Review of literature
2. REVIEW OF LITERATURE

Insect pests, diseases and weeds inflict enormous losses to the potential agricultural production. Anecdotal evidences also indicate a rise in the losses, despite increasing use of chemical pesticides. Simultaneously, there is a growing public concern about the potential adverse effects of chemical pesticides on the human health, environment and biodiversity. These negative externalities, though, cannot be eliminated altogether; their intensity can be minimized through the development, dissemination and promotion of alternative technologies such as biopesticides and bioagents as well as good agronomic practices rather than relying solely on chemical pesticides.

Over the next three decades, production of food grains in India has to increase by at least 2 million metric tons a year to meet the food requirement of the growing population (Paroda and Kumar, 2000). In the past, agricultural production increased through area expansion and increased use of high yielding seeds, chemical fertilizers, pesticides and irrigation water. Now, prospects of raising agricultural production through area expansion and application of existing technologies appear to be severely constrained. Land frontiers are closing down, and there is little, if any, scope to bring additional land under cultivation. Green revolution technologies have now been widely adopted, and the process of diminishing returns to additional input usage has set in.

Concurrently, agricultural production continues to be constrained by a number of biotic and abiotic factors (Fig.1). For instance, insect pests, diseases and weeds cause considerable damage to potential agricultural production. Evidences indicate that pests cause 25 percent loss in rice, 5-10 percent in wheat, 30 percent in pulses, 35 percent in oilseeds, 20 percent in sugarcane and 50 percent in cotton (Dhaliwal and Arora, 1996). The losses, though cannot be eliminated altogether, these can be reduced. Until recently, chemical pesticides were increasingly relied upon to set the production losses. Pesticide usage in India increased from a mere 15 g/ha of gross cropped in 1955-56 to 90 g/ha in 1965-66. Introduction of green revolution technologies in the mid - 1960s gave a fillip to pesticide use, and in 1975-76, it had increased to 266 g/ha, and reached a peak of 404 g/ha in 1990-91 (Birthal, 2003). Although, there is a paucity of reliable time-series data
on pest-induced yield losses, anecdotal evidences suggest an increase in losses (Pradhan 1983, Atwal 1986, Dhaliwal and Arora, 1996), despite an increase in the pesticide use. The paradox is explained in terms of the rising pest problem, technological failure of chemical pesticides and changes in production systems. However, pesticide use has started declining since 1990-91, reaching 265g/ha in 1998-99, without much affecting the agricultural productivity (Birmal, 2003).

2.1. Crop losses due to Pests

Insect pests, diseases and weeds are the major constraints limiting agricultural productivity growth. It is estimated that herbivorous insects eat about 26 percent of the potential food production. Emerging problems of insecticide resistance, secondary pest outbreak and resurgence further add to the cost of plant protection. Annual crop losses due to insect pests and diseases in India are estimated to be 18 percent of the agricultural output. Losses caused by specific pests may be higher. Helicoverpa spp. in cotton can cause losses up to 50 percent. According to Raheja and Tewari (1996), H. armigera (American bollworm) alone causes an annual loss of about Rs 1000 crores. The production losses have shown an increasing trend over the years. In 1983, the losses due to insect pests were estimated worth Rs. 6,000 crores (Krishnamurthy Rao and Murthy, 1983), which increased to Rs. 20,000 crores in 1993 (Jayaraj, 1993) and to 29,000 crores in 1996 (Dhaliwal and Arora, 1996). New pests have appeared due to the changes in the cropping patterns and the intensive agricultural practices.

![Figure 1. Abiotic and biotic factors causing crop losses.](image-url)
Crop losses may be attributed to abiotic and biotic factors, contributing to the reduction of crop performance and resulting in a lower actual yield than the site-specific attainable yield/production of crops (Figs 2 and 3). The attainable yield is defined as the site-specific technical maximum, depending on abiotic growth conditions, which in general is well below the yield potential, a rather theoretical yield level that cannot be realized under practical growth conditions.

(Source: Kodandaram et al., 2013)
2.2. Chemical-based Pest Management

Until the beginning of the 20th century, farmers relied exclusively on cultural practices such as crop rotation, healthy crop variety, manipulations in sowing dates, etc. to reduce the pest damage. Use of pesticides, although started in 1870s with the development of arsenic and copper-based insecticides, discovery of pesticidal properties of DDT during the World War II revolutionized pest control. DDT was effective against virtually all-insect species and was thought to be relatively harmless to the humans, animals, and plants. It was effective at low application rates and less expensive; hence the Indian industries too joined the race. Farmers were amazed by its effectiveness and started to use it increasingly particularly during the era of green revolution. As a consequence of rising demand, the pesticide industry rapidly expanded its research on synthetic insecticides as well as on other chemicals controlling the pests. The negative effects of chemical pesticides, however, started emerging soon after the introduction of DDT. Producers then turned to more toxic, organophosphates (OP) and pyrethroid insecticides, which resulted in the rapid development of resistant strains. Per capita usage of pesticide by country is depicted in Fig.4 and consumption of pesticide in India shown in Table.1

![Figure 4. Per capita usages of pesticide by country (Kg/Ka)](Source: Kodandaram et al., 2013)
Table 1. Consumption of Pesticides in India

<table>
<thead>
<tr>
<th>Year</th>
<th>Consumption (Tonnes Tech Grade)</th>
<th>Year</th>
<th>Consumption (Tonnes Tech Grade)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1965-66</td>
<td>14,630</td>
<td>1999-00</td>
<td>46,200</td>
</tr>
<tr>
<td>1975-76</td>
<td>45,613</td>
<td>2000-01</td>
<td>43,584</td>
</tr>
<tr>
<td>1985-86</td>
<td>61,881</td>
<td>2001-02</td>
<td>47,020</td>
</tr>
<tr>
<td>1990-91</td>
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<td>72,133</td>
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<td>70,794</td>
<td>2004-05</td>
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<td>61,260</td>
<td>2007-08</td>
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<td>41,824</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2010-11</td>
<td>55,542</td>
</tr>
</tbody>
</table>

(Source: Anonymous, 2002 and 2012)

Figure 5. Percentage share of different class of pesticides in India

(Source: Kodandaram et al., 2013)
Indian ‘Green Revolution’, one of the greatest success stories in the world, with dramatic impact on the food security, was based on principles of intensive farming. However, the intensive agriculture led to newer problems such as the excessive and untimely use of irrigation water, erosion of genetic resources caused by the substitution of the rich diversity of the traditional crop varieties with a few high yielding varieties, and inappropriate use of critical inputs such as chemical fertilizers and pesticides (Paroda, 1999). Thus, with the intensification of agriculture and consequent increase in genetic uniformity of crops, the incidence of insect-pests, diseases, nematodes and weeds has also increased. The pests that hitherto were of novelty have become the key pests affecting a number of crops. The consumption of pesticides in India is depicted in Fig.5 and 6.

![Figure 6. Consumption of pesticides (%) in different crops](image)

(Source: Kodandaram et al., 2013)

In the context of crop protection, sustainability refers to the substitution of chemicals and capital with farm grown biological inputs and knowledge, aimed at a reduction in the cost of production without taking down the yields (Swaminathan, 1995). Sustainability rests on the current agricultural achievements, adopting a sophisticated approach that can sustain high yields and farm profits without degrading the resources. Sustainable agriculture is a reality based on the human goals and on the understanding of the long-term impact of human activities on the environment and on other species. This
philosophy combines the application of prior experience and the latest scientific advancements to create integrated, resource-conserving, equitable farming systems. The systems approach minimizes environmental degradation, sustains agricultural productivity, promotes economic viability in both the short and long run, and maintains quality of life (Charles and Youngberg, 1990).

2.3. The study of insect biology

The order Coleoptera of class Insecta consists of beetles and is the largest order of insects. Beetles can vary in length from less than a millimetre to up to 75 mm, with the stored product beetles being usually less than 1 centimetre in length. Beetles are also characterized by the hardened covering over their abdomen which is actually a pair of wings. Beetles have 2 pair of wings. The front pair is hardened and leathery or thickened. These wings, which meet in a straight line down the middle of the back, are called elytra. The hind wings underneath the elytra are membranous, and are used for flying. When the beetles are not flying, the hind wings are folded under the elytra. Beetles have chewing mouthparts with well-developed mandibles. The weevil’s (which are also beetles) mouthparts are actually elongated and drawn out into a snout. Also, beetles develop by complete metamorphosis. That is, they have 4 developmental stages: egg, larvae, pupae and adult (Fig. 7)

![Figure 7. Life cycle of weevils (Coleoptera: Curculionidae),](source:www.everwoodfarm.com)
Identification of pests is an important aspect of integrated pest management and in order to identify them some knowledge of pest biology is necessary.

The main objectives of insect biology study were as follows,

- To able to describe the order of insects and their ecological importance.
- To describe the evolution of insects and their geological history.
- To explain insect morphological and physiological adaptations including: the exoskeleton, wings, compound eye, appendages, trachea, fat body, haemolymph, circulation, excretion and reproduction.
- To know the general external and internal anatomy of insects.
- To illustrate the basic principles of insect pest management, the major insect pests and the insect vectors of disease.
- To depict insect reproduction and life cycles.

Attaining the objectives as outlined above will facilitate new and refined methods of pest and crop management for improved crop productivity and quality.

The family Curculionidae appears to be the largest family of organisms and more than 47930 species in 4300 genera are currently recognized (Kuschel, 1995). The vast majority of weevil species are strictly phytophagous as adults and larvae although a few are saprophytic. Even those taxa that are hypogean in habits appear to feed primarily on roots (Osella, 1979). They are recognized by their distinctive long snout and geniculate antennae with small clubs. The genus *Myllocerus* are most important. As of date the following *Myllocerus* have been reported in South India *Myllocerus undecimpustulatus, M. discolor, M. sabulosus* (Butani, 1979). Among these *M. undecimpustulatus* is the most dominant in South India. *M. subsfasciatus* is a major problem on brinjal or eggplant. The *Myllocerus* spp are the “Important pest species of weevils” (Hill, 1987). The species in the genus *Myllocerus* spp are polyphagus (Hill, 1987). The adult weevils of *Myllocerus* spp feed on leaves, nibbling the leaves from the margins and eating away small patches of leaf lamina (Butani, 1979).
Marshall (1916) recorded it from the Punjab, Hafizabad, Lyallpur, Chiniot, Allahabad, Bengal, Madras and Bangalore. Several workers have reported it from Lahore, Delhi, (Fletcher, 1917). It has also been reported occurring in Amritsar, Dehradun, Baktiarpur, Manjre, Surat, Coimbatore (Misra, 1920). Siddapaji (1976) reported that the typical insect was noticed to be one of the widely distributed weevils in hilly, coastal and northern plains of Karnataka on a variety of crops and the three species namely, \textit{M. undecimpustulatus var. maculosus}, \textit{M. undecimpustulatus var. pistor} and \textit{M. undecimpustulatus var. marmoratus}, \textit{M. undecimpustulatus var. marmoratus} is most widely distributed, while other species were confined to specific areas of Karnataka as seasonal pests of crops.

Marshall (1916) reports the \textit{M. subfasciatus} species as occurring in Madras: Animal Hills, Ouchterlony Valley, Droog, Nadaumutam, Nilgiri, Hulikal, Utacamund, Coonoor, Mahe, Malabar, Pondichery; Bombay: North Canera, and Belgaum; Central Provenience: Chikalda, Berars. Senior-White (1920) records it from Ceylon, Burma, Saidapet, and Virajpet (South Coorg). Osman and Puttaradriah (1955) report it from Karnataka: Sakaleshpur; Subramaniam (1958) from Coimbatore, Singh (1960) from Dehra-Dun. The same species has been noted on brinjal, potato, apple and a variety of plant in the plains. Fletcher, 1914, 1917b; (Ayyar, 1920) on \textit{Acacia decurrens} (Rau, 1936) Senior-White (1920) observed the species damaging potato leaves, flower and buds of brinjal plants. Fletcher (1920) reports the species as causing appreciable damage. Ayyar (1922) stated that the species is found occasionally and is of minor importance. Katyanum (1967) listed it as one of the economical important weevils in India. Fletcher (1920) reported that the larvae abounded among the roots of wild and cultivated species of grasses in soils having a tolerance limit of moisture over a long period. Subramaniam (1958) studies life history of the species on brinjal.

An exotic weevil \textit{M. undecimpustulatus undatus} Marshall was first found in south Florida in 1995. The adults have a broad host range that includes foliage of fruit trees, ornamentals and vegetables, but little is known about their basic biology, including larval host plants. Adult females and the grubs do not select all hosts equally. Instead, preferences in both host finding and host acceptance behaviour are often shown towards
particular host species. Host preferences show a strong heritable component and are thought to represent the suitability of hosts for grubs’ survival (Singer 1983, Courtney et al., 1989, Singer et al., 1989, Thompson 1998). Suitability can depend upon a number of factors such as nutritional quality, host plant defense chemicals, prevalence of natural enemies or microenvironment (Thompson and Pellmyr 1999). Such preferences are not always fixed. Courtney and Kibota (1990) use existing theories of learning behavior to show how insect learning and the environment may interact to change an insect’s host selection behavior to favour one species. In their review they explain how the selection of a particular host species by an insect is probabilistic. When the abundance of one host plant is high, this will favour the probability that the insect lands on that plant.

