a	0.93 a	4.00 a
VI37401	0.00 a	0.43 a	2.92 a	1.31 a	2.33 ab
VI41182	0.00 a	0.17 a	2.15 a	1.48 a	1.63 ab
VI48139	0.00 a	0.00 a	0.75 a	1.93 a	1.59 ab
VI48315	0.00 a	0.00 a	0.29 a	2.57 a	0.32 b
F value	1.72	1.63	2.30	1.01	2.58
P value	0.18	0.19	0.07	0.47	0.05

Means followed by the same letter(s) in a column are not significantly different ($p < 0.05$) by Tukey's HSD.

TABLE 2

Effects of selected Indian mustard accessions as trap crop against major lepidopterans on cabbage (spring 2013)

Treatment	No. of DBM larve / Plant	No. of ICW larve / Plant	No. of CHC larve / Plant
VI33061 (15dB, 25dA)	2.04 a	1.56 a	1.29 a
VI33061 (15dB, 25dA, 50dA)	2.73 a	1.65 a	2.71 a
VI33129 (15dB, 25dA)	1.47 a	1.86 a	1.53 a
VI33129 (15dB, 25dA, 50dA)	1.73 a	1.73 a	0.85 a
VI36395 (15dB, 25dA)	2.80 a	1.21 a	1.87 a
VI36395 (15dB, 25dA, 50dA)	1.96 a	1.42 a	0.78 a
Check	2.71 a	1.81 a	0.84 a
F value	0.73	1.08	2.03
P value	0.63	0.43	0.10

Means followed by the same letter(s) in a column are not significantly different ($p < 0.05$) by Tukey's HSD

dB – days before transplanting main crop

dA – days after transplanting main crop

(4) Effectiveness of improved sex pheromone lures against* P. xylostella *for their potential in monitoring and/or mass-trapping

The improved pheromone lure from BCRL, India attracted significantly higher numbers of *P. xylostella* adults (154/week) compared to the untreated check (51/week) ($F=17.83$; $p=0.008$) in the trial conducted at AVRDC during autumn 2012 (Table 3). Similarly, the improved pheromone lures from India and Taiwan attracted almost an equal number of insects recording 344 and 331 insects/week, respectively, which were significantly higher than the untreated check (145/ week) ($F=16.21$; $p=0.0007$) (Table 4). Hence, we chose the Taiwan lures for subsequent trials.

TABLE 3

Effectiveness of improved sex pheromone lure (India) against P. xylostella *on cabbage (September – December 2012)*

Treatment	Total no. of *P. xylostella* / trap
Pheromone lure	1228.80 a (7.07)
Check	409.00 b (5.85)
F value	17.83
P value	0.008

Means followed by the same letter(s) in a column are not significantly different ($p < 0.05$) by Tukey's HSD

Figures in parentheses are log-transformed values

TABLE 4

Effectiveness of improved sex pheromone lures against P. xylostella on cabbage (Sept.–December 2012)

Treatment	Total no. of *P. xylostella* / trap
Pheromone lure (India)	1377.00 a (7.21)
Pheromone lure (Taiwan)	1323.00 a (7.11)
Check	581.00 b (6.17)
F value	16.21
P value	0.0007

Means followed by the same letter(s) in a column are not significantly different ($p < 0.05$) by Tukey's HSD
Figures in parentheses are log-transformed values

Both lures attracted a significantly higher number of *P. xylostella* adults compared to the untreated check ($F=32.56$; $p=0.0001$). However, it did not reduce the number of *P. xylostella* larvae ($F=0.45$; $p=0.66$) on

cabbage plants among the treatments (Table 5). In addition, *Pi. rapae* larvae were also present on cabbage plants in all the treatments. This led to significant reduction in marketable yield in both pheromone treated as well as control plots, which was not statistically significant among the treatments (F=0.29; p=0.76). The marketable yield was less than 4 t/ha in all the treatments (F=0.13; p=0.88). Hence, this suggests that pheromone lures can be used only as a monitoring tool in lowland cabbage production and not as a means of lowering the population.

TABLE 5

Effectiveness of improved sex pheromone lures against P. xylostella on cabbage (January – April 2013)

Treatment	No. of DBM adults / trap	No. of larvae / Plant		Yield (t/ha)	
		DBM	ICW	Marke-table	Un mar ket-able
TACTRI lure	185.80 ab	2.70	1.46	3.92	33.54
AVRDC lure	328.00 a	2.67	1.24	3.83	35.41
Check	131.00 b	3.14	1.52	3.20	33.02
F value	32.56	0.45	0.52	0.13	0.29
P value	0.0001	0.66	0.62	0.88	0.76

Means followed by the same letter(s) in a column are not significantly different ($p < 0.05$) by Tukey's HSD

(5) Development and evaluation of an insecticide window strategy against key lepidopteran pests

Among the chemical pesticides, spinetoram was the most toxic to *P. xylostella* (Table 6). This was followed by spinosad and emamectin benzoate, which were equally toxic because of their overlapping fiducial limits. Indoxacarb was also highly toxic to *P. xylostella* with an LC_{50} of 93.83 ppm. Chlorfenapyr, chlorantraniliprole and cartap were also on par in toxicity. Among the bio-pesticides, *B. thuringiensis* subsp. *aizawai* (Xentari®) was the most toxic, followed by *B. thuringiensis* subsp. *kurstaki* (Bt strain E-911) and *B. thuringiensis* subsp. *kurstaki* (Crymax®). Emamectin benzoate recorded the highest toxicity against *C. pavonana* (Table 7), which was followed by chlorantraniliprole and indoxacarb. Chlorfenapyr was also highly toxic to *C. pavonana* with a LC_{50} of 12.84 ppm.

Among the bio-pesticides, both *B. thuringiensis* subsp. *kurstaki* (Bt strain E-911) and *B. thuringiensis* subsp. *kurstaki* (Crymax®) were equally toxic. For *S. litura* larva also, emamectin benzoate recorded the highest toxicity (Table 8), which was followed by indoxacarb. Among the bio-pesticides, *B. thuringiensis* subsp. *aizawai* (Xentari®) recorded the lowest LC_{50}, followed by *B. thuringiensis* subsp. *kurstaki* (Crymax®). However, *S. litura* was not highly susceptible to *B. thuringiensis* subsp. *kurstaki* (Bt strain E-911).

The *P. xylostella* population was significantly higher when using the farmers' method which mainly involved calendar based spraying of chemical pesticides - chlorantraniliprole and fipronil (Table 9). Moreover, the population was significantly lower in the untreated check ($F=3.35$; $p = 0.04$). *Pi. rapae* population was significantly lowered in both window strategy and the farmers' method ($F=61.74$; $p < 0.0001$). Hence, the window strategy recorded significantly higher yield, followed by the farmers' method ($F=6.09$; $p = 0.04$).

In the second season, the pest population on cabbage did not differ significantly when the window strategy was deployed either alone or in combination with *P. xylostella* pheromone and/or trap crop (Table 10). Like the previous trial, *P. xylostella* population was significantly lower in untreated plots ($F=3.35$; $p=0.04$), which is mainly due to the presence of parasitoids. Both *Pi. rapae* ($F=36.72$; $p<0.0001$) and *C. pavonana* ($F=5.88$; $p=0.006$) population were significantly higher only in untreated plots. However, *S. litura* population did not differ among the treatments. The marketable yield was significantly lower only in untreated plots ($F=38.48$; $p<0.0001$).

TABLE 6

Toxicity of different chemical and bio-pesticides to Plutella xylostella *larvae*

Chemical or Bio-pesticide	**LC_{50} (ppm)**	**Fiducial Limits (95%)**	**LC_{90} (ppm)**	**Fiducial Limits (95%)**	**Slope (SE)**	**$\times^2$**	**df**
Spinetoram	4.79	(4.32 – 5.26)	12.43	(10.78 – 14.94)	3.09 (0.25)	2.17	3
Metaflumizone	2680.71	(2109.20 – 3589.94)	30672.02	(18046.19 – 64601.63)	1.21 (0.11)	5.09	4
Chlorfenapyr	249.09	(208.25 – 290.78)	1094.89	(883.86 – 1452.86)	1.99 (0.18)	6.48	3
Chlorantraniliprole	335.51	(201.17 – 531.62)	2418.89	(1852.71 – 8661.13)	1.49 (0.14)	13.39	4
Spinosad	27.54	(23.06 – 33.83)	173.18	(120.17 – 283.80)	1.61 (0.13)	3.72	4
Bacillus thuringiensis subsp. *aizawai* (Xentari®)	89.81	(75.35 – 107.08)	672.77	(487.41 – 1032.63)	1.47 (0.12)	5.86	4
B. thuringiensis subsp. *kurstaki* (Crymax®)	348.36	(190.53 – 793.25)	2999.04	(2776.19 – 24036.33)	1.37 (0.12)	20.64	4
B. thuringiensis subsp. *kurstaki* (Bt strain E-911)	270.64	(166.86 – 421.88)	1689.32	(1231.83 – 4653.46)	1.61 (0.13)	12.78	4
Tolfenpyrad	1137.49	(978.06 – 1336.48)	5905.19	(4470.61 – 8466.60)	1.79 (0.13)	6.51	4
Emamectin benzoate	33.09	(18.28 – 55.60)	178.51	(139.25 – 552.58)	1.75 (0.13)	21.09	4
Fipronil	582.40	(397.19 – 833.60)	3069.22	(2232.05 – 6245.39)	1.78 (0.13)	10.81	4
Cartap	334.40	(285.52 – 391.14)	1929.66	(1481.16 – 2711.81)	1.68 (0.13)	3.03	4
Indoxacarb	93.83	(63.24 – 130.47)	5638.77	(2353.90 – 25928.13)	0.72 (0.10)	0.78	4

TABLE 7

Toxicity of different chemical and bio-pesticides to Crocidolomia pavonana *larvae*

Chemical or Bio-pesticide	**LC_{50} (ppm)**	**Fiducial Limits (95%)**	**LC_{90} (ppm)**	**Fiducial Limits (95%)**	**Slope (SE)**	**$\times^2$**	**df**
Emamectin benzoate Emamectin benzoate	0.004	(0.003 0.005)	0.038	(0.027 – 0.057)	1.32 (0.09)	9.04	4
Indoxacarb	1.97	(1.17 – 2.97)	9.11	(6.84 – 21.00)	1.93 (0.14)	16.98	4
Chlorfenapyr	12.84	(9.21 – 18.39)	37.92	(29.37 – 75.71)	2.72 (0.20)	8.56	3
Cartap	194.40	(120.16 – 574.85)	931.82	(1164.64 – 9932.53)	1.88 (0.15)	30.50	4
Tofenpyred	188.96	(125.66 – 287.27)	1098.69	(793.78 – 2618.21)	1.68 (0.13)	12.13	4
Methomyl	273.79	(242.13 - 307.13)	733.71	(627.82 - 894.14)	2.99 (0.24)	6.11	3
Fenvalerate	326.75	(192.37 - 633.33)	1413.02	(1247.14 - 6920.17)	2.02 (0.17)	13.45	3
Chlorantraniliprole	1.58	(1.03 - 2.19)	69.32	(34.42 - 221.80)	0.78 (0.11)	2.45	4
B. thuringiensis subsp. *aizawai* (Xentari®)	386.16	(320.49 – 469.53)	2387.37	(1585.55 – 4591.66)	1.62 (0.20)	2.16	2
B. thuringiensis subsp. *kurstaki* (Crymax®)	187.94	(148.77 – 234.17)	2648.22	(1687.83 – 5047.28)	1.12 (0.11)	3.23	4
B. thuringiensis subsp. *kurstaki* (Bt strain E-911)	176.43	(151.81 – 205.00)	720.90	(548.85 – 1068.02)	2.10 (0.22)	1.31	2