Marshall (1916) reported *M. dentifer* species from Ceylon: Galle, Colombo; Madras, Madura, Vishakhapatnam, Sholapur, Malabar, Bengal, Orissa, Nagpur. Ayyar (1922) stated that this species is fairly well distributed throughout South India. The weevil was found feeding on paddy and groundnuts (Marshall, 1916). Fletcher (1917b) he stated that the species was probably quite a minor pest, often found intermixed with other species of *Myllocerus* on rice, but was not a serious pest. Ayyar (1940) recorded that other millets occasionally suffered from the attack of this species. Ayyar (1922) considered this as an economically important species in south India. He stated that the species occurred on rice and on groundnuts without apparently causing much injury even when found in large numbers.

Lefroy (1906) noticed that damage due to adults of the species and variety for the first time on cotton. He also studied the phenomenon of the adult “shaming death”. Stebbing quoted by Marshall (1916) recorded it on *Dalbergia sissoo*, strawberry, Lucerne, cotton, sunflower, sugarcane, pomegranate, *Zizyphus jujube* and mango. Bhasin et al., (1956) noted this species on *Casuarinas equisetifolia*, *Dalbergia paniculata* and fruit trees. Nangapal (1948) stated that the species has a special liking for *Cajanus indicus* and cotton. Subramaniam (1965) observed it on *Moringa oleifera*. Samuel (1969) and Rajagopal (1974) observed it on ragi and maize respectively. They stated that the pest occurs throughout the year on the above crops in varying intensities.
Lefroy (1906) stated no case is yet on record of this insect being sufficiently abundant to cause harm. Though the species was reported as the commonest of the leaf eating weevils at PUSA, it was considered as regular minor pest (Lefroy, 1906; Fletcher, 1917). Fletcher (1917) reported the larvae feeding on roots of various crops. Trehan (1929) reported that the larvae caused more damage to the cotton roots than the adults to foliage however the combined damage is far more serious. Sohi (1964) stated that the species is polyphagous and widely distributed in India and was first recorded at Kanpur in 1895.

Trehan (1929) studied the life history of the species. He indicated that the larvae and pupae completed their development in 16-35 days and one week respectively and the species completed five generation in a year. The first brood of adults as a rule appeared towards the end of April and fed on rain fed crop. The maximum infestation occurred in second or third week of June. The insect passed the winter in the grub stage in soil. Hibernating larvae were observed in soil during November-March and emergence of adults at the end of April. The adults had not been noticed to hibernate, but died about the middle of January as no adults could be discovered after the middle of January (Trehan, 1929).

No pesticides are listed for use for the control of *M. undecimpustulatus*. Severe infestations on ornamental plants may be controlled with insecticides containing carbaryl (Sevin), acephate (Orthene, formerly Isotox), or pyrethroid insecticides labeled for use on leaf-feeding insects. The *M. undecimpustulatus* is a weak flyer. When disturbed the weevil will move away, but when aggressively harassed it will flee by flying a short distance or drop to the ground and hide in grass, leaf litter or mulch under the plant it is infesting or exhibits feign death (http://www.floridagardener.com), but to this point no relief has been noted in the control of this beetle.

Apparently several insecticides are effective against adult weevils in the Genus *Myllocerus* (Budhraja et al., 1984; Singh et al., 1991; Sinha & Marwaha, 1995). However, the chemical treatment may be of little or no economic value because of the expense and the limited period of efficacy. The larvae will probably be protected while
feeding under the ground. However, methyl iodide is effective as a fumigant against insects (Waggoner et al., 2000) and soil fumigation by methyl iodide appears to be as effective as soil fumigation by methyl bromide (Eaves et al., 2000). Several insecticides are effective against larvae in the soil (Hill, 1987). Mahadeva Swamy et al., (2013) reported that toxicity of Bt protein was determined against *M. undecimpustulatus* Marshall (Coleoptera: Curculionidae) LC$_{50}$ 152 ng cm$^{-2}$. Cultural practices may be of value. Atwal (1976) reports that frequent hoeing and "interculture" disturb and kill the grubs of the cotton grey weevil, *M. undecimpustulatus* var. *maculosus*. Atwal (1976) also reports that the cotton grey weevil has a marked preference for *Cajanus cajan*, which can be sown as a trap crop.

The website of the CABI (CAB International) notes that *Steinina lunata* is a pathogen of *M. undecimpustulatus*. *Dinocampus mylloceri* Wilkinson (Braconidae) is a parasite of the adult weevils of *M. undecimpustulatus* (Pruthi & Batra, 1960).

Leather et al. (1998) studied the biology and ecology of the large pine weevil, *Hyllobius abietis* (Coleoptera: Curculionidae). The biology and pest status of *Hyllobius abietis* Linnaeus in Europe are critically reviewed. New data are presented and the relationships between the weevil and its host plants considered. In Europe, *H. abietis* is the major pest of establishment forestry causing millions of ECUs of damage annually and perpetuating the addition of insecticide residues to sensitive habitats. Predator and parasitoid complexes in Britain and Europe are compared and contrasted. The lack of knowledge of the processes involved in adult dispersal and longevity are highlighted as major areas of concern. The biology and behaviour of the adult and larval stages are reviewed and new data presented. The feeding preferences of the adult weevils are considered and the possibility of using deterrents as a pest management strategy discussed. The development of risk assessment and forecasting tools aimed at more effective deployment of pest-management options are discussed. Risk criteria have their origins in important ecological relationships which require new understanding, but the prospects for determining high-risk forest sites are promising. The options for biological control are evaluated, in particular the use of mycopesticides and increased larval predation. It is concluded that much more research into the biology and ecology of *H.*
Abietis is required before a successful Integrated Pest Management (IPM) programme can be initiated.

Reichert et al. (2010) reported the biology and host preferences of Cryptorrhynchus melastomae (Coleoptera: Curculionidae), a possible biocontrol agent for the weevil Miconia calvescens (Melastomataceae) in Hawaii. The introduced plant Miconia calvescens (Melastomataceae) poses a grave threat to Hawaii’s native ecosystems and biodiversity. One potential candidate for classical biological control is C. melastomae (Coleoptera: Curculionidae: Cryptorrhynchinae), a stem-boring weevil from Central and South America. This weevil feeds on M. calvescens in its native Costa Rica and has been successfully reared under greenhouse conditions. Comparison of its environmental conditions in Costa Rica with those in the Miconia infested areas of Hawaii indicates the latter is a suitable habitat for C. melastomae. C. melastomae has one or two generations per year. Adults feed on new stems, petioles, leaf buds, veins, and lamina, whereas larvae mine the stem until pupation. Adults appear to prefer saplings for oviposition and feeding. Under greenhouse conditions both adults and larvae can seriously damage and kill small M. calvescens. Preliminary host testing indicates that C. melastomae may be family specific on Melastomataceae. However, because Hawaii lacks native melastomes and has many other serious melastome weeds, a family specific insect may be suitable as a biocontrol agent in this case.

Furniss and Kegley (2008) studied the biology of Dendroctonus murrayanae Hopkins (Coleoptera: Curculionidae: Scolytinae) in lodgepole pine, Pinus contorta Douglas, in Idaho and Montana. The beetle was not a primary agent of tree mortality. Susceptible host trees were physically damaged, had thin foliage, or were otherwise predisposed to infestation. Beetles attacked individual trees, not in groups, near ground level and at low density. Life stages and their behavior are described. Egg galleries were constructed upward and usually had short spurs. Mating occurred in the egg gallery. Eggs were laid in an elongated group, not in niches, in a shallow excavation along only one side of the egg gallery. Larvae aggregated in a communal chamber, feeding side by side, but separated before pupation. D. murrayanae has four instars. One annual generation is indicated, overwintering as larvae. D. murrayanae occurred in some trees with Pseudips
(Ips) mexicanus (Hopkins), Ips pini (Say), and Hylurgops porosus (LeConte) but seldom with the mountain pine beetle, Dendroctonus ponderosae Hopkins. No natural enemy or commensal insect was observed in brood chambers.

Ekehukwu and Eluwa, (2008) reported the biological studies of Gasteroclisus rhomboidalis (Coleoptera: Curculionidae) on Amaranthus sp. In West Africa, the foliage of both wild and cultivated varieties of Amaranthus sp are used widely as vegetable and as fodder for cattle. This widely cultivated vegetable is severely attacked by the snouted beetle, Gastroelisus rhomboidalis. A comprehensive study on some aspects of the biology of G. rhomboidalis has been carried out. The full life cycle of the beetle (from copulation and oviposition through the various immature stages to the emergence of adult beetles) was investigated. It took on the average 40 days for the adult beetle to emerge from the day of oviposition. Studies on the feeding habit of the beetles revealed that the beetles fed heavily on the leaves and can inflict an enormous destruction on the host plant over a short period of time. Generally the studies have been able to confirm that the larva and adult beetles are the most potent destroyers of the host plant. While the adult beetle fed heavily on the leaves and tender plant stem, the larva when hatched in the plant stem, destroyed the host plant (particularly during the tender ages of between 2 - 3 weeks) with its boring activities causing the plant to wilt and die.

2.4. Molecular Systematics and Phylogenetics

"The species phylogeny is more like a statistical distribution, being composed of various trees (the gene trees), each of which may indicate different relationships."  
- Wayne Maddison, 1995

Molecular systematics is the use of molecular genetics to study the evolution of relationships among individuals and species. The goal of systematic studies is to provide insight into the history of groups of organisms and the evolutionary processes that create diversity among species. Insect molecular systematics has undergone remarkable growth in recent years. Advances in methods of data generation and analysis have led to the accumulation of large amounts of DNA sequence data from most major insect groups.
2.4.1. The rise of DNA barcoding

In 2003, Paul Hebert et al., from the University of Guelph, Ontario, Canada, published a paper in the Proceedings of the Royal Society stating that the mitochondrial gene COI could serve as a genetic barcode for all animal life (Hebert et al., 2003a). This was not the first time that DNA barcoding had been proposed as a concept. Using short DNA sequences to discriminate amongst microbial species was proposed as early as 1982 (Nannen 1982) and had subsequently been tested on a variety of taxa from nematodes to elephants and even that most famous of extinct species, the dodo (Eggert et al., 2002; Floyd et al., 2002; Shapiro et al., 2002).

Hebert et al., (2003a, b) went further than previous studies, however, proposing a single gene barcode locus as a silver bullet to identify species across the whole animal kingdom. More than that, the DNA barcoding system promised a better taxonomic resolution than that which could be achieved through morphological studies and a partial solution to the decline in traditional taxonomic knowledge. Thus, DNA barcoding was proposed as a tool not only to identify species but also to define species boundaries and aid in species delimitation (Hebert et al., 2003a). This promise to revolutionize taxonomy and species discovery received a mixed reception (Blaxter 2003; Janzen 2004; Prendini 2005). Nevertheless, today DNA barcoding is a global enterprise (Box 1), attracting large amounts of funding and operating three international websites encompassing a raft of partner organizations from around the world. The implementation of the idea has seen a rapid rise in that time (Fig. 8), and publications on the topic have been numerous with 411 papers published that mention DNA barcoding in their titles between 2003 and 2010 (Box 2). In its role as a species identifier, DNA barcoding has been used to tackle a wide variety of problems, from resolving adult and larval stages within species (Gossner & Hausmann 2009) to policing fish for sale in supermarkets (Rasmussen et al., 2009).

The practicalities of a universal barcode for all life have proved problematic, however, (Vences et al., 2005; Rubinoff et al., 2006; Eberhardt 2010) and the distance based methodologies employed in many barcoding studies have been repeatedly criticized (DeSalle et al., 2005; Kelly et al., 2007). It also remains unclear whether the
usefulness of DNA barcoding is restricted to species identification or if it is a general tool that can be used for species discovery and delimitation (Rubinoff, 2006a). More pertinently, although the concept and application of DNA barcoding are simple, there is, as yet, no solid system in place to deal effectively with the enormous volumes of data this field is generating in its attempts to create a genetic reference library (Lucking et al., 2008; Packer et al., 2009).

The main DNA barcoding bodies and resources

Consortium for the Barcode of Life (CBOL)

http://wwwbarcodeoflife.org

Established in 2004, CBOL promotes DNA barcoding through over 200 Member Organizations from 50 countries. Operates out of the Smithsonian Institution's National Museum of Natural History in Washington and is chaired by Dr. Scott E. Miller of the SI.

International Barcode of Life (iBOL)

http://www.ibol.org

Launched in October 2010, iBOL represents a not-for-profit effort to involve both developing and developed countries in the global barcoding effort, establishing commitments and working groups in 25 countries. The Biodiversity Institute of Ontario is the project's scientific hub and its director, Dr Paul Hebert, is also the scientific director of iBOL.

The Barcode of Life Data systems (BOLD)

http://www.boldsystems.org

The Barcode of Life Data system is an online workbench for DNA barcoders. Combines a barcode repository, analytical tools, interface for submission of sequences to GenBank, a species identification tool and connectivity for external web developers and bioinformaticians. Established in 2005 by the Biodiversity Institute of Ontario.
The advent of high-throughput next generation sequencing (NGS) technology is already ushering in a new era for molecular ecology (Ellegren et al., 2008) by making rapid access to fully sequenced genomes a distinct possibility. With such technology becoming increasingly accessible (Kircher et al., 2010), this availability of data from the entire genome has obvious implications for surrogate measures such as DNA barcoding and it will be important for its proponents to find a way to evolve their methods to encompass these new developments.