TABLE 8

Toxicity of different chemical and bio-pesticides to Spodoptera litura *larvae*

Chemical or Bio-pesticide	**LC_{50} (ppm)**	**Fiducial Limits (95%)**	**LC_{90} (ppm)**	**Fiducial Limits (95%)**	**Slope (SE)**	**$\times^2$**	**df**
Emamectin benzoate	1.24	(0.90 – 1.60)	16.75	(11.37 – 29.36)	1.13 (0.12)	9.40	4
Indoxacarb	23.54	(15.03 – 36.35)	83.50	(64.97 – 209.31)	2.33 (0.18)	10.72	3
B.thuringiensis subsp. *aizawai* (Xentari®)	369.03	(273.24 - 508.90)	1939.01	(1414.70 - 3449.30)	1.78 (0.12)	12.29	5
B.thuringiensis subsp. *kurstaki* (Crymax®)	544.23	(347.61 - 930.74)	4929.33	(3473.70 - 14951.46)	1.34 (0.10)	18.56	5
B.thuringiensis subsp. *kurstaki* (Bt strain E-911)	2183.41	(1875.06 - 2609.00)	8927.26	(6597.83 - 13548.68)	2.10 (0.19)	1.61	3

TABLE 9

Effectiveness of window strategy against P. xylostella *and* Pi. rapae *on cabbage (January – April 2013)*

Treatment	**No. of insects/plant/week**		**Yield (t/ha)**
	DBM	**ICW**	
Window strategy	1.93 ab	0.23 b	38.90 a (6.21)
Farmer's method	3.57 a	0.38 b	30.49 ab (5.56)
Check	1.31 b	2.46 a	20.89 b (4.57)
F value	6.78	61.74	6.09
P value	0.03	<0.0001	0.04

Means followed by the same letter(s) in a column are not significantly different (p<0.05) by Tukey's HSD

Figures in parentheses are square-root transformed values

TABLE 10

Effectiveness of window strategy against lepidopteran pests on cabbage (October – December 2013)

Treatment	**Mean larvae / Plant**				**Yield (t/ha)**
	DBM	**CAW**	**ICW**	**CHC**	
Window strategy	0.25 ab	0.02	0.12 b	0.00 b	37.52 a (6.16)
Window strategy + DBM pheromone	0.26 ab	0.06	0.09 b	0.00 b	36.87 a (6.10)
Window strategy + DBM pheromone + trap crop	0.26 ab	0.02	0.11 b	0.00 b	21.99 b (4.74)
Farmers' method	0.32 a	0.03	0.19 b	0.00 b	36.15 a (6.00)
Check	0.06 b	0.10	1.41 a	0.19 a	8.77 c (3.01)
F value	3.35	1.87	36.72	5.88	38.48
p value	0.04	0.18	<0.0001	0.006	<0.0001

Means followed by the same letter(s) in a column are not significantly different (p<0.05) by Tukey's HSD

Figures in parentheses are square-root transformed values

Discussion

Indian mustard has been suggested as a trap crop for effective management of one or more lepidopteran insects including *P. xylostella*, *C. pavonana*, *H. undalis* and *Trichoplusia ni* on cabbage in India (Srinivasan and Krishna Moorthy 1992), South Africa (Charleston and Kfir 2000) and Hawaii (Luther *et al.* 1996). However, it was not attractive for the pest population in west Texas (Bender *et al.* 1999) and Guam (Muniappan *et al*. 2001). Rather, the *P. xylostella* population in Guam and continental United States was attracted to collards, *Brassica oleracea acephala* cv. Vates. Although Muniappan *et al.* (2001) speculated that the population of *P. xylostella* in the New World and the Pacific are different from those of South Asia and Africa, the differential responses could also be attributed to the genotypic and/or phenotypic variations that may exist within the Indian mustard germplasm in different geographical locations. Some of the Indian mustard accessions from AVRDC's Gene Bank were found to be highly attractive to major lepidopteran pests of brassicas. However, they failed to reduce the larval population on cabbage, when deployed as trap crops. In fact, Indian mustard accessions in this study were not highly attractive to *P. xylostella* and *H. undalis*, but attracted *C. pavonana* and *Pi. rapae*. This is consistent with the observation of Luther *et al.* (1996) who found that Indian mustard was attractive to *H. undalis* and *T. ni*, but not to *P. xylostella*. In addition, the trap crops failed to increase the yield or marketability of cabbage despite their attraction to *H. undalis* and *T. ni*. Bender *et al.* (1999) also confirmed that Indian mustard trap cropping was ineffective in managing cabbage pests. In addition, one of the important practical constraints in the adoption of Indian mustard as a trap crop is its multiple planting. According to Srinivasan and Krishna Moorthy (1992), the trap crop should be planted at least twice. However, cabbage growers may not find it convenient or economical to plant the trap crops two or three times during the cabbage season. Hence, Indian mustard as a trap crop may not be an economically viable IPM component technology.

Sex pheromone lures have also been reported to be an effective component in managing *P. xylostella* in brassicas. Suckling *et al*. (2002) found that improved pheromone lures were effective in monitoring *P. xylostella* and thus minimizing the use of chemical pesticides in New Zealand. Synthetic sex pheromone and new attractant formulations were found to be highly effective in forecasting the occurrence of *P. xylostella* in cabbage and Chinese cabbage fields in Shanghai County, People's Republic of China (Dai *et al*. 2011). Besides *P. xylostella* population was reduced about 60 % by continuously using sex pheromone trap in a planting season in Yunnan, People's Republic of China (Chen *et al.* 2011). Despite the fact that *P. xylostella* pheromone lures in the current study were attractive to male moths, they failed to reduce the larval population on cabbage. Possibly, the lures may have attracted lesser moths. Interestingly, Evenden and Gries (2010) revealed that older lures were more attractive to male *P. xylostella* moths than fresh lures, and the pheromone release from aged lures was constant at very low release rates. They also found that even the most attractive commercially available sex pheromone lures attracted fewer *P. xylostella* males than calling virgin female moths. In the current study, we used freshly prepared lures. Hence, the age of the lures and the female moth population in field condition could have influenced the trap catches and the population build-up on cabbage plants. However, it is still possible that pheromone lures can be used as the monitoring tool for timely decision-making in brassica IPM programs.

The major lepidopterans were highly susceptible to spinosyn pesticides, emamectin benzoate, indoxacarb and chlorantraniliprole in this study. This result was consistent with earlier findings by Huang *et al.* (2011), Kannan *et al.* (2011) and Baker (2011) in different parts of the world. Among the biopesticides, they were susceptible to either *B. thuringiensis* subsp. *aizawai* or *B. thuringiensis* subsp. *kurstaki* or both. An earlier study by Srinivasan and Hsu (2008) has documented that *P. xylostella* was susceptible to both Cry1A and Cry1C toxins, whereas *C. pavonana* was susceptible only to Cry1A toxins and *H. undalis* was susceptible only to Cry1C toxins. Since *B. thuringiensis* subsp.

contain Cry1C as major toxin, it is not surprising that these pests are susceptible to either of the formulations in the current study. However, *S. litura* was also found to be susceptible to these formulations, although an earlier study found that *S. litura* was less susceptible to Cry1Ba2 and Cry1Ca4 toxins (Shelton *et al.* 2009).

The pesticide window strategy, which was developed using the most effective chemical and biopesticides showed significant effectiveness against the lepidopteran pests, especially *C. pavonana* and *P. rapae* during the first season. In the second season, the window strategy was combined with pheromone lures and/or trap crop. But neither the pheromone nor the trap crop offered any enhancement in the effectiveness of the window strategy. But the window strategy alone reduced the major lepidopteran pests significantly. In both seasons, window strategy was on par with the farmers' method, which predominantly relied on one or two chemical pesticides only throughout the season. Although the effectiveness was the same in both the treatments, the window strategy reduces the risk of resistance development, especially in *P. xylostella*. Carefully controlled greenhouse trials have demonstrated the value of the window strategy for *P. xylostella* (Zhao *et al.* 2010). It has already been proven that the inheritance of resistance mechanism was different for *B. thuringiensis*, chlorfenapyr and indoxacarb (Feng *et al.* 2011), which are the major control agents in this study. The pesticide window strategy has been proven effective for *P. xylostella* resistance management in different countries, including Australia (Baker 2011; Ridland and Endersby 2011), New Zealand (Walker *et al.* 2011), People's Republic of China (Feng *et al.* 2011) and Hawaii (Mau and Gusukuma-Minuto 2001). Thus, the two-window strategy is quite effective in curtailing the damage caused by major lepidopteran pests on cabbage in Taiwan.

An interesting observation in the window strategy experiment is the consistently lower *P. xylostella* population in untreated check plots. This might have been mainly due to the presence of *C. plutellae*, which kept the larval population in check. However, both the window strategy as well as farmers' method might have reduced the presence of *C. plutellae*, which slightly increased the *P. xylostella* population. However, the window strategy was found to have significant impact in reducing the population of *P. rapae* and *C. pavonana*, which reduced the marketable yield drastically in untreated check plots. Thus, the two-window strategy has an overall effect against key lepidopteran pests in lowland cabbage production systems.

In conclusion, Indian mustard was not an effective trap crop in managing the major lepidopteran pests on lowland brassicas, although it remained attractive. The sex pheromone lures of *P. xylostella* attracted the male moths, but failed to reduce the larval population on cabbage. Hence, it needs further improvement as a mass-trapping tool, although it could currently be used as a monitoring tool in IPM programs. The lepidopteran pests were highly susceptible to recent chemical pesticides, especially spinosyn insecticides and *B. thuringiensis* formulations. The two-window strategy based on these chemical and biopesticides offers significant protection of brassicas in tropical lowlands, where the diversity of *P. xylostella* parasitoids was quite low and the secondary lepidopterans such as *C. pavonana* and *P. rapae* lack effective bio-control agents.

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Review and Status of FAO-assisted DBM Biological Control Programs in the Asia Region

J.W. Ketelaar, A.L.M. Abubakar[1], A. Upanisakorn[2] and T.T.N. Le[3]
FAO IPM/Pesticide Risk Reduction Programme, FAO RAP, Bangkok, Thailand
E-mail: Johannes.Ketelaar@fao.org
[1]FAO IPM/Pesticide Risk Reduction Programme, FAO RAP, Bangkok, Thailand
E-mail: AlmaLinda.Abubakar@fao.org
[2]Biological Control Group, Department of Agricultural Extension, Ministry of Agriculture and Cooperatives, Bangkok, Thailand. E-mail: uareepan@yahoo.com
[3]Technical Division, Plant Protection Sub Department, Lam Dong, Vietnam. E-mail: thanhngadl@gmail.com

Abstract

FAO has supported member countries in the development and application of Integrated Pest Management (IPM) for key pests and diseases in brassica crops in Asia for the last two decades. Capacity building for biological control approaches for management of Diamondback Moth (DBM) and education of farmers in IPM Farmer Field Schools have played a central role in this assistance. Here we review assistance provided to date, assess current status, identify implementation challenges and propose recommendations for strengthening DBM management in selected FAO member countries in the Asia region.

Keywords: *Plutella xylostella*, *Diadegma semiclausum*, Farmer Field School, Integrated Pest Management, Southeast Asia

As the world population rises to a projected 9.2 billion in 2050, farmers must intensify crop production as to meet global food security. With declining yield growth rates, farmers face a series of unprecedented, intersecting challenges: increased competition for land, labor and water, rising fuel and fertilizer prices, and the impact of climate change. Induced by soaring commodity prices, national governments in the Asia region have promoted intensification of crop production in recent years. Incentives provided to farmers included subsidies for inputs, such as fertilizers and pesticides, which has induced wide-spread and indiscriminate use. Farmers have resorted to frequent calendar-based pesticide sprays and excessive amounts of chemical fertilizers in efforts to increase their yields.

Indiscriminate use of chemical inputs, both fertilizer and pesticides, puts agricultural production at risk. In particular, the overuse of pesticides is known to compromise vitally important ecosystem services provided by natural biological control, pollination and nutrient recycling systems. Indiscriminate use of insecticides can result in secondary pest outbreaks which jeopardizes national and regional food security. Intensive use of extremely hazardous chemicals by small-holder farmers continues to cause high incidence of farmer poisoning. Particularly, women and small children remain part of the most pesticide exposure risk-prone groups in rural communities. In addition, pesticide residues on harvested fresh produce regularly exceed Maximum Residue Levels (MRLs), a cause for domestic and international food safety concerns and jeopardizing export potential.

Given the future challenges to global food security, food safety and to the environment, sustainable intensification of agricultural production is emerging as a major priority for policy makers and international development partners. An ecosystem approach must underpin intensification of crop production and reduction of pesticide use. Adoption of Integrated Pest Management by smallholder farmers is deemed a vital component of such intensification efforts (FAO 2011).

The FAO Regional IPM Programme has been supporting governments through National IPM Programmes in the Asia region for over two decades as to address overuse and misuse of pesticides. National IPM Programmes implement farmer training in season-long Farmer Field Schools (FFS) to help farmers achieve more sustainable livelihoods through efficient, profitable, and ecologically-sound agricultural production using Integrated Pest Management. In tandem with such training efforts, FAO works with Governments to develop sustainable pest and pesticide management policies, to strengthen the regulatory framework to control the distribution and use of pesticides, and to enhance capacity for

implementation of these policies and enforcement of pesticide legislation.

Integrated Pest Management (IPM) is an approach that uses a combination of techniques to suppress pests effectively, economically, and in an environmentally sound manner. IPM is a decision-making process involving the identification and monitoring of pest populations, and using the monitoring information to decide the timing of treatments that integrate a variety of control measures, and finally, evaluating the results. One of the most important elements of any robust IPM strategy is making optimal use of biological control (BC), which includes the use of natural enemies to regulate pest populations to a level where these will not cause yield losses.

Within this context, the FAO Regional IPM/ Pesticide Risk Reduction Programme supported the introductions of the parasitoid *Diadegma semiclausum* (Hellén) (Hymenoptera: Ichneumonidae) to control the Diamondback Moth in cabbage in Thailand (2005) and Vietnam (1996-1998). The Diamondback Moth (DBM), *Plutella xylostella* (L.) (Lepidoptera: Plutellidae), is a major constraint to crucifer vegetable production in the highlands of Petchabun, Thailand and Dalat, Vietnam. The introduction of the parasitoid was done in conjunction with IPM Farmer Field School (FFS) training.

An FFS is a discovery-based group learning process. Usually, a group of 25-30 farmers meet one morning weekly for an entire crop growing season and engage in experiential learning activities to gain an ecological perspective of managing ecosystems and skills in informed decision making based on location-specific conditions. The learning process is facilitated by extension workers or trained farmers. Non-formal education methods are employed and the field is used as the primary resource for discovery-based learning. Hence, the FFS is often referred to as a "school without walls". The FFS aims to help small growers adopt IPM, grow healthy crops and produce more and safer food with less agro-chemicals. More specifically, the FFS on IPM in cabbage emphasized the reduction of chemical sprays and replacing these with effective and cost-efficient alternatives that would enhance the conservation and augmentation of natural enemies (i.e., the parasitoid *D. semiclausum*). In general, FFS develop groups of farmers to work together to address pesticide risk reduction in agriculture and deal with other broader rural community concerns (FAO 2013). The FFS is recognized as one of the best educational and capacity building tools for training farmers on acquiring complex and knowledge-intensive skills such as natural resource management (Swanson and Rajalathi 2010).

Materials and Methods

In January-April 2015, the FAO Regional IPM/ Pesticide Risk Reduction Programme carried out an Assessment Study to review and assess current status of DBM control and pesticide use in key highland brassica farming systems in two Asian countries. The study, done in collaboration with the Governments through the Ministry of Agriculture and Cooperatives (Thailand) and Ministry of Agriculture and Rural Development (Vietnam), aimed at formulating recommendations for strengthening IPM work relevant for farmers, government extension systems, regulatory systems and other stakeholders. The survey questionnaire included the key questions:

- What are key insect pests and what is current status of pesticide use in highland brassica farming systems?
- Have introduced parasitoids been established/ spread? How effective is natural biological control for population regulation of DBM and other key brassica pests?
- How effective have the FAO supported IPM interventions been in establishing IPM-based management systems? What, if anything, is needed to strengthen brassica IPM systems?

Two highland brassica farming systems were selected in Dalat, Vietnam and Petchabun, Thailand. A literature review was carried out in January 2015. Farmer surveys and field assessments, including rearing DBM larvae/pupae for assessment of parasitism rates, were conducted from February to March 2015. A total of 15 IPM FFS-trained and 15 non-IPM FFS-trained farmers were interviewed. Analysis of study findings, report writing and discussion of results with relevant stakeholders were carried out during the period March to April 2015.

Results and Discussion

In Thailand, 533 parasitoid adults (57 % female) were released and 100 cocoons left in FFS fields in

one site in November 2005. Patches of crucifers were planted during off-season periods to provide year-round food for DBM, the host for the parasitoids. In this site, farmers participated in two rounds of consecutive IPM FFS. From January to October 2007, another 3,600 parasitoids were released in seven sites to cover 400 ha (area-wide) along with scaling-up of IPM FFS training. Surveys carried out until 2010 consistently confirmed the spread and establishment of the parasitoid over 800 ha with parasitism rates at 80% in fields where farmers practiced IPM (Upanisakorn *et al.* 2011). Further releases of *D. semiclausum* were done in 2011.

Results of the survey taken at the end of the dry season in March 2015 showed that farmers practicing conventional methods for DBM management continue to apply 6-12 pesticide sprays/season. On the other hand, farmers trained in IPM FFS apply bio-pesticide (*Bacillus thuringiensis*) 1-2 times/season and report about 50-80% DBM parasitism (Fig. 1), primarily from *D. semiclausum.* White butterfly (*Pieris*), flea beetles (*Phyllotreta*) and aphids are present but do not cause any serious concern. IPM FFS-trained farmers produce high yielding, profitable and safe cabbages certified through GAP/food safety programmes, marketed locally and internationally (e.g. exported to Dubai and Brunei) by private sector companies such as SWIFT and Good Thai Food. IPM farmers receive 40-50 THB/kg for their cabbages compared to 5-30THB that non-IPM farmers receive for their produce.

In Vietnam, although *D. semiclausum* was imported in 1996, it was not until 1998 when the first release of 2,500 parasitoids was carried out in four sites in Dalat, Lamdong province. Training of farmers in IPM FFS in cabbage commenced in Dalat in 1996 and continued until 2002, whereas parasitoid releases were carried out from the period 1998 to 2001. Surveys carried out from 1998 to 2001 in the release sites showed fluctuating parasitism levels, the highest at 57 %. Surveys were also carried out in non-release sites from 1998 to 2006 and whereas the parasitism levels were fluctuating, these were consistently higher at 60 % in non-release sites. The timing of the surveys (i.e., late season) influenced the results of the survey.

Results of the survey taken at the end of the Winter season in March 2015 (Figure 2) showed that farmers practicing conventional methods for DBM management continue to apply 6 pesticide sprays/season (3-4 for DBM) and the rest for other target pests such as cut (*Agrotis*) and army (*Spodoptera*) worms, as well as flea beetles (*Phyllotreta*). On the other hand, farmers trained in IPM FFS apply bio-pesticide (*Bacillus thuringiensis*) 1-2 times/season and report about 60-75 % DBM parasitism, primarily from *D. semiclausum.* IPM FFS-trained farmers produce high yielding, profitable and safe cabbages in compliance with VietGAP standards for domestic markets. However, IPM farmers do not receive any premium for their produce. Farmer training through IPM FFS has continued, funded by local governments.

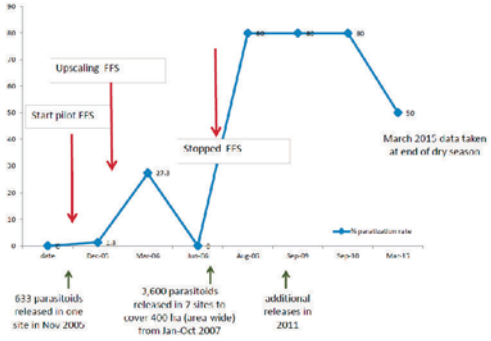

FIGURE 1

Percentage of parasitism by Diadegma semiclausum, *Petchabun Highlands, Thailand*

FIGURE 2

Survey on Diadegma semiclausum *on cabbage, Dalat, Vietnam*

CONCLUSION

Ten years after the introduction of *D. semiclausum* in Petchabun, Thailand (2005) and 17 years in Dalat, Vietnam (1998), the parasitoid is well established and has spread widely. Parasitism levels are established at 50-80 %. In tandem with other parasitoids such as *Cotesia* (providing parasitism at 2-3 % early season and 30-50 % in late season) and *Diadromus*, *D. semiclausum* is providing effective DBM biological control. The results are largely due to the combination of ecosystem-literacy education provided for smallholder farmers that enhances appreciation of biological control essential for the

conservation and sustainable management of the introduced parasitoids. Discovery-learning methods employed in Farmers Field Schools facilitate the development of farmer knowledge and skills for effective and sustainable IPM implementation. Local government buy-in to farmer education and policies in support of ecologically sound production practices contribute to successful and sustainable IPM for DBM. However, there is a need to continue support to strengthen biological control and IPM-FFS training and in particular continue to focus attention to IPM-based prevention and management for other brassica pests (and diseases). Policy support in terms of phasing out the registration and use of broad spectrum pesticides is needed to strengthen sustainable IPM for DBM. There is a need to provide advice to farmers for more effective and less toxic alternatives, where/when chemical pesticide use is still needed. This is important for the conservation and sustainable management of the introduced parasitoids. Access to markets that promote sustainable crop production and provide premiums to farmers facilitate the application and sustainability of ecologically sound practices, including biological control.