A simple literature search was conducted on Web of Science for all articles containing the phrase ‘DNA barcod*’ in the title of the paper. The asterisk was used to enable the return of results containing the words barcode, barcodes, barcoder, barcoders and barcoding. As no papers prior to 2003 featured the phrase ‘DNA barcod*’ in the title, the literature search was solely focussed on the period between 2003 and November 2010.

For articles in journals that were not open access or for which Manchester Metropolitan University did not hold a subscription, an email was sent to the correspondence author to request a copy. Where no response was received, any relevant information that could be extracted from the abstract was included in the analysis and the paper excluded from all analyses for which no information was available.

The initial search produced 525 hits that, after de-duplication and removal of meeting abstracts etc., were reduced to 411 published articles. Of these 411:1 321 focussed on a particular or several taxa rather than being more general reviews of the topic ‘DNA barcod*’. 2 296 were reports of the practical application of one or more DNA barcodes. 3 222 involved analysis of genetic barcode sequences (rather than simply a recording of the barcode).

As different numbers of papers were involved in different analyses for the review, each figure is labelled with the sample size for that statistic. It is appreciated that many papers on the topic of DNA barcoding do not feature the specific phrase ‘DNA barcod*’ in their title. A Web of Science search of papers containing ‘DNA barcod*’ in the topic
field returned 1192 results dating back to 1993. However, this wider search included a high number of irrelevant papers and the intention of this review is to provide a snapshot of the DNA barcoding landscape post Hebert et al. (2003a). It is felt that the most efficient way to proceed would be to limit the data search to those with ‘DNA barcode*’ in the title, but take care to refer to relevant papers that fell outside of this search within the body of the review. The major results of the literature review are summarized graphically below.

Figure 8. Number of papers published with ‘DNA barcode*’ in the title (N = 411).

2.4.2. Molecular Systematic Methods

2.4.2.1. Sampling

One of the key elements in designing a molecular systematic study is selecting ingroup and outgroup taxa that maximize the investigator’s chances of accurately answering the question being posed. While this issue has proven rather contentious, most studies agree that sampling can significantly affect phylogenetic inference. The primary point of contention has been whether better phylogenetic accuracy is attained by densely sampling the taxon of interest despite the fact that some of the sampled taxa may be extraneous to the question at hand, or whether more scant sampling may provide better estimates. The underlying agreement between these opinions is that it is important to sample the diversity of the group of interest evenly. It is well known that very short internal branches are difficult to reconstruct, and that long terminal branches tend to
attract each other, at least under the parsimony criterion. Therefore, when possible these long branches should be broken up by the addition of taxa or eliminated when not directly relevant to the study. Dense taxon sampling is likely to accomplish the first of these solutions and simulation studies have supported this as the most generally reliable strategy. Graybeal's et al., 1998 study further showed that addition of taxa to a problem may improve phylogenetic accuracy faster than addition of characters (i.e. more sequence). Beyond topological accuracy, taxon sampling is likely to affect the probability of correctly identifying the root of a tree (Sifnon et al., 1996) and the estimation of rates of evolution on the tree (Robinson et al., 1998). Thus, the optimal sampling strategy is inseparable from the goal of the study.

Concerns regarding branch lengths are especially relevant to insect molecular systematics, because major lineages of insects inherently have long branches. Particularly in higher-level studies, i.e. among tribes, families, and orders of insects, thorough taxonomic sampling will be of the utmost importance. Including single examples of highly divergent taxa in such studies is as likely as not to cause problems with phylogeny estimation. This point also serves to amplify our theme: ultimately coordinated efforts across many lower-level studies are more likely to solve higher level problems in insect systematics than sparse sampling across a diverse array of taxa.

2.4.2.2. Markers

The modern systematist has a wide variety of techniques at disposal. While DNA sequencing has justifiably become the method of choice for most molecular systematic studies, it should not be forgotten that many alternatives may be more appropriate or more practical for certain applications. The central guiding principle should be the question at hand. The importance of clearly outlining the objectives of a study is paramount. Sequencing is generally the most appropriate for studies at interspecific levels and higher (Avise 1994, Hillis et al., 1996). However, questions of intraspecific population structure, species limits, and species diagnosis often can be effectively addressed using time-honored techniques like allozyme electrophoresis, restriction fragment length polymorphisms (RFLP), or any of a variety of newer DNA-based
techniques such as single-stranded conformational polymorphisms (SSCP), amplified fragment length polymorphism (AFLP), or random amplified polymorphic DNA (RAPD). The best strategy for certain problems will often use one or more of these in combination with sequencing. Excellent discussions of the problem of choosing markers for a study can be found in Hillis et al., (1996). To their recommendations we would mainly add that consideration be given to maximizing compatibility with previous work. Choosing similar loci, primers, and analytical methods will ensure that a study adds to a body of accumulating knowledge, thus accomplishing more than merely solving the problem at hand. While in some cases it may seem that the objectives of the study are at odds with this goal—that popular markers may not be completely appropriate to the question—some simulation studies have shown that individual markers may be informative across broad ranges of divergence (Purvis et al., 1997). Furthermore, the more knowledge we have of particular loci, the more likely we are to be able to develop weighting schemes or models that make optimal use of observed variation.

2.4.2.3. DNA Sequences

It is obvious that DNA sequencing has become the dominant technique for generating comparative molecular data. The many desirable properties of sequence data have been reviewed at length elsewhere (Avise 1994, Hillis et al., 1996). We reiterate two of these properties here: (a) the inherent comparability of sequence data across studies—the “connectivity” of Avise (1994) and (b) a unique insight into the evolutionary processes driving the diversification of DNA itself. Automated sequencing facilities are accessible to most biologists within a reasonable budget, and appropriate markers have been developed for most evolutionary applications.

2.4.2.3.1. mtDNA

Mitochondrial DNA is by far the most widely used of DNA regions for insects as well as for animals in general (Hill et al., 1996). For detailed discussions of the general properties of mtDNA, see the reviews of Avise et al., (1994 & 1987), Harrison (1989) and Simon et al., (1994). The popularity of mtDNA markers derives in large part from its relative ease of isolation and amplification, even from marginally preserved specimens. It
is also amenable to straightforward analyses, individuals typically being homoplasmic and part of a strictly dichotomous lineage of haplotypes. Heteroplasmy has been reported only infrequently (Walton et al., 1997). One of the main developments in recent work on mtDNA is the documentation of paralogous nuclear copies of mitochondrial genes, or pseudogenes (Sunnucks et al., 1996, Zhang et al., 1996). These pseudogenes may seriously confound phylogenetic analyses. Given our near ignorance regarding their frequency and distribution in insects, Zhang & Hewitt's precautions (Zhang et al., 1996) should be kept in mind whenever one is working with mtDNA.

A synthesis of the information content of various mitochondrial loci is difficult for the reasons presented in the preceding section. Very few studies have explicitly compared the resolving power of different mitochondrial loci over a particular set of taxa. In those that have, topological incongruence is common (Barrio et al., 1994, Caterino et al., 1999, Vogler et al., 1997), sometimes significantly so (Baker et al., 1997). However, no clear patterns among loci have become apparent. What is clear is the degree to which variation depends on structural constraints in both protein coding (Caterino et al., 1999, Lunt et al., 1996, Simon et al., 1994) and rRNA genes (Flook et al., 1998). Thus, individual genes do not exhibit an overall rate of evolution, but comprise both conserved and variable regions. Variation in the mitochondrial control region (4AT rich region) has been reviewed (Caccone et al., 1996, Taylor et al., 1993, Zhang et al., 1997 and 1995) and has been found to be problematic for analyses at any level. Additional studies of variation patterns within other mitochondrial genes would be very useful.

2.4.2.3.2. Nuclear DNA

Due to the recognition that mitochondrial gene trees may represent only a partial and potentially biased view of organism phylogeny, numerous nuclear genes have been developed for arthropod phylogenetics (Brower et al., 1994 and 1998; Friedlander et al., 1992 and 1994; Graybeal et al., 1994; McHugh et al., 1998). Nuclear ribosomal RNA genes have been especially widely used owing to their abundance within a genome and concomitant ease of amplification and sequencing. The nuclear ribosomal repeat (an array containing the 18S, 5.8S, and 28S genes, separated by transcribed and no transcribed spacers) are mosaics of highly conserved and variable regions (Hillis et al.,
1991). While the conserved regions have been informative for resolving relationships at higher taxonomic levels, alignment of the variable regions is generally problematic (McHugh et al., 1998; Waegle et al., 1995; Winnepenninckx et al., 1996). With sufficient time, concerted evolution effectively homologizes the many copies of nuclear rDNA within a genome (Hillis et al., 1991) but some genomes have been shown to retain a considerable diversity of paralogous sequences (Buckler et al., 1997; Telford et al., 1997). Nonetheless, rDNA sequences have proven highly informative for phylogenetics. A compilation of sequences and secondary structures has recently been posted to the World Wide Web (http://rrna.uia.ac.be/ssu/; De Rijk et al., 1999, Van de Peer et al., 1999).

Nuclear protein-coding genes exhibiting a wide range of evolutionary rates have recently been assessed for phylogenetic utility. These loci show great promise for resolving relationships at levels too conserved to be easily examined with mitochondrial proteins. The inherent rate variation among codon positions in protein coding genes suggests that many of these loci may be effective for more recent divergences as well (Brower et al., 1998; Cho et al., 1995; Mitchell et al., 1997; Reed et al., 1999). Furthermore, the relatively unbiased nucleotide composition of most nuclear loci compared to mtDNA should lead to a higher saturation threshold. However, working with nuclear genes poses potential difficulties with heterozygosity, and low copy number may hinder amplification. In addition, many nuclear genes may contain large introns that necessitate reverse transcriptase PCR (RT-PCR; e.g. Fang et al., 1997), and many purportedly single-copy loci may be represented by one or more paralogues in other groups (for examples in EF-1α see Fritz et al., 1994; Walldorf et al., 1985).

2.4.2.3.3. Noncoding Markers

Seeking variation, which may often be elusive, systematists and population biologists are delving increasingly into the vast noncoding portions of the genome. Among the significant insights gained in this effort has been the extent to which noncoding regions comprise repetitive, or satellite, DNA: as much as 45% of the entire genome in some cases (Pons et al., 1997). Because much of this satellite DNA is
localized in the centromeric regions of chromosomes, and may exhibit highly conserved higher order structure, it is becoming apparent that satellite DNA plays an important role in chromosome structure and function (Plohl et al., 1998, Ugarkovic et al., 1996). Interestingly, the quantity of minisatellite DNA in the genome appears to vary with reproductive strategy. For example, parthenogenic *Bacillus* walking sticks harbor only 10% to 30% of the total satellite DNA of close bisexual relatives (Mantovani et al., 1997).

Microsatellites, named for the very short repeat units of which they are composed (dinucleotide repeat units being the most common), have come into widespread use for genetic analysis at or below the species level (Queller et al., 1993). Their generally high mutation rate ensures high levels of polymorphism (Choudhary et al., 1993; but see Schlo"tterer et al., 1998) and they have therefore been particularly useful for examining relationships among individuals and breeding groups within insect populations. Development of microsatellite loci for a particular organism is rather labor intensive, but there is some indication that loci may be more broadly applicable than originally thought (Ezenwa et al., 1998). In addition, methods have been developed recently to screen for microsatellite loci without creating a complete genomic library (Ender et al., 1996). Nonetheless, it appears that microsatellite loci may be substantially more difficult to develop in some insects, such as butterflies (Meglécz et al., 1998), than in others. Particularly exciting applications of microsatellites have focused on testing hypotheses of kin selection and behaviour as they covary with individual relatedness in social insects (Evans 1996, Oldroyd et al., 1997). A surprising result of these studies is that our ability to discern relationships in many cases exceeds that of the insects themselves and, therefore, behaviours predicted by kin selection are not realized (Are'valo et al., 1998, Solis et al., 1998). Microsatellites have also provided fascinating insights into sperm competition and paternity (Cooper et al., 1996), the origins of sociality (Chapman TW et al., 1996), and population structure (Lanzaro et al., 1998). A recent study of the four species of the *Drosophila melanogaster* species complex (a phylogenetic enigma on par with the gorilla-chimpanzee-human trichotomy) indicates excellent promise for the resolution of interspecific relationships using multiple microsatellites (Harr et al., 1998).
Increasingly, introns within single-copy nuclear protein-coding loci are being applied to systematic studies. Though these noncoding regions are generally perceived to be highly variable relative to the coding portions of their host genes (Palumbi 1996), this is, in fact, a controversial point. Moriyama and Powell 1996 found lower average polymorphism in noncoding introns than among silent sites in adjacent coding regions (across 24 loci). However, Villablanca et al., 1998 have demonstrated significantly higher levels of variation in introns than in either isozymes or mitochondrial DNA for invasive Mediterranean fruit fly (Ceratitis capitata) populations. The disagreement may simply be a result of the more variable introns having been developed and used by systematists. It should, nonetheless, be emphasized that higher variability may not be a general property of introns. Palumbi (Palumbi 1996) offers a selection of potentially useful intron loci and primers. In addition to Villablanca et al., 1998 study of the population structure of a recently invading species, introns have been successfully applied to problems of native population structure in yucca moths (Leebens-Mack et al., 1998) and interspecific phylogeny in Spodoptera (Noctuidae; 1).