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Towards Developing an Integrated Pest Management Strategy for Striped Flea Beetle on Radish

R. Srinivasan, Y.-C. Hsu[1], M.-Y. Lin[2], F.-C. Su[3] and C.-C. Huang[4]

Entomology Unit, AVRDC – World Vegetable Center, Shanhua, Tainan 74151, Taiwan, Republic of China

E-mail: srini.ramasamy@worldveg.org; [1]E-mail: yun-che.hsu@worldveg.org; [2]E-mail: mei-ying.lin@worldveg.org

[3]E-mail: fu-cheng.su@worldveg.org; [4]E-mail: amy.huang@worldveg.org

Abstract

Radish is an important crop in Taiwan. Striped flea beetle, *Phyllotreta striolata* is a major pest of vegetable brassicas including radish. Resistant or tolerant varieties form the core of an integrated pest management (IPM) strategy. Hence, we screened 35 radish accessions from AVRDC's Genetic Resources and Seed Unit for their resistance to *P. striolata* infestation. Only one accession (VI039717) had a lower number of, and amount of damage by, *P. striolata*. The susceptible accession (VI045960) had almost twice the amount of glucosinolates compared with the moderately resistant accession (VI039717). In earlier studies, Chinese cabbage, pak-choi and winter rape were identified as the most attractive crops for *P. striolata*. However, the effect was not sufficiently strong for them to be used as trap crops. Subsequent studies evaluated their use in combination with non-host repellent crops in a 'push (repellent crop) – pull (trap crop)' strategy. Two field experiments were conducted with radish as a main crop, and tomato and winter rape as repellent and trap crops, respectively. However, the results have shown that the push-pull strategy did not reduce the *P. striolata* damage on radish. The push–pull strategy was subsequently combined with allylisothiocyanate (AITC), a glucosinolate hydrolysis product used in traps, and validated against *P. striolata* on radish. The results confirmed that the push–pull strategy combined with AITC was also not effective against *P. striolata*. Therefore, we currently do not have any effective and appropriate pest management strategy against *P. striolata*, except host plant resistance. However, recent research has indicated the promise of male borne aggregation pheromone either alone or in combination with host plant volatiles. In the future, this component technology may be combined with resistant or tolerant cultivars for the sustainable management of *P. striolata* on radish.

***Keywords*:** Radish, *Phyllotreta striolata*, trap crops, push – pull strategy, AITC, aggregation pheromone

Radish is an important crop in Taiwan, and is cultivated on an area of 2,650 ha with an annual production of over 101,000 t (Council of Agriculture, 2014). Striped flea beetle, *Phyllotreta striolata* (F.) (Coleoptera: Chrysomelidae) is one of the specialist insects feeding on brassica vegetables, including radish, and can cause severe crop damage even inside protective structures. Although *P. chotanica* Duvivier has recently been recorded for the first time in Taiwan (Lee *et al.* 2011) as a serious pest in organic farms, *P. striolata* still remains the key pest on vegetable brassicas in Taiwan.

Phyllotrata striolata often lacks parasitoids or other bio-control measures. For instance, natural parasitism of *P. striolata* is uncommon in Taiwan, although *Microctonus vittatae* Mues. (Hymenoptera: Braconidae) has been reported as the parasitoid of *P. striolata* in North America (Wylie 1982). The literature on the effectiveness of *Bacillus thuringiensis* against *P. striolata* is also very scanty. Wongnikong (2007) found that *B. thuringiensis* subsp. *tenebrionis* effectively controlled *P. sinuata* (a junior synonym of *P. striolata*) either alone or in combination with *Steinernema siamkayai* KB strain or chemical pesticides on Chinese radish in Thailand. Hence, radish growers predominantly rely on chemical insecticides to control this insect. However, *P. striolata* has developed resistance to insecticides in several countries including Taiwan (Feng *et al.* 2000), which prompts farmers to spray more insecticides to control the pest. Environmental degradation, human health impacts, resource loss and economic concerns due to pesticide misuse and/or overuse have triggered a growing interest in developing safer management techniques for this pest on radish.

Resistant or tolerant varieties form the core of an integrated pest management (IPM) strategy. However, there are no reported radish varieties with strong levels of resistance to *Phyllotreta* spp. flea beetles. Hence, it becomes imperative to identify if there is any resistant radish accessions available from the gene bank collection(s).

In earlier studies, we identified Chinese cabbage, pak-choi and winter rape as the most attractive crops for *P. striolata*. However, it is highly uncertain whether these attractive host plants can serve as an effective trap crop, since trap cropping has not been found effective in earlier studies. For instance, treatment of the trap crop, turnip rape (*Brassica rapa* L.) with insecticide had little effect on cabbage stem flea beetle, *Psylliodes chrysocephala* (L.) (Coleoptera: Chrysomelidae) on oilseed rape (*Brassica napus* L.) (Barari *et al.* 2005). Chinese cabbage as a trap crop failed to reduce the damage caused by flea beetles on white cabbage (Trdan *et al*. 2005). However, subsequent studies evaluated the use of trap crops in combination with non-host repellent crops in a 'push (repellent crop) – pull (trap crop)' strategy, which was proven effective against the pollen beetle (*Meligethes aeneus*) on oilseed rape (Cook *et al.* 2007). Hence, it is worth investigating the effects of a push-pull strategy against *P. striolata* on radish.

Phyllotreta cruciferae Goeze and *P. vittula* Redtenbacher males were reported to produce a major pheromone compound, which was attractive when combined with host plant volatiles such as 3-butenyl isothiocyanate or allylisothiocyanate (AITC) (Toth *et al.* 2005, 2007, 2012). A male-borne aggregation pheromone has shown enhanced attraction when combined with AITC under laboratory conditions (Beran *et al*. 2011). Hence, traps baited with aggregation pheromones and host plant volatiles may provide useful trapping of *P. striolata* in radish production systems. Thus, the current study was carried out to identify *P. striolata* resistant radish accessions, and investigate a push-pull strategy and aggregation pheromone based trapping strategy.

Materials and Methods

(1) Screening of radish germplasm against P. striolata, and estimation of total glucosinolates in the least damaged accession

Radish germplasm (*Raphanus sativus*) used in this study was obtained from the Genetic Resources and Seed Unit, AVRDC – The World Vegetable Center, Taiwan. Eight commercially available varieties in Taiwan were also included. Preliminary screening was conducted in replicated trials. Four preliminary screening trials *viz.*, spring (March-April) 2009 (15 accessions), autumn (November-December) 2009 (13 accessions), spring (March-April) 2010 (12 accessions) and autumn (November-December) 2010 (56 accessions) in Shanhua, Taiwan were conducted. The trials were carried out in one field in each season. The trials were maintained following customary cultural practices, and without any pesticide application to control flea beetles. The trials were exposed to the natural infestation of flea beetles and the feeding damage was measured as shot-hole area per plant at weekly intervals starting from the first weeks after transplanting in the field for four or five weeks. Five plants of each accession were randomly selected for damage measurement. The mean *P. striolata* damage of the screened accessions was subjected to a statistical analysis based on mean (*m*) and standard deviation (*sd*) (AVRDC 1979), and categorized as follows: the accessions that had mean damage score (n) less than m-2sd were considered highly resistant; between m-1sd to m-2sd as resistant; between m to m-1sd as moderately resistant; between m to m+1sd as moderately susceptible; between m+1sd to m+2sd as susceptible; and more than m+2sd as highly susceptible.

The selected resistant radish accessions with the known susceptible check from preliminary screening trials were screened in an advanced screening trial during March – April 2011. This advanced trial was conducted using a randomized block design with three replications as a confirmatory screening. The crop management and screening method were similar to the preliminary screening trials. Three plants were randomly selected in each replication to record the flea beetles' population and feeding damage. Data obtained on flea beetle population were subjected to analysis of variance (ANOVA) with the Proc GLM procedure of SAS, version 9.1 (SAS Institute, Cary, NC, USA). Least significant difference (LSD) test was used to separate the means at 5 % significance level of probability.

One of the least damaged accession was selected to be compared with a susceptible accession. These two accessions were compared for their total glucosinolate content in both leaves and roots. Total glucosinolate content was determined using microcolumn-enzyme procedure described in Kershaw and Johnstone (1990) with minor modification. Briefly, total glucosinolates in 0.3 g of fine powder were extracted two times with 5 mL of 50% methanol in water in a water bath at 80°C. The extracts were stored at -20°C until analyzed. Columns

with pasteur pipettes (150 mm) and 4-cm bed of DEAE–SephadexTM A-25 (Amersham Biosciences, Sweden) were prepared. The column was equilibrated by passing the following solutions successively: 1 mL of dH2O, 1 mL of 0.5 M pyridine acetate (PA) buffer (pyridine:glacial acetic acid:water, 40:30:930), 1 mL dH2O, 1 mL 0.02 PA buffer. Plant extracts were applied to the columns after pH equilibration. The columns were washed twice with 1 mL dH_2O and 1 mL 0.02 M PA buffer. Myrosinase (thioglucosidase; E.C. 3:2:3:1, from *Sinapsis alba*, Sigma–Aldrich Chemie, Germany) solution was prepared by diluting 0.5 mL myrosinase (10 units/mL) to 9.5 mL 0.02 M PA buffer. An amount of 0.25 mL of the myrosinase solution was added into each column, while 0.02 M PA buffer was added onto the control columns. The columns were immediately plugged with rubber stoppers to create positive pressure to force the enzyme into, but not through, the columns. The columns were left for 16 h at room temperature of around 24 °C to allow enzyme reaction. After the enzyme reaction, 0.5 mL of dH_2O was passed through the columns and force-eluted using compressed air. A total of 1.25 mL of solution for each extract was collected. The glucose content of the aliquot was estimated using a glucose diagnostic kit (Diagnostic Chemicals Limited, USA). Absorbance was determined at 340 nm in a spectrophotometer (Hitachi U-2001, Japan). A calibration curve was made to calculate the amounts of total glucosinolate content.

(2) Evaluating a push-pull strategy against **P. striolata**

Winter rape, which was identified as one of the most preferred host plants of *P. striolata* from our earlier studies (unpublished) was used as the trap crop. A non-host crop (tomato) was used as the repellent crop. The trial conducted during spring 2009 included four treatments *viz*., push-pull, push, pull and the check. Each treatment was replicated three times, and the design was a randomized complete block design (RCBD).

(i) The push-pull plots had eight beds of radish, one bed of tomato after four beds of radish, and one bed of winter rape each before the first bed and after the eighth bed of radish.

(ii) The push plots had eight beds of radish, one bed of tomato each before the first bed, after the fourth and eighth beds of radish.

(iii) The pull plots had nine beds of radish, one bed of winter rape each before the first bed and after the eighth bed of radish.

(iv) The check plots had eleven beds of radish only.

Ten radish plants were randomly selected in each replication, and the total number of *P. striolata* on each plant as well as the number of shot holes on two selected leaves in each plant were counted at weekly intervals for five weeks starting from two weeks after transplanting. The mean number of *P. striolata* and shot holes per plant were compared between the treatments using ANOVA.

A second trial conducted during autumn 2009 included five treatments, in which a second pull treatment using buckwheat was included besides the four treatments used in the spring. However, the number of shot holes in a 1-cm X 1-cm area were counted on two selected leaves, because of the large number of damage holes. The remaining details were similar to the spring trial.

The third field trial was conducted during spring 2011, and was exactly similar to the spring 2009 trial. However, a fifth treatment included a chemical pesticide targeting *P. striolata* control. In addition, the chemical pesticides (profenofos, cartap and abamectin) were also applied on the trap crop (winter rape) to prevent the movement of *P. striolata* to the main crop (radish). Because of the large number of beetles and feeding damage, we measured the shot-hole area using a leaf area meter, instead of counting the number of shot holes. The remaining details were similar to the spring 2009 trial.

(3) Evaluation of AITC and aggregation pheromone against **P. striolata**

During the trials in spring 2009, five different AITC-doses (0.2 ml, 0.4 ml, 0.8 ml, 1.6 ml and 3.2 ml) were compared to a check (without AITC) for attracting the *P. striolata*. The six treatments were applied in four replications in a RCBD. For each replication, two sticky traps baited with the AITC lures were erected. The number of *P. striolata* in each trap was collected and counted, and the AITC was filled to the original level every week. The experiment was continued till the harvest of radish. The data on trap counts, number of *P. striolata* on radish and mean number of shot holes per plant were analyzed by ANOVA to identify the effective doses of AITC.

A second field experiment was conducted during autumn 2009 with the chosen AITC-dose (0.4 ml/trap) to confirm its effectiveness in attracting striped flea beetle. AITC was mixed with an equal quantity (0.4 ml) of liquid paraffin and it was compared to the control treatment (0.8 ml liquid paraffin). Liquid paraffin was added to reduce the evaporation of AITC. The experiment was conducted using a RCBD with seven replications. For each plot, two sticky traps baited with the AITC lures were erected. The number of striped flea beetle in each trap was collected and counted, and the AITC was filled to the original level every week. The experiment was continued till the harvest of radish. The data on trap counts, number of *P. striolata* on radish and mean number of shot holes per plant were analyzed by ANOVA to confirm the effectiveness of AITC.

During the trials in autumn 2011, the aggregation pheromone was used to develop the lures and tested against *P. striolata* on radish. In addition, the lures were also developed by combining the AITC with the male-borne aggregation pheromone of *P. striolata*, and tested against *P. striolata* on radish under field conditions. There were six treatments: (i) AITC – 400 µl, (ii) aggregation pheromone – 250 µg, (iii) AITC – 200 µl + aggregation pheromone – 125 µg, (iv) AITC – 400 µl + aggregation pheromone –250 µg, (v) chemical pesticides (profenofos and abamectin applied alternatively), and (vi) untreated check. The six treatments were applied in three replications following RCBD. The remaining details were similar to the above experiment.

(4) Evaluation of integrated management approaches against P. striolata

A field trial based on the integration of 'push-pull' strategy and AITC was conducted in spring 2010. There were eight treatments: (i) push-pull plots with tomato and winter rape, as repellent and trap crops, respectively, (ii) push plots with tomato, (iii) pull plots with radish, (iv) plots with AITC traps, (v) plots containing both tomato and AITC traps, (vi) plots containing both winter rape and AITC traps, (vii) chemical pesticide (profenophos), and (viii) check plots which had radish only. Each treatment was replicated three times, and the design was a RCBD. Fifteen plants were randomly selected in each replication, and the total number of *P. striolata* on each plant as well as the number of shot holes on two selected leaves in each plant were counted at weekly intervals for four weeks starting from two weeks after transplanting. The mean number of *P. striolata* and shot holes per plant were compared between the treatments using ANOVA. The yield was also recorded during harvest.

Results

(1) Screening of radish germplasm against P. striolata

Of the 15 accessions screened during spring 2009, no accession was rated as highly resistant or resistant. However, eight accessions were selected as moderately resistant (data not shown). Similarly, no accession was rated as highly resistant or resistant out of 13 accessions screened during autumn 2009. Another eight accessions were selected as moderately resistant from this trial (data not shown). Of the 12 accessions screened during spring 2010, no accession was rated as highly resistant, resistant or moderately resistant. However, of the 56 accessions screened during autumn 2010, 33 accessions were rated as moderately resistant (data not shown). Thus 49 moderately resistant accessions were identified from four preliminary screening trials in 2009 and 2010. However, only 35 least damaged accessions from this list of 49 accessions were selected for further advanced screening trial. The results revealed that 22 accessions were confirmed to be moderately resistant based on the *P. striolata* damage (Table 1). However, based on the least feeding damage as well as lowest number of *P. striolata* adults per plant, only one accession (VI039717) was selected. Of the moderately susceptible accessions, VI045960 was selected as the susceptible check, because of the highest number of *P. striolata* adults per plant and more feeding damage on this accession.

Since glucosinolates are important feeding stimulants for *P. striolata*, the total glucosinolate content in VI039717 was compared with that of VI045960. VI045960 had significantly higher glucosinolates (113.17 µmole/100g) in its roots than VI039717 (61.98 µmole/100g) ($t=22.24$; $p=0.002$). Similarly, the glucosinolate content in the leaves of VI045960 was also significantly higher (104.93 µmole/100g) than VI039717 (48.40 µmole/100g) ($t=56.54$; $p=0.0004$).

TABLE 1

Resistance reaction of selected radish accessions to striped flea beetle (March – April 2015)

Accession	Mean no. of *P. striolata* / plant	Mean hole area (cm^2)/leaf	Resistance rating
VI034560	19.30 (4.37) a-d	12.28	MS*
VI034594	12.00 (3.53) b-f	11.13	MS
VI038232	17.97 (4.26) b-d	9.48	MS
VI040000	14.22 (3.80) b-e	8.73	MR**
VI040031	14.36 (3.84) b-e	8.60	MR
VI040082	15.53(3.90)b-e	10.43	MS
VI039717	5.36 (2.30) f	5.28	MR
VI039718	9.03 (3.05) d-f	7.23	MR
VI039720	16.03 (4.03) b-e	8.01	MR
VI039722	9.36 (3.12) d-f	7.13	MR
VI041125	9.72 (3.15) d-f	6.57	MR
VI041164	16.00 (3.99) b-e	9.37	MS
VI041175	15.58 (3.96) b-e	9.84	MS
VI043327	11.53 (3.38) c-f	7.43	MR
VI043328	15.92 (3.95) b-e	7.72	MR
VI043330	16.45 (4.06) b-e	7.81	MR
VI045960	33.22 (5.68) a	12.75	MS
VI046024	14.75 (3.89) b-e	10.74	MS
VI046025	17.84 (4.23) b-e	9.12	MS
VI046029	22.58 (4.73) a-c	8.87	MS
VI046031	13.97 (3.66) b-f	7.56	MR
VI046226	9.11 (3.10) d-f	5.82	MR
VI046227	11.33 (3.39) c-f	6.59	MR
VI046610	12.00 (3.45) c-f	13.07	MS
VI046616	18.08 (4.30) b-d	7.43	MR
VI046728	15.81 (3.93) b-e	8.08	MR
VI047201	12.36 (3.58) b-f	8.55	MR
VI047221	14.72 (3.77) b-e	8.53	MR
VI047243	9.86 (3.15) d-f	6.27	MR
VI047367	11.89 (3.42) c-f	12.89	MS
VI047694	8.31 (2.86) ef	7.63	MR
VI048157	10.28 (3.19) d-f	7.72	MR
VI050748	15.92 (4.04) b-e	9.12	MS
VI050750	13.36 (3.64) b-f	7.92	MR
VI050752	23.97 (4.89) ab	12.01	MS
• ‡ • •	13.17 (3.69) b-e	7.42	MR
F value	1.62		
P value	0.04		

(2) Evaluating a push-pull strategy against **P. striolata**

None of the treatments including the trap crop (winter rape) alone, repellent crop (tomato) alone and both trap crop and repellent crop together did not reduce the incidence and damage of *P. striolata* significantly during the spring season (Table 2). Similarly, none of the treatments including the trap crop (winter rape), non-host repellent crops (tomato and buckwheat) failed to reduce the incidence and damage by *P. striolata* since there was no significant difference in mean number of *P. striolata* and shot-holes among the treatments during the autumn season, as well (Table 3). Hence, the yield also did not vary significantly among the treatments.

TABLE 2

Evaluation of 'push-pull' strategy against striped flea beetle on radish (May – June 2009)

Treatment	Mean no. of *P. striolata*/leaf	Mean no. of shot holes/leaf
Check (only radish)	1.73	8.05
Push (tomato with radish)	1.54	8.29
Pull (winter rape with radish)	1.51	7.60
Push-Pull (tomato & winter rape with radish)	1.44	7.60
F value	0.14	0.34
P value	0.94	0.80

TABLE 3

Evaluation of 'push-pull' strategy against striped flea beetle on radish (November – December 2009)

Treatment	Mean no. of *P. striolata*/leaf	Mean no. of shot holes/cm^2	Yield (t/ha)
Check (only radish)	1.15	1.63	8.81
Push (tomato with radish)	1.07	1.47	11.38
Push (buckwheat with radish)	0.62	1.42	7.63
Push-Pull (tomato & winter rape with radish)	1.27	1.35	10.49
Pull (winter rape with radish)	0.62	1.21	7.07
F value	1.53	0.41	1.29
P value	0.27	0.80	0.34

The third trial conducted during spring 2011 also confirmed the earlier results. Although chemical pesticide was used on the trap crop to curtail the movement of *P. striolata* from winter rape to radish.

TABLE 4

Evaluation of 'push-pull' strategy against striped flea beetle on radish (March – May 2011)

Treatment	Mean no. of *P. striolata*/plant	Mean shot hole area (cm^2)/leaf	Yield (t/ha)
Check (only radish)	18.44	11.16	11.94 b
Profenofos	14.70	8.91	19.31 a
Pull (winter rape with radish)	18.50	10.93	10.82 b
Push (tomato with radish)	23.55	13.30	9.38 b
Push-Pull (tomato & winter rape with radish)	21.83	13.44	10.15 b
F value	0.94	1.08	20.22
P value	0.47	0.40	<0.0001

Means followed by the same letter(s) in a column are not significantly different ($p < 0.05$) by Tukey's HSD

(3) Evaluation of AITC and aggregation pheromone against P. striolata

The total number of *P. striolata* trapped over six weeks was significantly higher in traps baited with AITC @ 0.4 ml and 3.2 ml per lure (Table 5). Other two doses of AITC also attracted higher number of beetles than untreated check (F=2.94; p=0.028). However, it did not reduce the incidence and damage by *P. striolata*, since there was no significant difference in mean number of beetles per plant and shot holes among the treatments. The AITC treatment @ 0.4 ml with liquid paraffin also attracted significantly more striped flea beetle than untreated check (F=71.80; p=0.0001) (Table 6). However, this also did not result in a significantly lower beetle population on the plants (F=1.78; p=0.23). Unfortunately, the AITC treatment slightly increased the damage compared to the untreated check (F=6.74; p=0.04). Hence, AITC alone may not be effective in reducing the striped flea beetle damage.

TABLE 5

Evaluation of allyl isothiocyanate (AITC) against striped flea beetle on radish (February – April 2009)

Dose (ml/lure)	Mean no. of *P. striolata* / trap	Mean no. of *P. striolata* / plant	Mean no. of shot holes /leaf	Yield (t/ha)
Check	252.00 (5.52) c	34.84	14.51	9.29
0.2	324.70 (5.57) bc	20.44	11.30	6.53
0.4	548.20 (6.18) a	24.29	14.49	7.34
0.8	480.70 (6.15) ab	28.85	13.39	7.73
1.6	400.00 (5.98) abc	23.67	12.07	4.99
3.2	541.30 (6.24) a	31.68	13.84	1.28
F value	2.94	1.18	1.02	1.12
P value	0.028	0.33	0.41	0.40

Means followed by the same letter(s) in a column are not significantly different ($p<0.05$) by Tukey's HSD
Figures in parentheses are log-transformed values

TABLE 6

Evaluation of allyl isothiocyanate (AITC) against striped flea beetle on radish (October – November 2009)

Treatment	Mean no. of *P. striolata* / trap	Mean no. of *P. striolata* / plant	Mean no. of shot holes / leaf
0.4 ml AITC + 0.4 ml liquid paraffin	125.33 (4.82) a	2.18	2.78 a
0.8 ml liquid paraffin	19.42 (2.93) b	1.83	2.20 b
F value	71.80	1.78	6.74
P value	0.0001	0.23	0.04

Means followed by the same letter(s) in a column are not significantly different ($p < 0.05$) by Tukey's HSD
Figures in parentheses are log-transformed values

The aggregation pheromone alone was unable to attract significantly higher number of beetles when it was tested in the trial in 2011. The number of beetles attracted in traps baited with aggregation pheromone was on par with traps without any lures (Table 7). Similarly, the aggregation pheromone did not have any synergistic or additive effects, since the number of beetles attracted in traps baited with AITC alone or AITC combined with aggregation pheromone (either half-dose or full dose) remained non-significant ($F=7.25$; $p=0.002$). However, as in the above experiments, higher attraction of beetles did not reduce the incidence and damage of *P. striolata,* since there was no significant difference in mean number of beetles on the leaves ($F=1.42$; $p=0.28$) and shot holes ($F=0.96$; $p=0.48$) among the treatments.