2.5. Gene Arrangements

As large amounts of DNA data have accumulated it has become apparent that the organization of the genome itself may contain substantial phylogenetic information. The value of mitochondrial gene rearrangements in arthropods was first pointed out by Boore et al., (1995) in a study that appeared to support monophyly of the chelicerates, crustaceans, hexapods, and myriapods to the exclusion of the Onychophora. Such rearrangements are likely quite rare and thus would seem to be excellent potential characters, though some parallelisms have been shown (e.g. Dowton et al., 1999; Mindell et al., 1998).

2.6. Analytical Issues

2.6.1. Phylogenetic Methods

Methods for reconstructing phylogenies have been reviewed at length, most cogently by Swoford et al., (1996). A few advances that are especially pertinent to insect phylogenetics, however, are worth highlighting. Sequence alignment constitutes the
primary assessment of homology among sequence data sets and has accordingly figured prominently in discussions of phylogenetic methodology. In most studies of protein coding genes, alignment proves straightforward as long as amino acid sequence is conserved (though introns pose potential problems). However, the variable regions of ribosomal genes may be substantially more difficult. Methods developed to deal with this problem include incorporating information on phylogeny (Mindell 1991; Wheeler et al., 1994) and secondary structure (Kjer 1995) during alignment.

Many analytical advances derive from our increasing ability to incorporate knowledge of molecular evolution in tree reconstruction. While some have argued that it is best to let the data itself guide our analyses (Brower et al., 1994), this goal can be achieved through many analytical techniques. Insect DNA exhibits many potentially confounding features (e.g. AT bias in mtDNA; rate heterogeneity in rDNA evolution). The ability to accommodate, as well as gain a deeper understanding of, these biases in the process of tree reconstruction is an important strength of model-based methods such as maximum likelihood and distance methods. Because model-based methods can account for unobserved substitutions, these techniques may also extend the range of phylogenetic utility of particular loci.

The tremendous increase in computational power available to the systematist has itself been an important advance. The ease of implementation of many methods through programs like Phylogenetic Analysis Using Parsimony (PAUP; Swofford 1993; Swofford 1998), the Phylogenetic Inference Package (PHYLIP; Felsenstein 1993), MacClade (1992), and Phylogenetic Analysis by Maximum Likelihood (PAML; Yang 1997), has been an important factor in broadening our analytical perspectives. Continued disagreement over the merits of various approaches is in part rendered moot by the ease of performing multiple analyses and comparing the results. Indeed, concordance among analyses has been considered strong support for tree topologies (Cunningham 1997).

2.6.2. Gene Trees Versus Species Trees

Phylogenetic analysis of a particular locus produces a so-called gene tree which may or may not agree with the species phylogeny (Avise, 1994, Doyle, 1997, Maddison,
1997). Although this concern may have been overly emphasized (Brower et al., 1996), incongruence among gene trees is expected under certain circumstances: horizontal transfer (including hybridization), lineage sorting (deep coalescence), and gene duplication coupled with extinction (Doyle, 1992; Maddison, 1997). Due to their smaller effective population sizes, mitochondrial genes may more reliably track short internodes than nuclear autosomal genes for recent divergences (Moore, 1995). However, it should be kept in mind that in species with polygynous mating systems and sex-biased dispersal (e.g., female philopatry), mtDNA may exhibit longer coalescence times than nuclear alleles do (Hoelzer, 1997; Moore, 1997). Specific methods have been proposed to accommodate a variety of such complications under the view that a species tree may be considered to be a cloud of gene histories (Maddison, 1997). Reconciliation methods for inferring species trees from multiple gene trees have been implemented in the program GeneTree (Page, 1998).

2.7. OTHER ISSUES

2.7.1. Preserving Specimens for Molecular Study

A substantial amount of discussion has centered on how best to preserve nucleic acids for molecular study. Comparative studies on insect DNA, in addition to our own experiences, have shown that ultracold freezing (1808 C) of live specimens is by far the most effective method (Dillon, et al., 1996; Post, et al., 1993; Reiss, et al., 1995). Single-copy nuclear genes are easily amplified from such frozen specimens. Proteins for allozyme analysis are preserved as well. Preservation with ethanol (100%), as well as fast-drying methods using silica gel, critical-point drying, or air-drying in a dessicator, have also proven to be effective, although they are questionable for long-term storage. Ideally, some part of the specimen should remain frozen (or otherwise preserved) to permit replication of the entire experiment, if necessary. There has been some discussion regarding museums' roles in molecular systematics (Whitfield, et al., 1994) and we agree there is a need for development of a general policy among collections with respect to frozen collections, voucher deposition, and destructive sampling, especially given the possibility that museums may house species that no longer exist in nature.
2.8. DNA barcoding of Coleoptera

Coleoptera is the most diverse order of insects in the world (Foottit, 2009) and dominates many ecosystems in terms of individual abundance and niches occupied. Despite "the Creator's inordinate fondness for beetles" (Haldane, 1959), Coleoptera have not been favoured to date by barcoders. For example, using the public data portal available through BOLD3 (accessed June 24, 2013, Woodcock et al., 2013), there were ca. 82 K public barcode records for beetles representing ca. 19 K provisional species. By comparison, the others of the top four most diverse insect orders are represented by approximately 2.5-fold (Hymenoptera), 3.5-fold (Diptera), and 8.5-fold (Lepidoptera) more public records. Moreover, several important studies on genetic variability within and between Coleoptera species have largely employed genetic regions other than the standard animal barcode region e.g. (Monaghan et al., 2005, Bergsten et al., 2012). Thus, the DNA barcoding of Coleoptera is in its infancy, especially when considering their described (Foottit, 2009) and projected (Odegaard, 2000) global diversity.

Woodcock et al. (2013) studied DNA barcodes from 3203 specimens representing 302 species or provisional species (the latter quantitatively defined on the basis of Molecular Operational Taxonomic Units, MOTUs) in 31 families of Coleoptera. Of the 184 taxa identified to the level of a Linnaean species name, 170 (92.4%) corresponded to a single MOTU, four (2.2%) represented closely related sibling species pairs within a single MOTU, and ten (5.4%) were divided into two or more MOTUs suggestive of cryptic species. The most diverse families were the Dytiscidae (63 spp.), Staphylinidae (54 spp.), and Carabidae (52 spp.), although the accumulation curve for Staphylinidae suggests that considerable additional diversity remains to be sampled in this family. Most of the species present are predatory, with phytophagous, mycophagous, and saprofagous guilds being represented by fewer species. Most named species of Carabidae and Dytiscidae showed a significant bias toward open habitats (wet or dry). Forest habitats, particularly dry boreal forest, although limited in extent in the region, was under sampled.
Ahrens et al. (2007) studied DNA-based taxonomy for associating adults and larvae in multi-species assemblages of chafers (Coleoptera: Scarabaeidae). They sequenced ca. 1600 bp of mitochondrial cox1 and rml and 700 bp of nuclear 28S rRNA from 250 larval and adult specimens. Individuals were grouped into putative species using statistical parsimony analysis and population aggregation analysis (PAA), whereby specimens from each locality were grouped according to the presence of diagnostic nucleotides. In addition, species membership was determined based on shifts in branching rates on clock-constrained trees to detect the putative transition from speciation to population coalescence patterns. Using these two methods we delineated between 48 and 56 groups, of which 16–20 were composed of larval and adult individuals. Nuclear and mtDNA-based groups were highly congruent; variation of 28S rRNA within groups was very low, while one widespread 28S rRNA genotype was universally found in a paraphyletic group of five mtDNA clusters. Linnean names could be assigned to 19 groups, and hence between 86.1% and 92.7% of larval specimens could be associated to species by their membership in clearly delineated groups that contained fully identified adults. The remaining larvae were delineated as five species, four of which could be assigned to Anomala or Adoretus based on their phylogenetic position. We conclude that the sequence variation was highly structured in this complex assemblage of chafers and that any given individual (larva or adult) can be readily associated with a particular DNA group using the criterion of diagnosability. The association of different developmental stages therefore becomes a matter of determining the extent of the DNA-based groups, rather than matching of sequences from adult and larval individuals. This indicates the need for a purely sequence-based taxonomic system when associating different life stages via DNA.

2.9. Eco-friendly approaches for the management of M. undecimpustulatus and M. subfasciatus

Unlike the biology, ecology and chemical control strategies, availability of literature on naturally occurring semiochemicals/synthetic pheromones, interaction and behavioral studies of M. subfasciatus are rather scanty. Literature pertaining to the present subject matter is reviewed elaborately. Besides the reports on M. subfasciatus and
relevant literatures related with the present subject in other crops is also given due importance. Semiochemicals, that evoke both behavioral and physiological responses in insects, play a major role in the insect pest management programme in several crop plants.

2.10. Scope of insect Pheromones study

Since the first identification of a pheromone fifty years ago the world of chemical signals has received much attention from scientists in biology, chemistry and agriculture/forestry. Many of the findings have come into practical use, mainly for monitoring or suppression of insect pests. Yet, a very small fraction of crop protection is based on semiochemicals, despite their obvious advantages over conventional insecticides. Why is this and what can be done to increase the applied use of these sustainable alternatives? More recently other possibilities to use odour signals have become obvious for example in detection of rare species. Can this be developed and used more widely in conservation biology?

The field of chemical ecology rapidly improved with the advent of recent chemical technologies particularly for the past thirty years. Chemical communication is generally accepted as an area of chemical ecology, considering a broad definition for communication. Organisms interact with the same and with different species where the communication may be different in nature. Law and Rignier (1971) proposed the term semiochemicals (Gk. Semeon, a mark or signal) for the chemicals that mediate interactions between organisms. Semiochemicals are sub divided into two groups i.e., pheromones and allelochemics on the basis of interactions, particularly, interspecific or intraspecific. Pheromone was first proposed by Karlson and Butenandt (1959) but their definition of the term pheromone was restricted to animals. Nordlund and Lewis (1976) broadened the definition as “a substance secreted by an organism to outside that causes a specific reaction in a receiving organism of the same species”. Pheromone was then classified on the basis of type of interaction mediated such as sex pheromone, alarm pheromone and epidetic pheromone. The term allelochemics were first proposed by Whittaker (1970 a & b) and he described them as chemicals which mediate interspecific
interactions. At present, the four types of allelochemics recognized are allomone, kairomone, synomone and apnuemone. Brown (1968) and Lewis et al., (1975) described allomones as substance produced or acquired by an organism which in contact with an individual of the same or different species in the natural context evokes a behavior physiological response that is adaptably favourable to the emitter but not to the receiver. Kairomones are chemicals beneficial to the receiver than to the emitter in interspecific interactions. The term kairomone (Gk. Kairos, opportunistic) was proposed by Brown et al., (1970). Synomones are chemicals which mediate mutualistic interactions and were first proposed by Nordlund and Lewis (1976). Apnuemones are chemicals which originate from a non-living material and mediate interaction between the individuals of different species (Nordlund and Lewis, 1976).

2.11. Semiochemicals

Law and Regnier (1971) proposed the term semiochemicals (Gk, semen, a mark or signal) for chemicals that mediate interactions between organisms. Semiochemicals are divided into allelochemicals and pheromones, depending on whether the interactions are interspecific or intraspecific (Fig. 9).

![Figure 9. Classification of semiochemicals](image)

2.12. Allelochemicals

The term allelochemical was first proposed by Whittaker (1970a and b), as “a chemical that is significant to organisms of a species different from its sources for reasons other than food as such”. These in turn are subdivided into allomones,
kairomones, synomones and apneumones. Allomones are those chemical signals that give advantage to the emitter (e.g. defensive secretions) but not to the receiver. Numerous types of interactions are mediated by allomones. Venoms for example, are used in prey capture and defence (Brown, 1968). Rhoades and Cates (1976) reported that allomones also serve plants as defence mechanisms against herbivores and reduce competition from other plants. Defence is not the only role allomones play, as certain predators are able to use allomones to attract or manipulate prey (Holldobler, 1971 and Weaver et al., 1975).

2.13. Kairomones

Brown et al., (1970) proposed the term kairomone (Gk, kairo, opportunistic) to cover chemicals produced or acquired by an organism that, on contact with an individual of another species in the natural context, evokes in the receiver a behavioural or physiological response that is adaptively favourable to the receiver but not to the emitter. Examples are the chemicals emitted by host animals which attract blood-feeding tsetse flies (Hall et al., 1984 and Bursell et al., 1988), and chemicals used by many beneficial insects in their host or prey selection behavior (Lewis and Jones, 1971). In a few cases, it has been shown that prey use kairomones to detect the presence of a predator (Tinbergen, 1951).

2.14. Synomones

Nordlund and Lewis (1976) proposed the term synomone (Gk. syn, with or jointly) for chemical signals that give advantages to both sender and receiver, as in the case of floral scents which indicate a nectar source to insects and ensure pollination of the flowers producing them. Synomones play important roles in maintaining the species specificity of response to certain pheromones (Vite et al., 1972).

2.15. Apneumones

The term apneumone (Gk, a-pneum, breathless or lifeless) was proposed by Nordlund and Lewis (1976) to cover chemicals, mediating interactions between different species, which derive from a non-living source. For example, Thorpe and Jones (1937)
reported that the *Ichneumonid* parasitoid *Venturia canescens* is attracted to the odor of oatmeal, its host's food.

2.16. Pheromones

The term pheromone (Gk. Phereum, to carry; hormone, to excite or to stimulate) was originally proposed by Karlson and Butenandt (1959) and Karlson and Luscher (1959) to cover "substances which are secreted to the outside by an individual and received by a second individual of the same species, in which they release a specific reaction, for example, a definite behaviour"

Pheromones and other semiochemicals are found throughout the plant and animal kingdoms. They are particularly well developed in insects where they often affect behaviour in a way that is essential to the maintenance of the individual and/or the species. Chemical stimuli that produce an immediate and reversible change in the behavior of the receiving individual are called releasers and most insect pheromones fall into this category. In contrast, primer pheromones are those which act through long-term physiological changes in receiving organisms, probably involving hormones (Wilson, 1965). Pheromones are classified according to the type of behavior they influence.

2.17. Sex pheromones

Based on Karlson and Butenandt's (1959) definition of a pheromone, a chemical or a mixture of chemicals, secreted to the outside by an individual that produces immediate response of sexual behavior in the receiving individual, has been called a sex pheromone. These are the most widespread and widely documented types of pheromones, which are used to increase the probability of successful mating. Sex pheromones may be produced by females or by males, according to the species and in some cases both sexes contribute to the chemical communication involved in mating. Since the first successful identification of bombykol in the silk moth Bombyx mori (Butenandt et al., 1959), pheromones have been identified in several hundred species of Lepidoptera (Arn et al., 1997) and other insect orders (Hardie and Minks, 1999).
2.18. Aggregation pheromones

Aggregation pheromones are generally produced by one sex and attract both sexes to a food source as well as attracting the opposite sex for mating. Aggregation pheromones are found in many species of Coleoptera (Borden et al., 1982). Examples include aggregation pheromones produced by the male coconut rhinoceros beetles, Oryctes rhinoceros (Hallett et al., 1995) and Rhynchophorus ferrugineus weevils (Hallett et al., 1993).

Pheromones have four main uses in insect pest control:

- use of pheromone-baited traps for monitoring insect populations,
- use of traps for control by mass trapping,
- use of pheromones combined with insecticide in a “lure and kill” approach,
- use of sex pheromones to disrupt mating of the target species,

(Jutsum and Gordon, 1989, McVeigh et al., 1993 and Hall, 1995)

However, the selectivity of pheromones is a disadvantage in situations where several pest species co-exist. Moreover, they may be expensive to manufacture and the optimal blend of pheromone components may be difficult to obtain or dispense. Many pheromone components are unstable and decompose in the presence of light or air. To overcome this problem a variety of formulations and dispensers have been developed (Jutsum and Gordon, 1989).

2.19. Pest monitoring

Sex and aggregation pheromones are species-specific attractants, and traps baited with these can be used as specific monitoring tools for the target pest species (Inscoc et al., 1990, Cunningham et al., 1990 and Jones, 1998). Traps of many varieties have been devised for different pests, most involving some form of adhesive surface, water or dry funnel as the trapping medium. The pheromone is formulated in a slow-release dispenser, which remains active for several weeks in the field. Several types of pheromone dispensers have been developed for use in traps. They include rubber septa, polythene vials, polythene tubes, polyvinylchloride lures, dental roll, cigarette filters and others.
(Weatherston, 1989). These traps are thus relatively simple and cheap, robust in field use and do not require trained operators to sort the catch as they are specific for the target pest unlike light traps.

However, much work is required to interpret catches in pheromone traps for a number of reasons. Pheromone traps generally attract adults whereas it is often the larval stages that do the damage. Furthermore, traps baited with sex pheromones often attract male insects whereas it is the female insects that lay the eggs producing the next generation of larvae. Catches in pheromone traps are often significantly affected by environmental conditions such as wind speed, temperature, rainfall and humidity and even moonlight. Trap efficiency can also vary with population density, generally decreasing as density and competition from natural pheromone sources increases (Miller and McDougall, 1973). Nevertheless pheromone traps are now widely used for monitoring in both open field and storage situations (Wall, 1989, Ridgeway et al., 1990, Jones, 1998 and Hodges and Pike 1995).

2.20. Mass trapping

Mass trapping of pest insects is an alternative to mass killing by using insecticides. The control measure is directed against the adult insect (Bakke and Lie, 1989). The intention is to catch a pest species selectively and thereby suppress the population to a level below the threshold of damage. Mass trapping is especially applicable:

- where the pest is widely dispersed
- where control by conventional pesticides is inapplicable;
- where resistance has developed to conventional insecticide and no other form of control is available or most importantly;
- where it can be an economic form of pest control.

Method involved in behaviour modifying chemical starts with extraction, identification and quantification of volatile chemicals. Historically, volatile semiochemicals were extracted by solvent and steam extraction and the same method is
used for isolation of semi- and non-volatiles also (Millar and Sims, 1998). Major disadvantages for these extraction methods are firstly, the extracts are complex mixtures of volatile and non-volatile compounds and numerous fractionation steps required to isolate pure compounds and secondly, the profile of volatiles obtained is often not representative of the blend released by the intact living organism (Lorbeer et al., 1984; Teranishi et al., 1993; Takeoka et al., 1988; Tollsten and Bergstrom, 1988). To outwit these problems most of the recent work has focused on collection of volatiles onto an activated adsorbent emitted from the system (insects/plants) into the airspace inside a closed container (Sham, 1990; Heath and Manukian, 1992; Plaza et al., 2000). A variety of adsorbents have been used and among them the most versatile and commonly used are Porapak Q (Ethyl vinyl benzene - divinyl benzene - copolymer), Tenax GC (2, 6-diphenyl-p-phenylene oxide polymer), and activated charcoal (Millar and Sims, 1998). Identification and quantification of the volatile compounds were usually done using GC-MS and GC-FID (Cork et al., 1991; David et al., 1996).

Scanning electron microscopic study of the insect antenna is very important for the behavioral studies. Most of the olfactory sensilla are located in the paired antennae pointing out from the forehead on an insect. The classification of sensillum types in insects are based on the morphology and supported by studies of the ultra structure and electro physiology. Schneider (1964) classified the insect sensilla into ten different types which are widely followed universally till date.

Behavioral response of an insect towards a volatile chemical was usually tested in the laboratory using olfactometers, wind tunnels and electro antennogram. Liendo et al., (2005) proved through olfactometric and EAG experiments that Steirastoma breve (Coleoptera: Cerambycidae) olfactory behaviour is highly influenced by odour emitted by cocoa plants. Behavioural bioassay measure is a change in behaviour in response to the test material and it is generally of three types of equipments viz., static cages/arenas, olfactometers or wind tunnels (Baker and Carde, 1984).

Electro antennogram, developed by Schneider (1957) is extremely useful for detecting various attractive components in fractionated extracts or individual compounds.
In general, insect antennae possess olfactory sensillae, which contain receptor cells that respond to the behaviour modifying compounds. Schneider (1957) showed that by inserting microelectrodes into the base and tip of an insect’s antenna it was possible to record a slow depolarization across the antenna in response to stimulation by volatile compounds. After the invention of EAG many studies have been executed on various insects.

When a single component of volatile is presented, either it does not elicit behavior response to source location/upwind flight or only elicits response at very high dosages that too only in a very small numbers (Linn et al., 1986). It is mandatory that all the volatiles identified have to be tested individually and as blends to determine their role in evoking behavioural response. Bhasin et al., 2000 determined the sensitivity of Culicoides impunctatus to individual components by testing the nine host kairomones where only three compounds found to evoke significant behavioural activity. Many insects respond only to semiochemicals over a certain concentration range or require exposure to a defined blend (Suckling and Karg, 1999). In scolytid beetles, except for some individual pheromone compounds, a blend of chemicals especially monoterpenes are found to the cue for attraction (Sun et al., 2003; 2004; Poland et al., 2003). In many instances, monoterpenes served as synergists with host volatiles and pheromones (Byers, 1992; Byers et al., 1990, 1998; Millar and Borden, 2000; Nadir et al., 2003).

Cotton is an important cash crop in many countries. Even with use of insecticides, many insects cause extensive damage to crops. The boll weevil, Anthonomus grandis Boheman, is a serious pest in the USA. The male-released pheromone (grandlure) provides an effective attractant compared to traps baited with cottonseed meal, molasses and cotton plant extracts (Tumilinson et al., 1969). The male produced pheromone acts as an aggregation pheromone to attract both the sexes. Based on field evaluation results on control of boll weevil, Hardee (1982) reported that it was possible to demonstrate an effect on the insect population using pheromone baited traps by mass trapping but the suppression was not sufficient to avoid insecticide use. The Pink bollworm, Pectinophora gossypiella, is an important pest causing great damage in many cotton growing areas. Control of pink bollworm by using female sex pheromone (Shani, 1982) baited traps
proved to be too labour-intensive on a large scale. Beevor et al., (1993) reported that control of the cocoa pod borer, *C. cramerella*, in East Malaysia could be achieved with pheromone traps at 4-8 per ha, but early attempts to control the cotton leafworm, *Spodoptera littoralis*, with pheromone traps in Egypt were unsuccessful (Campion and Hosny, 1987). Recent attempts to control sweet potato weevils, *Cylas spp.*, with traps baited with the female sex pheromone caught large numbers of male weevils but did not significantly reduce damage in Indonesia (Braun and van de Fliert, 1998).

On the other hand, semiochemicals that attract exclusively female insects have much better potential for the control of insect pests by trapping. For example the sex pheromone produced by the male coffee white stemborer, *X. quadripes*, has recently been identified (Hall et al., 1998) and traps baited with the synthetic pheromone shown to attract females. Similarly, aggregation pheromones, which attract both male and female insects, can be used for control by mass trapping, for example, *Rhynchophorus palmarum* (Oehlschlager et al., 1993). The few successful attempts at mass-trapping with other types of pest include stored product pests, e.g. the Mediterranean flour moth, *Ephestia kuehniella* (Zeller) (Trematerra and Battaini, 1987) and horticultural pests, eg. the citrus flower moth, *Prays citri* (Sternlicht et al., 1981).

Brinjal is a native of India and one of the most popular vegetables grown throughout the country especially in North East Region. The vegetable brinjal, *Solanum melongena* L. (Solanaceae), is often referred to as eggplant or aubergine in other regions of the world and is grown for its fleshy fruit. Two crops are typically grown per year in South Asia and because fruits can be harvested every week farmers are provided with an assured income and resource-poor consumers have access to a much-needed, nutritious vegetable, in the summer months when other vegetables are in short supply.

In India alone over 500,000 ha of brinjal is cultivated per annum with yields of typically 20-40 tonnes/ha/crop. In Bangladesh vegetables are grown on 2% of the available agricultural land and yield 4% of the produce, unlike rice that uses 72% of the land and yields 51% of the food by weight. Brinjal is by far the major vegetable
representing some 41% by weight of all vegetables produced, occupying 19% of the land used to cultivate them.

As with every crop farmers have to cope with a range of pests, diseases and crop nutrition issues in order to produce a healthy brinjal crop. Brinjal is affected locally by whitefly (Bemisia tabaci), ash weevil, (M. subfasciatus), red spider mite (Tetranychus curcurbitae) and little leaf disease (transmitted by jassids) and soil pathogens such as bacterial wilt (Ralstonia solanacearum) are increasing in prevalence. In some areas of Gujarat parasitic plants such as orobranche are endemic. Nevertheless, the most serious pest of brinjal is without doubt the brinjal fruit and shoot borer, Leucinodes orbonalis, a lepidopterous pest whose larvae are well protected from pesticides and natural enemies once they have entered the fruit or young shoots.

Farmers have depended mostly on insecticides to control of M. subfasciatus but the sustainability of this approach is in question as farmers in some areas of South India and Tamil nadu are finding that even multiple applications do not provide effective control. Indeed farmers in some areas of Tamil nadu are applying carbofuran 3 G @ 15 kg/ ha, 15 days after planting and spray carbaryl 50 WP @ 3g + wettable sulphur 50 WP @ 2g/lit. For this reason theses prioritized research effort on this key pest and much of the IPM package developed was concerned with this issue (http://susveg-asia.nri.org/).

Among the pests, the leaf notcher and root feeder M. subfasciatus (Coleoptera: Curculionidae) is a serious pest of brinjal in India. Even though the occurrence of M. subfasciatus of brinjal was reported in Florida on numerous ornamental species and fruit crops, in which it causes damage to the foliage and possibly root systems (Thomas 2005; NPGA 2000; O'Brien et al., 2006), it gained major pest status in India.

At present, infestation by the weevil is known from most areas of south India. Achievement of control is moderate due to the peculiar habitat of these weevils. Increasing concern on the adverse effect of usage of pesticides on the environment and the presence of residues in made brinjal have precipitated a strong decision for limited
and discriminate use of pesticides. This led to the development of an integrated pest management strategy in brinjal.

The use of attractant trap has long been practiced by the entomologists in general and applied entomologist in particular (Nordlund et al., 1981). Though the progress from a suction trap to more sophisticated trap was rapid and one of the greatest advantages of attractant trap is the specificity to the target insect. In course of time, many investigations had been made on different orders, genera and species that have attraction towards their own species (sex pheromones) or to other species (kairomones). The first isolation and identification of a pheromone was reported by Butenandt et al., (1959).

2.21. Uses of Pheromones in Pest Management

The timeline outline the chain of events has led to use of pheromones in pest management is depicted in fig. 10. The use of pheromone for controlling pest insects requires three items: a pheromone chemical, a trap, and a support to hang the trap in the field. Technically sex pheromones can be used in three principal ways:

2.21.1. Detection and Monitoring

The principle use of insect pheromones is to attract insects to traps for detection and determination of temporal distribution. In most instances, the males are responders to female-produced pheromones. Trap baits, therefore, are designed to closely reproduce the ratio of chemical components and emission rate of calling females.