TABLE 7

Evaluation of allyl isothiocyanate (AITC) and aggregation pheromone against striped flea beetle on radish (November – December 2011)

Treatment	Mean no. of *P. striolata* / trap	Mean no. of *P. striolata* / leaf	Mean no. of shot holes / cm^2
Check	5.00 (2.30) b	1.85 (1.52)	5.73 (2.49)
AITC (400 µl)	30.67 (5.55) a	1.31 (1.33)	6.06 (2.50)
Aggregation pheromone (250 µg)	6.67 (2.62) b	0.94 (1.18)	4.82 (2.27)
AITC (200 µl) + Aggregation pheromone (125 µg)	54.67 (6.90) a	1.52 (1.42)	4.80 (2.28)
AITC (400 µl) + Aggregation pheromone (250 µg)	59.33 (7.68) a	2.56 (1.73)	5.56 (2.46)
Profenofos	7.00 (2.72) b	1.13 (1.23)	4.08 (2.14)
F value	7.25	1.42	0.96
P value	0.002	0.28	0.48

Means followed by the same letter(s) in a column are not significantly different ($p<0.05$) by Tukey's HSD
Figures in parentheses are square-root transformed values

(4) Evaluation of integrated management approaches against P. striolata

Two results have shown that the number of *P. striolata* beetles ($F=0.78$; $p=0.61$), its damage ($F=0.30$; $p=0.95$) and yield ($F=1.27$; $p=0.32$) did not differ significantly among the treatments (Table 8). Thus the AITC alone or in combination with trap crops and/or repellent crops was unable to reduce the incidence of *P. striolata* on radish.

TABLE 8

Evaluation of 'push-pull' strategy alone and in combination with allyl isothiocyanate (AITC) against striped flea beetle on radish (April – May 2010)

Treatment	Mean no. of *P. striolata*/leaf	Mean shot hole area (cm^2)/leaf	Yield (t/ha)
Tomato and winter rape	4.06	9.67	2.22
Tomato as a repellent crop	3.79	8.74	2.09
Winter rape as a trap crop	3.76	11.61	2.11
AITC trap	4.75	8.68	1.41
Tomato and AITC trap	6.58	10.28	1.29
Winter rape and AITC trap	4.05	10.52	1.33
Profenofos	5.44	10.88	3.17
Control (only radish)	3.73	8.73	1.67
F value	0.78	0.30	1.27
P value	0.61	0.95	0.32

DISCUSSION

The screening results confirmed that there are no highly resistant radish accessions against *P. striolata*. Availability of resistant germplasm within Cruciferae to *P. striolata* is very limited. *Sinapis alba* cv. Ochre was reported to have high levels of resistance to *P. cruciferae* feeding (Bodnaryk and Lamb 1991), and antixenosis as well as tolerance were identified as the major mechanisms of resistance in this cultivar. In our study, glucosinolate content was associated with susceptibility of radish accession(s) to *P. striolata*, since it was found to be higher in the susceptible accession. However, further work is needed to determine whether it is a cause and effect relationship. *Phyllotreta* beetles are closely associated with glucosinolate-containing plants in the plant families including Brassicaceae, Resedaceae, Tropaeolaceae, and Capparaceae, on which they feed and complete their life cycle (Feeny *et al.* 1970; Nielsen 1988; Vig 2004). Since brassica crops with the highest as well as the lowest total glucosinolate content are equally preferred by *P. striolata*, Beran *et al.* (2008) assumed that the total glucosinolate content is not critical for their interaction. Gavloski *et al.* (2000) found that resistance to flea beetles within the Brassicaceae is a genetic trait and can be transferred by interspecific hybridization. Further work is needed to determine which specific glucosinolate is most closely related to resistance and whether radish accessions with lower glucosinolate content could be used in *Phyllotreta* resistance breeding programs. However, glucosinolates have been reported to lower the risk of certain types of cancers, and thus have potential health benefits. Hence, the nutritional importance of glucosinolates and their role in enhancing the susceptibility of crops to flea beetles should be thoroughly weighed.

Although several brassica crops such as Chinese cabbage, pak-choi and winter rape were found to be attractive to flea beetles, they failed to reduce the beetle damage on the main crops, when they were deployed as the trap crops. Trdan *et al.* (2005) also confirmed the inefficiency of Chinese cabbage as a trap crop in reducing flea beetle damage on white cabbage. Historically, trap crops showed variable efficiencies against various brassica pests in different locations. For instance, Indian mustard was proven to be an effective trap crop for diamondback moth in India (Srinivasan and Krishna Moorthy 1992) and South Africa (Charleston and Kfir 2000), however, it was not attractive for the population in the Pacific (Muniappan *et al.* 2001). Hence, we attempted a modified version of trap cropping in the current study. Recently, the deployment of repellent crop – trap crop in a 'push-pull' strategy has proven to be effective in managing devastating pests including corn stem borer (Cook *et al.* 2007). However, when we deployed tomato and buckwheat as 'push' crops and winter rape as a 'pull crop', it proved ineffective in curtailing the *P. striolata* infestation on radish.

Allylisothiocyanate (AITC), a mustard oil, is a breakdown product of glucosinolates, which is commonly present in brassicas. AITC is known to be attractive to various flea beetles including *P. striolata* (Pivnick *et al.* 1992; Tóth *et al.* 2007). Although AITC was found to be attractive in the current study, it failed

to translate the trap catches into reduced feeding damage on radish and thus improving the yield. Hence, we attempted testing the aggregation pheromone, which was recently reported by Beran *et al*. (2011) in *P. striolata*. However, aggregation pheromone alone was found to be only slightly attractive to *P. striolata*. Similar results have already been reported for *P. cruciferae*. For instance, the synthetic compounds were not or only slightly attractive although six male specific sesquiterpenes were identified. Only, (+)-(6R,7S)-himachala-9,11-diene was identified as attractive to *P. cruciferae* (Tóth *et al.* 2005). However, aggregation pheromone was found to significantly enhance the attractiveness of AITC (Soroka *et al.* 2005; Tóth *et al.* 2005). We found a similar synergistic attractiveness in the current study. However, AITC when combined with the 'push-pull' strategy failed to reduce the flea beetle damage. Thus, utilization of AITC in combination with aggregation pheromone could be considered in the management of *P. striolata* on radish, as one of the components in an IPM program.

In conclusion, we failed to identify a highly resistant radish accession or cultivar for managing *P. striolata*. However, partial resistance is a possibility and it has to be integrated with other management strategies. Although lower glucosinolates may be associated with plant resistance to *P. striolata*, it might also reduce the nutritional value of an accession or cultivar since glucosinolates reduce the risk of certain cancer types. Our tests on trap cropping and the 'push-pull' strategy were not effective in managing *P. striolata* on radish. However, additional research may identify stronger repellent crops; but these repellent crops should have commercial value, which would guarantee the adoption of this technology by the small-holder brassica growers. Traps based on host plant volatiles and aggregation pheromone show promise in managing *P. striolata*, although this needs further refinement and validation.

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Plantwise- a Center for Agriculture and Bioscience International (CABI)–led Extension Program for Farmers

A. Sivapragasam and W. H. Loke[1]
CABI Southeast Asia Regional Center, Building A19, MARDI, Serdang, Selangor, Malaysia
E-mail: a.siva@cabi.org; [1]E-mail: w.loke@cabi.org

Abstract

This paper described the broad aspects of the Center for Agriculture and Bioscience International (CABI)-led *Plantwise* program which supports and complements national plant health system including extension programs in developing countries. Essentially, *Plantwise* provided smallholder farmers with better access to advice and information - and thus losing less of what they grow due to plant health problems. The three key components of *Plantwise* were briefly discussed, *viz*., plant clinics run by trained plant doctors and supported by a range of stakeholders including agro-dealers, the Knowledge Bank and Monitoring and Evaluation. Some aspects of these components were illustrated using brassicas and the concomitant pests and diseases. Aspects examined included the types of brassica crops that the farmers bring to the plant clinics, the key problems diagnosed by plant doctors and their management recommendations. The paper also included examples of farmer-friendly extension materials such as pest and disease factsheets and the pest management decision guide. The opportunities and challenges to implementing *Plantwise* in target countries are also addressed.

Keywords: *Plantwise*, brassica farmers, plant clinics, pests, diseases

Many plant health problems threaten crop production. These include pests which are estimated to destroy 30-40 % of smallholder farmers' produce. Helping farmers to reduce losses can therefore make significant improvements in their livelihoods and family food security. In this context, effective management of plant health problems is crucial towards the many depending on it for their livelihoods.

Shelton (2017) in his keynote address presented in this Conference titled: *The Talekar Challenge: What Have We Learned and Where Are We Going with Practical DBM Research and Extension since 1985?*, highlighted the continuing challenges for the management of the diamondback moth (DBM), the key pest of brassicas worldwide causing billions of dollars in losses. Amongst these challenges were "*creating practical outreach programs that enable farmers to manage DBM in a more sustainable manner"* and the bridging of the *'disconnect between the Science and Service to Farmers*". Friis-Hansen and Egelyng (2007) (as quoted by Danielsen *et al.* 2011) made a comparative study of five major funds created to support local innovations, and revealed that there is a tendency for projects to focus on technologies, farmer learning, experimentation and farmers' motives, with less emphasis on innovations in delivery of services and information to farmers. This happened despite the historic failures with establishing advisory services. Danielsen *et al.* (2011) also suggested that the emphasis on projects has limited institutional innovations needed to create systemic changes in service delivery and integration of effort across the plant health spectrum *e.g*., research, extension, input supply and regulatory services, which remain largely disconnected in many countries.

Issues in the current extension systems

Amongst the key issues that pervade many extension systems in countries include: (i) Weak horizontal and vertical integration of stakeholders across the plant health spectrum *viz*., policy makers, research, extension, input supply and regulatory services. Essentially, many of the stakeholders remain largely disconnected, (ii) Lack of resources and access to provide basic services especially for marginal or peripheral rural communities, (iii) Poor accessibility of advisory services which is generally based on a 'as and when' basis. Currently, government-based extension services provide advisory services to farmers who bring their problems to them. However, not all farmers, especially those in the deep rural landscape, are able to access such services, and (iv) Inadequate or incomplete record keeping for future referencing and data mining for strategic and tactical interventions.

Cabi-Led Plantwise Program

Plantwise (PW) is a global alliance, led by the Centre for Agriculture and Biosciences International (CABI), working together with various partners to improve food security and the lives of the rural poor by providing them knowledge they need to feed more and lose less to pests. The program helps countries to develop sustainable national plant health systems where community-based plant clinics provide practical advice to smallholder farmers when they need it. Thus, the PW program is about building crop production resilience, enhancing equity of smallholders and it integrates, both horizontally and vertically, with various stakeholders involved with the wellbeing of farmers, providing equal opportunity in terms of gender, accessibility and quality of service through effective farmer-friendly communication channels. The plant clinics play a pivotal role in providing plant health advisory services to farmers as well as inserving as an entry point for the Plantwise approach and catalyzing new patterns of interaction between stakeholders (Figure 1). *Plantwise*, through the knowledge bank, also strengthens the availability and exchange of knowledge, data and information among plant health system stakeholders. Thus, the CABI-led PW program provides a strengthened extension approach, supported by the knowledge bank (KB) and diagnostics components. PW is functional through three key components, *viz*., (i) plant clinics (PCs) supported by a range of stakeholders including agro-dealers; (ii) a comprehensive Knowledge Bank (KB) (www.plantwise.org/Knowledge Bank) and (iii) Monitoring and Evaluation system in place to assess the quality of PCs implemented. The key entry point relies on the establishment and operation of plant clinic (Figure 2) networks which provide primary health care and are run by trained 'plant doctors' (PDs) supported by the global knowledge bank. Generally, PDs are local extension staff with agriculture and/or plant protection background. The KB is made up of an open-access area, which is freely-accessible online for anyone with an internet connection (and offline as a USB) wanting to access information about pest and disease identification and management and a closed access area in the Plantwise Online Management System (POMS), where data from clinics is kept under secure access control for viewing only by the in-country stakeholders. POMS providesdata management support across *Plantwise*, including analytics. For more salient details of the PW program, refer to the following website: www.plantwise.org.