Trap baits of many designs have been tested over the years. Trap design is also critical to effective use of traps for monitoring insect populations. Traps vary in design and size dependent on the behavior of the target insects. The information from trap catches can be very useful for decision making on insecticide applications or other control measures. For example, trap catches may indicate a loss of effect of pheromone on mating disruption and the need to reapply a pheromone treatment. Careful monitoring and experience in interpreting collected data are important for success. Traps may also be placed with the objective of destroying males for population control.
**Figure 10.** Timeline outlines the chain of events has led to use of pheromones in pest management. Fortunately the development of benign pheromone-based alternatives for pest management has given effective new options in their endless battle with the bugs. As we have seen here, many of the scientific breakthroughs that led to these options stemmed from fundamental research, conducted by curious people who merely wanted to understand how nature works.

2.21.2. Mass trapping

Sex pheromone baited traps can capture male moths continuously, thus preventing mating and multiplication of the pest. This approach has proven to be particularly efficient and economical. *Rhynchophorus palmarum* is the primary pest of oil, coconut and palm in Central and South America. By 1994 the number of trees needing to be felled was reduced to less than 3,000 per annual demonstrating that mass trapping can be highly effective in controlling palm weevil populations (Alpizar *et al.*, 2002, Hallett *et al.*, 1999, Oehlschlager *et al.*, 2002). Highest mass trapping of males of *Macroscelies japonica* reported by lure baited with E2, Z13-18: Ald and E2, Z13-18:OH (Islam *et al.*, 2007). Examples of mass trapping are lures baited by Z7, 9-10:OH were examined on nettle moth, *P. lepida lepida* (Islam *et al.*, 2009), cotton weevil (*Anthonomus grandis*) successfully baited with its aggregation pheromone (Cork *et al.*, 2003), Japanese strain of *Phyllocnistis citrella* trapped only the lure containing Z7,Z11-16:Ald (Vang *et al.*, 2008).

Brinjal (*Solanum melongena* L.) is an economically important crop throughout South and South East Asia. Fruit losses in excess of 50% are commonly reported due to the boring activity of larvae of the brinjal shoot and fruit borer, *Leucinodes orbonalis* (Cork *et al.*, 2005). Zhu *et al.* (1987) reported (E)-11-hexadecenyl acetate as the pheromone of *L. orbonalis* and traps baited with up to 500 µg attracted more male moths than six virgin females. Subsequently Attygalle *et al.* (1988) identified (E)-11-hexadecen-1-ol in addition to the related acetate using i sects obtained from Sri Lanka. In field trials conducted in India where blends containing between 1 and 10% E11-16: OH caught even more male *L. orbonalis* than E11-16: Ac alone. At the 1000 µg dose,
addition of 1% E11-16: OH to E11-16: Ac was found to be significantly more attractive to male *L. orbonalis* than either 0.1 or 10% E11-16:OH. Trap catch was found to be positively correlated with pheromone release rate, with the highest dose tested, 3000 µg, catching significantly more male moths than lower doses (Cork *et al.*, 2001). In order to reduce the cost of pheromone based technologies for control of *S. incertulas*, a programme of research to develop an effective mass trapping system. This proved to be highly effective using indigenous traps and lures at a density of 20 traps ha⁻¹ (Cork and Krishnaiah, 2000).

### 2.21.3. Mating disruption

Sex pheromone can be used for disruption of mating, which is achieved by placing high concentrations of pheromone at regular intervals throughout the field. The high concentration of pheromone saturates the area resulting in males failing to find females, which produce very minute quantities of these chemicals, thus preventing mating and multiplication of the pest. The major pest of cotton in Egypt in the early 1980's was the pink bollworm (PBW), *Pectinophora gossypiella*. The female sex pheromone was identified by workers in the USA (Bierl *et al.*, 1974). The diversity of mating communication system in lepidopteran insects was also reported (Islam *et al.*, 2008). The economic importance of *P. gossypiella* and the fact that its pheromone is relatively cheap and chemically stable, the decision was made to try to control it using mating disruption (Fig. 11). It has also been identified as a pest control method in which the insect does not become resistant.
2.22. Pheromone traps

Various types of traps are available commercially, while others can be made by farmers inexpensively at home. A pheromone baited lure inside the trap will bring male moths inside the trap. Proper trap design is critical to kill the pest once it enters the trap. The type of trap to be used depends on the behaviour of the target insect. Various research works showed the most effective traps in pest control are delta traps, winged traps and funnel traps. Different types of traps are available and relatively low in cost (Fig. 12). Orientation of bark beetles *Pityogenes chalcographus* and *Ips typographus* to pheromone-baited puddle traps placed in grids: a new trap for control of scolytids is depicted in fig. 13. Pheromone traps are very sensitive, meaning they attract insects present at very low densities. They are often used to detect presence of exotic pests, or for sampling, monitoring, or to determine the first appearance of a pest in an area.
Figure 12. Different types of traps used for insect pest management—an ecofriendly approach (Source: jenny.tfrec.wsu.edu)

Figure 13. Puddle trap constructed of wire hoop for support of plastic sheet containing water pool and wire arch for holding a plastic cup (covered with aluminum foil) containing pheromone dispensers. A depression, as indicated, is dug in the forest duff to form and support the water pool held by the plastic sheet.
2.23. Chances for success

2.23.1. Restrictions of insecticide use

Detrimental health effects, environmental issues, insect resistance, or marketing opportunities for organically-produced food are well-known arguments against the use of insecticides. Increasing restrictions in the use of conventional insecticides in Europe further emphasize the need for new, environmentally safe methods of pest control. The "Environmental Action Programme" adopted by the European Union for the year 2000 aims at "the significant reduction in pesticide use per unit of land under production, and conversion to methods of integrated pest control, at least in areas of importance for nature conservation". The five-year-programs and resolutions adopted by European authorities are not always entirely realistic, but it remains a fact that a number of neuroactive insecticides have been or are being deregulated in several countries. In Germany, for example, the number of insecticides available for control of fruit insects has been decreasing steadily over the past decade, from 40 compounds in 1988 to 24 compounds in 1998 (Koch and Schietinger 1999).

Resistance against the available insecticides continues to be an important issue. In codling moth, for example, insecticide resistance is increasing all over Europe (Sauphanor & Bouvier 1995, Charmillot et al., 1999). In addition, it is a well-known fact that the overuse of insecticides induces outbreaks of secondary pests such as phytophagous mites, which are difficult to control with pesticides (Knisely and Swift 1972, Tanigoshi et al., 1983). Replacing insecticide with pheromone treatment against codling moth renders treatments against phytophagous mites superfluous, which, for example in South Tyrolian orchards, compensates for the cost of the pheromone treatment (Waldner 1997). Insecticides do not work as well as often believed, and a long-term population decrease has never been achieved, not in any species. In comparison, a well-implemented mating disruption programme may clearly be more efficient than insecticides in some species. An observation shared by many working with mating disruption is that its long-term use results in continuously decreasing populations of the target species. This may be due to recovery of the beneficial insect fauna and an overall increasing stability of the orchard ecosystem.
Insecticide sprays harm the terrestrial and aquatic fauna. This is true even for chitin synthesis inhibitors, which have been long thought to be environmentally rather safe. In Scania (Southern Sweden), the contamination of ground water resources with agrochemicals has risen to such a pitch that protected areas need to be established, where all use of insecticides is prohibited in order to conserve drinking water resources. In such areas, farmers are forced by law to turn to organic production methods from one year to another.

It then is plain to see what we know, even though we are not always aware of it that environmentally safe control methods are available only against a precious few insect species, and that biological control methods other than pheromones are often even more expensive or less reliable than pheromones. It then also becomes apparent that there is great potential in pheromone-based methods, once the "tools" have been developed properly. The tools are the knowledge of the pheromone chemistry and biology of a given species, economic synthesis, appropriate dispensers and the knowledge and experience of how to use them in the field. It was possible to elaborate pheromone-based methods against several species, and with the database on insect pheromones which is available today, it will certainly be possible to extend the use of pheromones to other species.

2.23.2. Economic aspects

The current conflict in Scania between cheap and rather efficient plant protection by insecticides and contamination of water resources by the same insecticides offers the trivial, but nevertheless important insight that the price of insecticides or pheromones should not be confounded with economy of use. Simple equations such as "insecticides are less expensive than pheromones - therefore, insecticide use is more economic" are no longer valid. The economic importance of horticultural production in Scania is ever decreasing. More precisely: the revenue from the produced commodities is on the decline. As a result, the area covered by orchards has significantly decreased in the past decade. However, fruit production is, beyond fruit sales, of great significance for the attractively of this region.
The farmer is becoming a landscape gardener and "landscape" is turning into "viewscape". A pleasant environment will not only attract tourists, but also new enterprises and investors, and people who wish to inhabitate this land. The character of the Scanian fruit-growing region will change and most likely suffer if the orchards are replaced with agricultural crops. This could accentuate already existing structural problems in this area. Today, both the growers' economy and Scanian orchards are threatened by cheap fruit imports. At the same time, the consumer demand for unsprayed fruit has steadily increased over the past decade but cannot be met by the local production. A leading Swedish supermarket chain has based its marketing campaign on a green image, and all attempts to cut down on pesticide use receive much attention in local and national news media. Employment of safe insect control techniques will improve fruit quality. This will be rewarded by the consumers and strengthen the competitiveness of local fruit production. The farmers' efforts to establish sustainable production methods will thus contribute to rural development.

2.24. Reasons for lack of success

2.24.1. Motivation

The main applications of the mating disruption technique in Europe are against codling moth on >10,000 ha and the grape berry moths on >30,000 ha (Arn and Louis 1996, Waldner 1997, Kast 2001, Zingg 2001). This clearly demonstrates the potential of the mating disruption technique for insect control and the figures are encouraging, until we take a closer look and realize that the area under mating disruption represents only a fraction of the several millions of hectares of European orchards and vineyards on which conventional insecticides are being used.

One reason for the comparatively limited use of mating disruption is that the technology currently in use must become more reliable. The most urgent shortcomings have been outlined in detail by many authors, and will be shortly mentioned below. However, one most important factor is certainly motivation and determination. A growers' association in Northern Italy has been determined to implement mating disruption against codling moth and has been successful (Waldner 1997). The conditions
in Northern Italy for using pheromones are as favorable or poor as in most other European growing areas, but the main difference is that growers were determined to cut down insecticide use.

A similar situation is encountered with the grape berry moths. A significant part of German and Swiss vineyards are being treated with pheromones since many years (Kast 2001, Zingg 2001), but the river Rhine in Germany and the Jura mountains in Switzerland are natural borders for pheromone use. Admittedly, the climatic, economic and faunistic conditions may be less favorable for mating disruption in Southern European vineyards. However, pheromone use in grapes is rapidly increasing - in Northern Italy (Varner et al., 2001).

Motivation as a main factor for pheromone vs. insecticide use obviously concerns not only the farmers, but also researchers and chemical industries. Pheromone research, funded by the public hand over four decades, has provided the basic knowledge for the development of new pest control techniques. It is important to realize that only goal-oriented research will lead to reliable and more widespread applications. However, for many researchers at academic institutions it may instead be more rewarding to explore new directions and to point out the potential, future use of knowledge yet to be acquired. Some companies in the agrochemical sector may have used pheromones mainly as a public relation tool, whereas other chemical companies have made a most significant contribution to pheromone synthesis and dispenser systems. Work done by chemical companies was again driven by individuals motivated and determined to make pheromones work.

2.24.2. Integrated research

For future development, stronger emphasis on a multidisciplinary approach is stressed. Clearly, there is a need for intensifying communication and collaboration, and for coordinating activities between academic research institutions, chemical industries and extension organizations. Insecticides are at least as complex chemicals as pheromones and they are difficult to formulate, market, and difficult to apply. However, in contrast to pheromones, the entire development, from research to application, is
carefully planned and has often been done under one roof. Already existing knowledge on pheromone technology can be contributed from various disciplines to achieve improvements or to rapidly establish new methods. Unfortunately, lab people all too often lack understanding for the difficulties associated with field implementation work. Chemical companies sometimes tend to believe that they "know all about pheromones" and that access to published information is sufficient. And farmers' organizations rarely manage to establish a dialogue with people from the academic circuit. Summer fruit tortrix *Adoxophyes orana* is the most important leafroller moth in European orchards under mating disruption for codling moth and provides a good example of how people from different disciplines view the same problem and how lack of integration can become an important obstacle. A detailed account of female-produced compounds and their effect on male behaviours was published in 1986 by Guerin and coworkers, showing that the main pheromone compound of *Summer fruit tortrix* is (Z)-9-tetradecenyl acetate and that it must be blended with additional minor pheromone compounds to attract males. Nonetheless, dispensers for *S. tortrix* control were formulated to contain (Z)-11-tetradecenyl acetate (e.g. Neumann 1997, Rama 1997) and it comes to no surprise that satisfactory control was not achieved.