The early proponents of PW-based clinics (PCs) (Boa 2009; Bentley and Boa 2011) suggested that plant clinics are a catalyst, an entry point for improving and widening access to extension services. The overarching goal is to achieve sustainable productivity increases by making advisory services more effective, increasing outreach and providing timely, reliable and regular information. PCs are regular clinics (= visible extension) linked with diagnostics (= quality control) connected to in-country labs with international lab back-up with remote microscopy, consolidated support data (knowledge bank), which are country-specific. Although the plant clinics are usually run in a fixed location to meet the convenience of the farmers, there are also mobile-based services provided *e.g.*, in Thailand and Vietnam due to poor accessibility of farmers to the fixed PCs.

Based on the summary statistics from the Plantwise Annual Report 2014 (www.plantwise.org), the *Plantwise* programme is operating in 33 countries by the end of 2014. Nearly 2 million households (estimate: 1.9 million) were reached through direct and indirect reach of plant clinics and complementary activities. *Plantwise* Partnership Agreements signed with partners in Asia, Africa and Latin America. *Plantwise* training courses on various topics (field diagnosis, giving advice, producing extension materials, data management, monitoring and evaluation) delivered to 4,400 personnel from partner organizations. A total of 661 plant clinics were newly established in 2014 (1,413 plant clinics in total). Successful trials of using digital tablets at plant clinics in Kenya and India for more efficient information exchange with and among plant doctors were conducted and over 75,000 plant clinic records from 20 countries were deposited in the POMS.

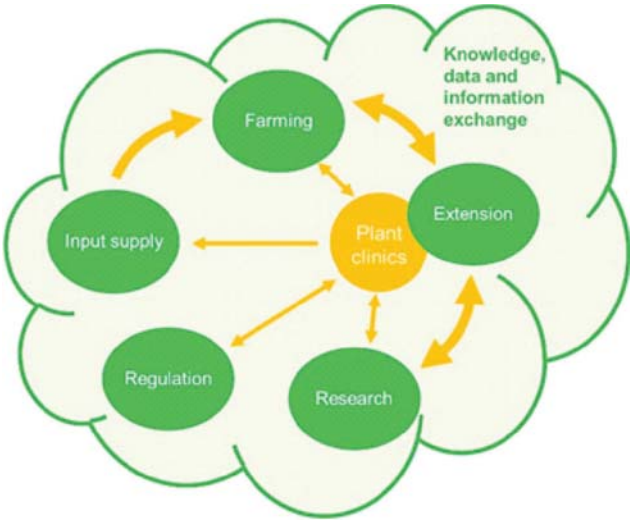


FIGURE 1
A plant health system is defined by the set of all national plant health stakeholders and their linkages.This diagram illustrates with orange arrows which stakeholder linkages Plantwise most effectively strengthens through plant clinics and other activities

FIGURE 2
Examples of plant clinics in action attended by plant doctors (in uniform) in Myanmar (top) and Thailand (bottom)

PLANTWISE AND BRASSICA PESTS & DISEASES

Here, we briefly describe how the *Plantwise* program assists farmers against brassica pests and diseases. It should be mentioned that the paper outlined only some aspects of the *Plantwise* program. There are many other areas of activities, such as plant health rallies which are conducted as a mass communication approach to inform farmers on specific problems or issues, elements of the M&E component such as PC monitoring for performance, cluster meetings held with PDs and relevant stakeholders to discuss issues, improvements of PC performance, *etc.*

Use of data records

To elicit information on brassica pests and diseases, we examined data entries from the POMS. Essentially, each country's PC data are entered into POMS after checking on the accuracy of the data entries for symptoms, diagnosis and recommendations. We obtained the following data: (i) List of Brassica crops that were brought to the PCs by farmers, (ii) Key pests and diseases, and (iii) List of recommendations given by PDs for managing the diamondback moth, a key pest of brassicas worldwide.

(i) Brassica crops brought to the PCs by farmers

Of the over 75,000 plant clinic records from 20 countries deposited in the POMS, more than 4000 records are of various species of Brassicas grown globally. The number of brassica records increased gradually from 45 (2011), to 672 (2012), 1269 (2013) and 1825 (2014). The crops recorded and examined in POMS included head cabbage (*Brassica oleracea* var. *capitata*), green or Indian mustard *(B. juncea* (L.) , broccoli (*B.oleracea* var. *italica*), cauliflower (*B. oleracea* var. *botrytis*), tronchuda cabbage or Portuguese kale (*B. oleracea* var. *acephala*), Chinese kale or kailan (*B. oleracea* var. *alboglabra*), kohlrabi (*B. oleracea* var. *gongylodes*), Brussels sprouts (*B. oleracea* var. *gemmifera*), canola (*B. napus*) and Chinese turnip (*B. rapa* var. *rapa*). For example, the distribution map for plant clinics in Cambodia and Vietnam with brassicas reports is shown in Figure 3.

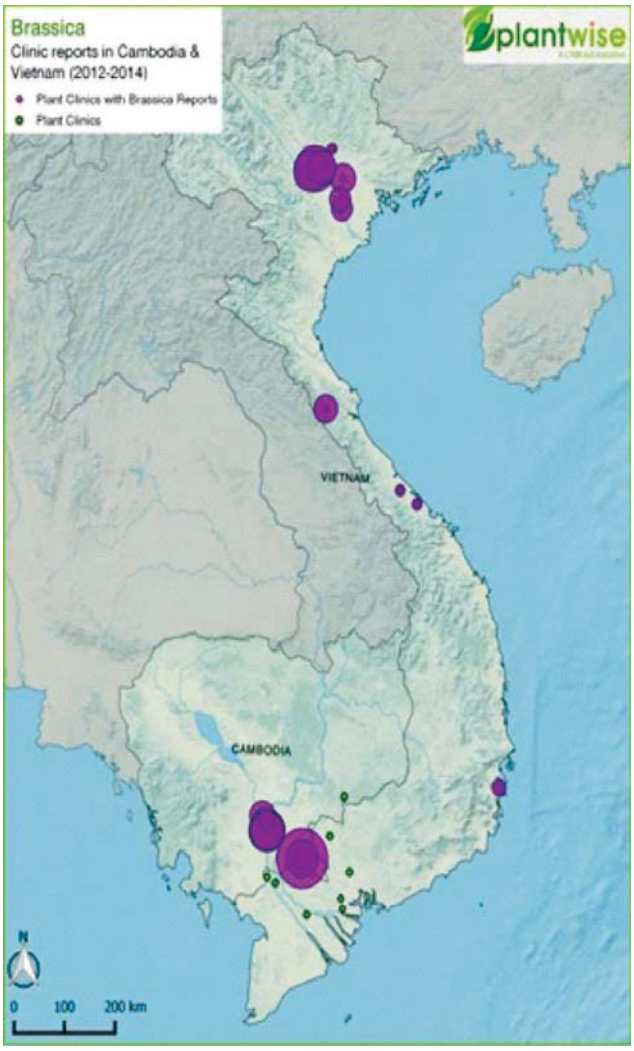


FIGURE 3

Map showing distribution of plant clinics showing brassica reports in Cambodia and Vietnam

(ii) Key pests and disease problems

Table 1 provides a sample crucifer pest and disease records from various countries. The majority of the problems (> 90%) addressed at the PCs by the PDs were biotic (*e.g*., pests and diseases), whereas the rest were abiotic (*e.g*., nutrient deficiency) problems. Amongst the key pests recorded by PDs were the diamondback moth (DBM), armyworm, aphids, cutworm, leaf-miners and the diseases included soft rot, black rot, and leaf spot. Globally, the DBM infested crops based on the number of records (in parentheses) were as follows: cabbage (128), Chinese kale (46), Chinese cabbage (11), rape (9), mustard (7), cauliflower (4) and kohlrabi (3). Figure 4 shows an example of a map on the distribution of plant clinics and DBM reports in Cambodia and Vietnam.

TABLE 1

Sample crucifer pest and disease records from various countries

Damping off disease	Bangladesh
Diamondback Moth (DBM)	Cambodia
Cabbage head caterpillar	Cambodia
Cabbage looper	Cambodia
Cabbage webworm	Cambodia
Chinese kale - downy mildew	Cambodia
Chinese kale - striped flea beetle	Cambodia
Diamondback Moth	China
Downy Mildew of Chinese Cabbage	China
Rape Sclerotium	China
Soft Rot of Chinese Cabbage	China
Aphid in cabbage	Grenada
Cabbage aphid	India
Alternaria	Kenya
Aphids	Kenya
Armyworms on Brassica	Kenya
Bacterial leaf spot on Brassica	Kenya
Bacterial soft rot on Brassica	Kenya
Bagrada bug on Brassica	Kenya

(iii) Recommendations/Management options

At the PCs, the pest management recommendations for DBM were largely aligned towards the use of insecticides (54 %), followed by cultural (40 %) and biological control (5 %). Suggestions were also provided to monitor the problem. In addition to the recommendations given in a PC prescription form, where appropriate, farmers are also provided with farmer-friendly factsheets (FS) (examples on DBM and black rot of cabbage; Figures 6 and 7) and Pest Management Decision Guides (PMDGs) (example of DBM; Figure 8). The *Plantwise* PMDGs are based on the Green and Yellow Lists of Plant Protection Measures based on the IOBC IP tool box that enables and supports the implementation of Integrated Production (IP) into practice (Source:https://www.iobc-wprs.org/ip_ipm/IOBC_IP_Tool_ Box.html#4). To further demonstrate the use of the Knowledge Bank, one

could also access some of the KB content. For example, search related to DBM has 43 records which include FS (indicate number of records) for farmers (12); PMDG (7); Technical FS (9); External FS (14) and video FS (1). The FSs are written in various languages (indicate number of records) which include English (27), Chinese (2), Spanish (2), Portuguese (1), Tamil (1) and Vietnamese (1). The KB also has news articles, pest alerts, images and global distribution map for DBM.