2.24.3. *Field implementation*

Field implementation is the focal point of mating disruption research. All available knowledge of an insect's biology, its pheromone chemistry and biology, and dispenser technology needs to be integrated. Lack of success is often equivalent to lack of know-how, or the lack of knowledge transfer. Heinrich Arn, the former convener of the Pheromone Working Group, emphasized the need for field measurements on insect behaviour and pheromone dispersal as he coined the title of the Neustadt meeting: "*Mating Disruption - The behaviour of Moths and Molecules*" (Arn 1987). This meeting reflected the optimism that the necessary tools would soon be at hand. A quantum leap was then achieved by Uwe Koch and his working group, who managed to measure ambient pheromone concentrations using an insect antenna (Koch *et al.*, 1992, 1997), and several behavioural studies, including work in field wind tunnels (Cardé *et al.*, 1993,
1997), made a significant contribution to our understanding of insect behaviour in a pheromone-permeated atmosphere.

2.24.4. Dispenser technology

Pheromone synthesis, dispensers and dispenser application dictate the cost of mating disruption. Discussions of behavioral mechanisms may turn out to be futile if only we were able to afford or to achieve sufficiently high aerial pheromone concentrations. Major achievements have been made with large-scale industrial synthesis of insect pheromones (Yamamoto and Ogawa 1989). It is important to realize that commercial use of codling moth mating disruption became possible only after economic synthesis of codlemone became available. The price for this compound has been diminished by a factor of 50 to 100 since the eighties.

A major flaw of currently used commercial pheromone dispensers is that they are "passive" and that pheromone release depends on ambient temperature. In Swedish apple orchards, ca. 90% of pheromone applied is released outside codling moth diel flight period, mainly during daytime at peak ambient temperatures (Witzgall et al., 1999). In addition, dispensers must be applied early in season when population densities are still low, while their release rates decrease during the season, as population densities start to increase (Ogawa 1997). These problems are circumvented by using active pheromone "puffers", releasing large amounts of pheromone when the insects are active (Shorey et al., 1996, Baker et al. 1997). However, their commercial use is still limited. Hand-application of dispensers does not pose a problem in labour-intensive horticultural crops, but precludes the use of mating disruption in most field crops. The formulation of sprayable pheromone dispensers has been attempted in the past (Hall et al., 1982, Weatherston 1990), and is subject of recent efforts (Polavarapu et al., 2001).

Present work is aimed to develop a protocol to extract, isolate and identify the pheromone from *M. subfasciatus* to construct an efficient pheromone trap for *M. subfasciatus*. Laboratory and field evaluations were carried out with these compounds singly and in combinations to monitor their efficiency in trapping of *M. subfasciatus* weevils. Optimization of dispenser, suitable attractant blends and their optimum amount
required per dispenser for efficient trapping were also carried out. Determination of suitable trap, position and height were evaluated. Field trials on the most effective trap with the most promising blend were carried out to determine the number of traps required per hectare. Using all these findings, large scale field experiments were conducted to generate information on the efficiency of attractant trap which can further improve the integrated pest management schedule for *M. subfasciatus*.

2.25. *Bacillus thuringiensis* – as a biological control agent

Microbial insecticides containing δ-endotoxins (Cry proteins) from the bacterium *Bacillus thuringiensis* (*Bi*) have been used as an alternative to conventional chemical insecticides for almost 70 years. Natural isolates of *B. thuringiensis* have been used as a biological pesticide since the 1950s for the control of certain insect species among the orders Lepidoptera, Coloeptera and Diptera. The genes of *B. thuringiensis* coding parasporal crystals are also a key source for transgenic expression which provides pest resistance in plants (Schnepf *et al.*, 1998). This feature makes *B. thuringiensis* the most important biopesticide on the world market (Bernhard *et al.*, 1997). The feasibility of using *B. thuringiensis* for insect control has been increased by advances in recombinant DNA technology. The use of this technology has facilitated the cloning of the toxin genes and their expression in plants, seeds, and in plant and soil-inhabiting bacteria, thereby providing novel means of toxin delivery to insects. These new developments in *B. thuringiensis* insect control, although safe for humans, environment and compatible with other agricultural practices, will likely encounter problems with insect resistance in the near future (Anon and Tipvadee, 2008). Recent progress in the introduction of *B. thuringiensis* toxin genes into plants and other organisms will be of little use if the potential problems of insect resistance are not understood. As a consequence, *B. thuringiensis* products must be developed within the framework of managing the development of insect resistance prior to the widespread use of *B. thuringiensis*. When compared with the chemical pesticides, *B. thuringiensis* has the advantages of being biologically degradable, selectively active on pests and less likely to cause resistance. The safety of *B. thuringiensis* formulations for humans, beneficial animals and plants explains the replacement of chemical pesticides in many
countries with these environmentally friendly pest control agents and technologies (Mario and Alejandra, 2008).

The first report on the crystalline parasporal body in the bacterium that might be associated with the insecticidal activity appeared by 1953 (Hannay, 1953). Angus (1954) demonstrated that this crystal contains an alkaline-soluble toxin for insects. *B. thuringiensis* produces a -exotoxin known also as the fly-toxin, thermostable toxin, or thuringiensin, but this toxin was not approved for use in agriculture because its toxicity was not limited to insect pests (Sebesta *et al.*, 1981). The -endotoxin showed the most promising characteristics of an insect-specific bioinsecticide. By the end of the 1950s, the toxicity of the spore-crystal complex was classified into 'insect types' (Heimpel and Angus, 1959) - an early indication that the spore contributes to the insecticidal power of *B. thuringiensis*. The genetic diversity and distribution of cry genes in *B. thuringiensis* strains vary based on geographical location. Each habitat may contain novel *B. thuringiensis* isolate that have more toxic effects on target spectra of insects. To obtain novel *B. thuringiensis* strains for the production of Cry proteins, isolation of numerous new *B. thuringiensis* strains is becoming a routine activity in many industries. The characterizations done for most of the collections were based on bioassays against different insect larvae without identification of the cry genes present in the *B. thuringiensis* strains. Occurrence of *Bt* in different environmental niches depicted in Fig. 14.
Vertebrates and invertebrates are only examples of a much wider range of possible hosts. The soil (1) is usually the largest reservoir of \textit{Bt}, because it receives the highest amount of propagules from other environments. From it, \textit{Bt} can colonize the rhizosphere (2) feeding on roots exudates. If eaten by soil invertebrates such as worms, insects and nematodes (3), \textit{Bt} can infect in a paratenic way, colonizing the gut and feces, or in a pathogenic way, killing the host and growing in the cadaver. Thus \textit{Bt} is re-introduced into the environment through these two ways. Rhizosphere colonization favors endophytic colonization (4) which protects the plant from some herbivores, while helps \textit{Bt} to proliferate in plant tissues and infecting herbivores in paratenic (5) or pathogenic (6) ways. Besides endophytism, \textit{Bt} can reach the surface of the plants from the soil due to the germination process, by splashes of rain water, and through the feces of animals that carry it, such as insects and birds (7). The infected fallen leaves can re-introduce \textit{Bt} in soil and water (8). The rain may also carry the \textit{Bt} to water bodies from soil and plants (9). In water the \textit{Bt} can infect and proliferate in vertebrates or
invertebrates and persist in this environment by associating with substrates as aquatic plants and sediments (10). Faeces from animals that feed on contaminated plants or insects can serve as a source of nutrients for Bt growth, and they can act as a source of infection for coprophagous (11). It is known that ticks and mites are also Bt hosts (12), but the natural mechanism of infection is unknown. It is possible to observe a wide range of strategies for Bt occupy different niches and disperse in environment with or without causing disease. *(Source: Insects 2014, 5, 62-91; doi:10.3390/insects5010062)*.

2.26. Mode of action of Cry toxin

Crickmore *et al.*, 1998 defined Cry Protein as a parasporal inclusion (crystal) protein from *B. thuringensis* that exhibits some experimentally verifiable toxic effect to a target organism or any protein that has obvious sequence similarity to a known Cry protein. Similarly Cyt protein denotes a parasporal inclusion protein from *B. thuringensis* that exhibit haemolytic activity or any protein that has obvious sequence similarity to a known Cyt protein. Cry toxins are synthesized as protoxins. When susceptible larvae ingest the protoxin, it is solubilized and activated by gut proteases, generating a toxic fragment. The activated toxin then binds to two different receptors in a sequential manner. Both receptors are localized in the microvilli membrane of cells that form the midgut epithelium. The first contact of the toxin is with the cadherin receptor. This interaction induces a conformational change in the toxin, cleaving a small fragment from the amino-terminal region, the helix α-1. This cleavage exposes previously buried hydrophobic regions and triggers the formation of a tetrameric oligomer structure. The oligomer then has an increased affinity to the second receptor, aminopeptidase N (APN). APN facilitates the insertion of the oligomer into the membrane, forming a lytic pore that leads to cell disruption and ultimately insect death. The first symptoms of intoxication are the immediate paralysis of intestinal movement and feeding cessation. Then, the midgut cells are disrupted and the insect dies from destruction of the midgut tissue (Mario and Alejandra, 2008). Local defense mechanisms of an insect model against pathogenic bacteria depicted in Fig. 15 and Mode of action of Bt toxins are depicted in Fig. 16. *(Source: Insects 2014, 5, 62-91; doi:10.3390/insects5010062)*
**Figure 15.** Schematic diagram of local defense mechanisms of an insect model against pathogenic bacteria.

The cuticle is a first barrier, which can be overcome through spiracles or injury. To cause infection, ingested pathogens must overcome various physical barriers, such as peritrophic membrane, epithelium, peristalsis and commensal microbiota, as well as chemical defences present in the digestive system as pH, proteases, cell receptors and antimicrobial compounds. In addition, the commensal microbiota provides a competitive environment for the pathogen establishment, and also produces antimicrobial compounds that hinder the pathogenic action. Much of the toxins secreted by pathogens require specific receptors to perform their functions; changes in these receptors allow development of insect resistance to pathogens. Finally, after overcoming all these defenses, the pathogen must still deal with the innate immune system and circulating haemocytes to succeed with an infection. *(Source: Insects 2014, 5, 62-91; doi:10.3390/insects5010062)*
**Figure 16.** Models of the mode of action of Cry toxins from *Bacillus thuringiensis*.

Pore formation model and signal transduction model. Arrow number 1 shows general steps: crystal production during sporulation of *Bt*, solubilization and activation by midgut proteases. Arrows number 2 show the different steps in the pore formation model: Binding to cadherin receptor, cleavage of helix α-1, oligomerization of the toxin, binding to aminopeptidase and membrane insertion to form a pore in lipid rafts that ends with cell death. Arrows number 3 show the steps involved in signal transduction model: binding to cadherin, activation of a protein G that increase activity of adenyl cyclase resulting in increased levels of camp that triggers activity of protein kinase A that induce cell death. Finally, arrows number 4 show the mechanism of the CryMod toxins that were deleted of helix α-1 and skip cadherin interaction, forming pores after binding to aminopeptidase receptor and killing the cells by pore formation.
2.27. Bioassay Methods for Quantification of *Bacillus thuringiensis* δ-Endotoxin

Biological assays (bioassays) are a set of techniques relevant to the comparisons between the strength of alternative but similar biological stimuli (a pesticide, a fungicide, a drug, a vitamin, plant extract, etc) based on the response produced by them on the subjects. Typically, a bioassay involves (i) stimulus, (ii) subject, and (iii) response, the change produced on the subject due to application of stimulus.

Normally, two preparations of the stimulus, one of known strength (standard preparation) and another of unknown strength (test preparation), both with quantitative doses, are applied to a set of living organisms. The general objective of the bioassays is to draw statistically valid conclusions on the relative potency of test preparation with respect to standard one. If $ds$ and $dt$ denote the doses of the standard and the test preparations respectively such that each of them produces a pre-assigned response in living organism, then the ration $\rho = ds/dt$ is called the relative potency of the test preparation. If $\rho$ is greater than unity, it shows that a smaller dose of the test preparation produces as much response as relatively larger dose of standard preparation. Similarly, $\rho$ is less than unity; the potency of the standard preparation is greater than that of the test preparation. Naturally, such statistical procedures may depend on the nature of the stimulus and response as well as on other extraneous experimental considerations.

The need for new products and pest control technologies will continue to accelerate due not only to demands for safer pest control technologies, but to the continuing increase in the world’s populations. The aim of using bioassay was to provide the worker with a rapid, standardized and simple procedure for estimating the activity of a microbial strain.

In the 1960s, initial efforts were made to quantify the spore-crystal mixtures by means of standardized bioassays (Bonnefoi *et al.*, 1958; Burgerjon, 1959; Meinl, 1960; Mechulas and Anderson, 1964). The introduction of commercial *B. thuringiensis* products by the agrochemical industry and the growing knowledge of the insecticidal toxin led to an urgent need to replace the spore count that had labelled the commercial products with a standardized value of insecticidal power. The idea of using a standard
microbe for potency determination of the bioinsecticide product was accepted at the 1966 Colloquium of Insect Pathology and Microbial Control at Wageningen, The Netherlands. The E-61 formulation from the Institute Pasteur, Paris, France, with an assigned potency of 1000 IU mg⁻¹, was first adopted as the standard (Burges, 1967), but was later replaced in 1971 by the HD-1-S-1971 with an assigned potency of 18,000 IU mg⁻¹. As from 1980 the HD-1-S-80 standard with a potency of 16,000 IU mg⁻¹ has been available from international *B. thuringiensis* cultures. The standardized bioassay procedures proposed by Dulmage *et al.* (1970) were reviewed (Dulmage *et al.*, 1981; Beegle, 1986) and further standardized for several insects species (Navon *et al.*, 1990) in parallel (Dulmage *et al.*, 1981) is useful for measuring differences between the tested microbe powder and the standard. Their potencies may be identical in one insect but different in another insect species. This ratio for any two insect species can be compared between laboratories, provided that the microbe powder is the same.