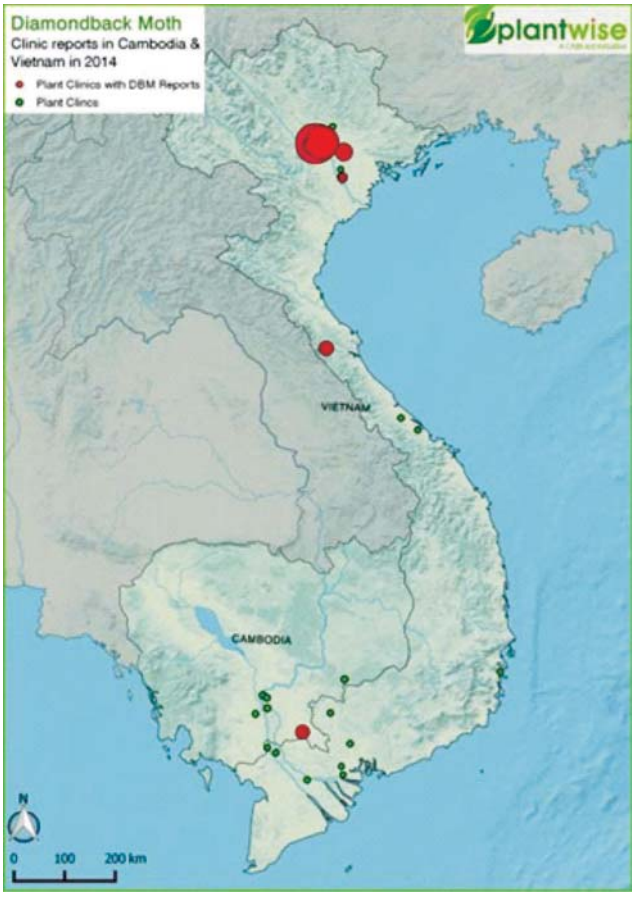


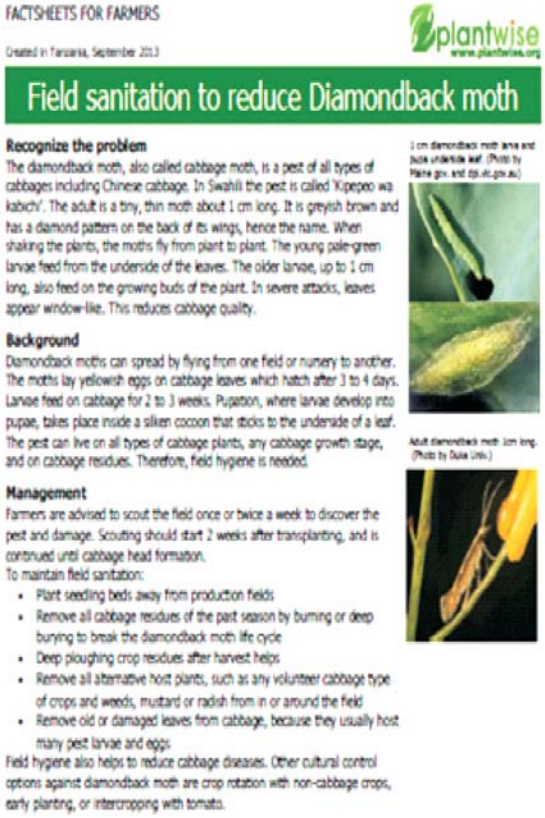
FIGURE 4

Map showing distribution of plant clinics showing diamondback moth reports in Cambodia and Vietnam

For identification of unknown pests or disease problems, the PW program also has a Directory of Diagnostic Services (DODS) both within and outside the country, to help the PDs for special or new problems. This is in addition to the referencing made using the KB as a source of diagnosis and identification. Where necessary, technical back-stopping visits (Figure 5) and plant health rallies are conducted to monitor and create awareness of key problems diagnosed in the PCs.

FIGURE 5

Cabbage with a club root problem (left) and (right) CABI experts helping with the diagnosis and explaining to a farmer on the management of the problem


FACTSHEETS FOR FARMERS

Created in Tanzania, September 2013

plantwise

Field sanitation to reduce Diamondback moth

Recognize the problem

The diamondback moth, also called cabbage moth, is a pest of all types of cabbages including Chinese cabbage. In Swahili the pest is called 'Kipepeo wa kabichi'. The adult is a tiny, thin moth about 1 cm long. It is greyish brown and has a diamond pattern on the back of its wings, hence the name. When shaking the plants, the moths fly from plant to plant. The young pale-green larvae feed from the underside of the leaves. The older larvae, up to 1 cm long, also feed on the growing buds of the plant. In severe attacks, leaves appear window-like. This reduces cabbage quality.

Background

Diamondback moths can spread by flying from one field or nursery to another. The moths lay yellowish eggs on cabbage leaves which hatch after 3 to 4 days. Larvae feed on cabbage for 2 to 3 weeks. Pupation, where larvae develop into pupae, takes place inside a silken cocoon that sticks to the underside of a leaf. The pest can live on all types of cabbage plants, any cabbage growth stage, and on cabbage residues. Therefore, field hygiene is needed.

Management

Farmers are advised to scout the field once or twice a week to discover the pest and damage. Scouting should start 2 weeks after transplanting, and is continued until cabbage head formation.

To maintain field sanitation:

- Plant seeding beds away from production fields
- Remove all cabbage residues of the past season by burning or deep burying to break the diamondback moth life cycle
- Deep ploughing crop residues after harvest helps
- Remove all alternative host plants, such as any volunteer cabbage type of crops and weeds, mustard or radish from in or around the field
- Remove old or damaged leaves from cabbage, because they usually host many pest larvae and eggs

Field hygiene also helps to reduce cabbage diseases. Other cultural control options against diamondback moth are crop rotation with non-cabbage crops, early planting, or intercropping with tomato.

FIGURE 6

An illustration of a factsheet for farmers on field sanitation for the diamondback moth

Discussion and Conclusion

Plantwise is a dynamic program that fits well within and strengthens the extension framework of various countries. As for vegetable brassicas, about 70% of which comes from Asia, the impact of pests and diseases on the livelihoods of the resource-striven Asian farmers cannot be overemphasized. Thus, a concerted focus on pests, such as the DBM, which is one of the world's significant agricultural pests costing farmers billions of dollars every year, the PW program could bring sustainable returns to investments based on its holistic approach. We already see some trends in certain aspects. For example, in PW countries, as result of the use of the PMDGs by PDs, we currently see evidence of a tactical shift of pest management recommendations to more 'greener' approaches (*e.g*., cultural and biological control measures) from the unilateral use of pesticides.

In terms of the over-riding policy implications, PW offers the message that systematic, cogent and responsible crop health advice and information is the key to sustainable agriculture and rural development. However, there are challenges that need to be addressed as PW evolves. Currently, many of the PW projects implemented in various countries are in the pilot phase, and as with any innovation, PW is still being refined and improved with the final objective of making the program a much broader holistic framework and an effective interface for total crop health i.e. *any crop, any problem*- beyond its current focus on pests and diseases. Danielsen *et al.* (2014) cautioned that for PCs, which are central to PW, to succeed, the fundamental issues of governance, resources and implementation structure need to be considered. They also underscored the importance of understanding not only the policy and institutional frameworks in which plant clinics operate, but also the effects of political imperatives and donors on policy implementation.


FACTSHEETS FOR FARMERS

plantwise

Black rot of Cabbage

Recognize the problem

Black rot of cabbage is a common disease affecting cabbage and related vegetables. It is caused by germs (Bacteria). Once your cabbages have the disease it is very difficult to control it. Plants can be affected at any time and all parts are affected.

Background

On older plants it begins with V-shaped light brown areas on the edges of the leaves. On severely infected plants a dark ring appears within the stem of the plant. On seedlings a yellowish spot/blotch appears on the leaf which causes it to drop off.

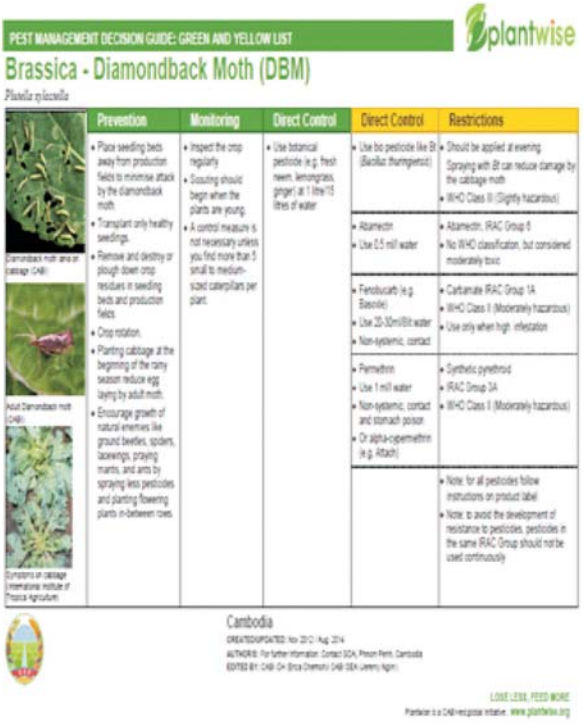
The bacteria live in the affected areas of the plant and like wet conditions. They enter plants through damaged areas, even tiny amounts of damage (such as from insects chewing or sand blown against the leaves) can let bacteria in. They can also live in the soil and seeds of the plant. The bacteria cannot move themselves. Insects, rain splash and weeding tools, can all move the bacteria to healthy plants which will then become infected.

Management

- Seeds should be disease free, disease tolerant varieties should be selected.
- Don't crop cabbages or plants of the same family when black rot has occurred.
- Don't grow cabbages on the same land year after year even if they are healthy.
- When symptoms are seen the infected plants should be carefully removed.
- Diseased plants contains large amounts of bacteria ready to infect the next crop of cabbages so try to reduce the amount of diseased material being returned to the soil.
- Mulching the cabbage crop will reduce rainwater splashes to the plants, thus reduce spread of the infection/disease.
- Avoiding weeding during damp conditions, accidental damage to the plants will allow the bacteria into the plants.
- Copper oxychloride will protect the plants from further infection but will not control disease within the infected plants.

Figure 7

An Illustration of A Factsheet For Farmers on Black Rot of Cabbage


PEST MANAGEMENT DECISION GUIDE: GREEN AND YELLOW LIST

plantwise

Brassica - Diamondback Moth (DBM)

Plutella xylostella

Prevention	Monitoring	Direct Control	Direct Control	Restrictions
• Place seedling beds away from production fields to minimise attack by the diamondback moth • Transplant only healthy seedlings. • Remove and destroy or plough down crop residues in seedling beds and production fields • Crop rotation. • Planting cabbage at the beginning of the rainy season reduce egg laying by adult moth. • Encourage growth of natural enemies like ground beetles, spiders, lacewings, praying mantis, and ants by spraying less pesticides and planting flowering plants in-between rows.	• Inspect the crop regularly • Dosuing should begin when the plants are young. • A control measure is not necessary unless you find more than 5 small to medium-sized caterpillars per plant.	• Use botanical pesticide (e.g. fresh neem, lemongrass, ginger) at 1 litre/15 litres of water	• Use bio pesticide like Bt (Bacillus thuringiensis)	• Should be applied at evening Spraying with Bt can reduce damage by the cabbage moth • WHO Class III (Slightly hazardous)
			• Abamectin • Use 0.5 ml/l water	• Abamectin, IRAC Group 6 • No WHO classification, but considered moderately toxic
			• Fenobucarb (e.g. Bassolie) • Use 20-30ml/8lt water • Non-systemic, contact	• Carbamate IRAC Group 1A • WHO Class 1 (Moderately hazardous) • Use only when high infestation
			• Permethrin • Use 1 ml/l water • Non-systemic, contact and stomach poison • Or alpha-cypermethrin (e.g. Attack)	• Synthetic pyrethroid • IRAC Group 3A • WHO Class 1 (Moderately hazardous)
				• Note: for all pesticides follow instructions on product label • Note: to avoid the development of resistance to pesticides, pesticides in the same IRAC Group should not be used continuously

Cambodia

LOSE LESS, FEED MORE

Figure 8

An illustration of a Pest Management Decision Guide (PMDG) for farmers e.g. the diamondback moth

Acknowledgement

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Naik Bhavan

An administrative fulcrum of University