So far, however, the possibility of using new international standardization of *Bt* has not been accepted after discussing this issue in a recent Society for Invertebrate Pathology (SIP) meeting (Navon and Gelernter, 1996), mainly because: (i) insect strains and qualities differ between laboratories, and therefore mortalities are not comparable; and (ii) each of the *Bt* manufacturers has developed its own bioassay procedures for labelling the *Bt* products and changing the protocols will not be feasible for commercial considerations. Moreover, in the last two decades, additional bioassay protocols had to be designed in view of the following new developments and discoveries: (i) identification of new Cry proteins and their use in genetically engineered products (Baum *et al.*, 1999); (ii) development of new conventional formulations (Burges and Jones, 1999); (iii) an accumulating evidence of insect resistance to δ-endotoxin and Cry proteins (see also S. Sims in Subchapter 1B); and (iv) analytical assays for the δ-endotoxin, Cry proteins and insecticidal crystal protein (ICP) genes (Cannon, 1993; Plimmer, 1999), as complementary information on the activity of *Bt* proteins in the microbe isolates.
These developments and the new knowledge acquired challenged us to describe a comprehensive collection of bioassay protocols and procedures for quantifying activities of conventional \textit{B. thuringiensis} products. Bioassays of genetically engineered \textit{B. thuringiensis} plant products are also described in this.

\textbf{2.28. Types of Bioassay}

\textbf{2.28.1. Dose-response}

Dose-response is the most common type of bioassay among those used to determine biological effects of the bioinsecticide or the entomopathogen. In this bioassay, mortality is counted after a single period of time. For neonate larvae it is limited to 1-3 days. In the official bioassay with third instar larvae, the bioassay time is 7 or 8 days. The potency bioassay of \textit{B. thuringiensis} is a special case of dose-response bioassay where the mortality of the experimental microbe is referenced against the international standard and expressed in international units per mg (Novan, 2000).

\textbf{2.28.2. Time-response}

Time-mortality bioassay are suitable mostly for second and third instar larvae, since the mortality of neonate larvae occurs too quickly to obtain time mortality slope. In order to obtain good slopes with first instar larvae, it is advised to make counts of dead larvae every 6 or 8 hours. In comparison, the bioassay with older larvae can be prolonged to several days. This is explained in part by the fact that, unlike hatching larvae, third instars survive for several days without feeding owing to the fat body that is used as an energy reserve when the larva cannot feed. Also, larval recovery from microbe intoxication increases survival (Dulmage \textit{et al}., 1983). In time mortality bioassay with third insears, most larval death occurs in the first 4-8 days, during which time-mortality is counted on successive days or every other day.

\textbf{2.28.3. Feeding inhibition}

These bioassays are conducted with second and third instars larvae. Larvae are weighed on an analytical balance (0.1 mg accuracy). The mode of diet preservation should be considered carefully because of exposure to airborne contamination during
larval handling. The concentration that causes a 50% reduction in larval weight as compared with that of the control larvae (EC50) is the most common quantitative character is this type of bioassay. It is recommended as a criterion in comparing activities of *B. thuringiensis* preparations, since, for instance, weight reduction in young larvae caused by Cry1A(c) proteins is more sensitive parameter than the LC50 mortality in insect species susceptible to *B. thuringiensis subsp. kurstaki* (MacIntosh *et al.*, 1990).

2.28.4. Sublethal effect

Sublethal effects of *B. thuringiensis* are assessed in insects in two ways.

2.28.4.1. Continuous Exposure

When larvae are exposed continuously to low levels of the pathogen, the follow
up of the symptoms caused in the insect by the microbe preparation is extended to a full insect generation. Dulmage and Martinez (1973) demonstrated that sublethal doses of the microbe had marked effects on insect development. In bioassays for recording these effects, records of the following parameters are collected: larval period, percentage pupation, pupal weight, percentage adult emergence, adult fecundity and fertility.

2.28.4.2. Short-Term exposure

In these assays, feeding of the larvae on dietary *B. thuringiensis* is discontinued after one of the exposure times, 24, 48 or 72 h, and the larvae are transferred to diets without the microbe. The control larvae are fed on untreated diets from the start of the bioassay. The microbe concentration, the length of exposure and combinations thereof determine the rate of reduction in the insect quality, including moth reproductive capacity (Salama *et al.*, 1981). These bioassays reflect pest management situation in the field when: (i) the larvae feed for a short time on the microbe and then escape feeding by penetrating into inner tissues of the plant; (ii) the microbial product covers the plant canopy only partially, so that defoliators feed on plant parts devoid of *B. thuringiensis*; and (iii) the product’s activity at lethal concentration is lost rapidly under direct sunlight and phylloplane effects.
2.29. Types of preparations

Two types (i) Non-formulated and (ii) Formulated

2.29.1. Spore-crystal mixtures

Most commercial products are based on the spore-crystal complex. This mixture is the natural product obtained from the fermentation process. In products used in Japan, spores are inactivated to avoid detrimental effects to the silkworm, which is used commercially for the production of silk. The crystal of B. thuringiensis is responsible for the insecticidal effect in the majority of agricultural pests, but spore effects have been determined against several insects.

2.29.2. Spore

Spores are plated on nutrient agar to determine viability. Dilution series of spores for the bioassay are prepared as for potency assay but without the B. thuringiensis standard. The insecticidal activity of B.thuringiensis spores has been investigated less in recent years, primarily because genetic manipulations for pest control are based solely on the crystal toxins. However, spore bioassays are useful in developing microbial pest management strategies with B. thuringiensis products containing both spores and crystals.

2.29.3. Crystals

Official potency bioassays are limited to the spore-crystal mixtures and therefore cannot be used for purified crystals. However, mortality, feeding inhibition and sublethal effects can be determined with dietary bioassays as was described for the spore-crystal mixtures and the commercial B. thuringiensis products. Spore irradiation with a low dose of γ-rays (3.0 Mrad) and addition of streptomycin to the diet were used to avoid effects of spores remaining as impurities in the crystal preparations (Li et al., 1987). Adequate concentration of surfactant for homogeneous dispersion has to be added to the purified crystal mixture. This dispersion has to be confirmed by microscope observations. Nanogram amounts of the purified crystals per gram of diet are needed for the neonate bioassay.
2.29.4. Cry proteins

Dilution series are used in the diets as described for the potency bioassays, but without using the *B. thuringiensis* standard. Both mortality (LC$_{50}$) and feeding inhibition (EC$_{50}$) parameters are useful to describe the protein activities in the insect larvae (Macintosh *et al.*, 1990).

2.29.5. Formulated

2.29.5.1. Conventional - Liquids

Liquid concentrates and aqueous mixtures of wettable powders have to be well shaken to disperse the active ingredients homogeneously in the aqueous phase before their use in laboratory assays or in the tank mix in the field.

2.29.5.1.1. Granules.

The spore-crystal mixtures encapsulated in starch (Dunkle and Shasha, 1988) or embedded in wheat flour granules (Navon *et al.*, 1997) have to be released from the coating matrix so that the *B. thuringiensis* preparation will be dispersed homogeneously in the diet. In corn starch preparations, digestion with amylase has been recommended to release the *B. thuringiensis* materials (McGuire *et al.*, 1997). In wheat flour-embedded products, the microbe materials are recovered from the granules by blending the product in 0.5% aqueous Tween 80 solution for 30 s (Navon, unpublished). The spore-crystal mixture is then separated from the starchy or granule material by centrifugation. This is necessary in order to avoid undesired feeding effects of those materials which can be separated from the spores and crystals by centrifugation.

2.29.5.1.2. Genetically Manipulated

CellCap®. The δ-endotoxin is encapsulated in the dead walls of *Pseudomonas fluorescens* through transgenic manipulation. By this encapsulation the crystal toxins are protected from undesired environmental effects (Gelernter and Schwab, 1993). These transgenic commercial products are active against Lepidoptera by means of the Cry1A(c) protein, and the Colorado potato beetle (CPB) by means of the CryIIIa protein from *B.
*thuringiensis* subsp. *san diego* (Herrnstadt *et al.*, 1986). The plant and field bioassays of these products against CPB are described by Zehnder and Gelernter (1989).

Transconjugalional. These products are based on combinations of two delta endotoxin proteins that together expand the activity against insects that are not affected by either of the toxic proteins when expressed alone. This combination was produced by transconjugation of a plasmid bearing a gene of one *B. thuringiensis* subspecies into the cells of another subspecies of the microbe (Carlton *et al.*, 1990). In addition, the activity of each of the transferred proteins was increased by genetic manipulation. Using this strategy, commercial products have been developed that are active against both lepidopterans and coleopterans or with a widened host range against lepidopterous pests (combining *B. thuringiensis* subsp. *kurstaki* and subsp. *aizawai*).

Bioassays of the transconjugalional products are based on using two insect species in parallel, each susceptible to one of the two crystal proteins in the product. For example, *Helicoverpa armigera* larvae or other heliothine species can be used to assay the activity of the CryIA(c) toxin of *B. thuringiensis* subsp. *kurstaki*, whereas *S. littoralis* larvae or other *Spodoptera* species are used to determine the activity of CryIC toxin from *B. thuringiensis* subsp. *aizawai*. The transconjugant with both coleopteran and lepidopteran activity is used against the CPB and a *kurstaki*-sensitive lepidopterous species. Recombinant *Bt* products were highly active against a broad insect host range (Baum *et al.*, 1999).

### 2.30. Preparation of Spores and Crystals of *B. thuringiensis*

In some bioassays spores and crystals may be tested separately. For potency, parasporal crystals can be physically separated from spores, residual vegetative cells and cell debris by a variety of different methods (Goodman *et al.*, 1967; Delafeld *et al.*, 1968). They are based upon various principles such as differences in buoyancy, hydrophobicity or surface charge. The degree, by which such differences appear between parasporal crystals on the one hand, and spores, vegetative cells and cellular debris on the other hand are strain specific. Therefore, none of the many methods published over the
years give satisfactory results with all strains. The use of different methods may be necessary to isolate parasporal crystals from a variety of strains.

2.31. Bioassays of *B. thuringiensis* against Coleopteran insects

The *B. thuringiensis* subsp. *tenebrionis* and subsp. *san diego* (Herrnstadt *et al.*, 1986) are active against coleopteran insects belonging mostly to Chrysomelidae species, of which the CPB, *Leptinotarsa decemlineata*, is the most important target insect for these microbes. Lower susceptibilities were recorded in other beetle species (Keller and Langenbruch, 1993). LC₅₀ determinations in the CPB were made with leaf disc bioassays based on spore count of the microbial strain using a reference standard preparation (Keller and Langenbruch, 1993). Potency bioassays were developed for the CPB based on a standard *B. thuringiensis* subsp. *san diego* with an assigned potency of 50,000 CPB international units (Ferro and Gelernter, 1989). In these bioassays the full insecticidal power (i.e. the spore-crystal mixture) of this microbe was determined.

Sauka *et al.* (2010) reported the Induced-feeding bioassays for detection of *Bacillus thuringiensis* insecticidal activity against *Epilachna paenulata* (Coleoptera). Larvae were induced to swallow high concentrations of spore-crystal suspensions of seven exotic and 30 Argentine *B. thuringiensis* strains. The great majority of strains showed no toxicity to *E. paenulata* larvae, and observed mortality was lower than 30%. Induced-feeding bioassay is feasible, and should be used for prospecting strains that produce right combinations of Cry proteins needed to an efficient pest control. This study is the first report of an induced-feeding bioassay where the toxic activity of *B. thuringiensis* strains to *E. paenulata* is screened. Although the susceptibility of *E. paenulata* larvae to *B. thuringiensis* did not prove to be high, this method is feasible, and may be used for prospecting strains with the right combination of Cry proteins needed to control this or other pests.

Sezen *et al.* (2010) studied the highly pathogenic *Bacillus thuringiensis* subsp. *tenebrionis* from European shot-hole borer, *Xyleborus dispar* (Coleoptera: Scolytidae). Based on various morphological, physiological, biochemical, and molecular characteristics, the bacterial isolate was identified as *B. thuringiensis* subsp. *tenebrionis*.
(morrisoni) serovar H8a8b. This isolate was compared with the reference strains by scanning electron microscopy, SDS-PAGE analysis, cry gene content, and insecticidal activity. Isolate Xd3 forms a flat-square inclusion containing a protein component of c. 70 kDa. PCR analysis showed that the Xd3 has a cry gene, cry3. Toxicity tests were performed against coleopteran species. One hundred percent mortality was observed against larvae of Agelastica alni (Coleoptera: Chrysomelidae). The others were 90% for Amphimallon solstitiale (Coleoptera: Scarabaeidae), and Melolontha melolontha (Coleoptera: Scarabaeidae). Our results indicate that B. thuringiensis subsp. tenebrionis (Xd3) may be valuable as a biological control agent for coleopteran insects.

López-Pazos et al. (2009) reported the presence and significance of Bacillus thuringiensis Cry proteins associated with the Andean weevil Premnotypes vorax (Coleoptera: Curculionidae). The Andean weevil P. vorax represents an important cause of damage to Colombian potato crops. Due to the impact of this plague on the economy of the country, we searched for new alternatives for its biological control, based on the entomopathogenic bacteria Bacillus thuringiensis. A total of 300 B. thuringiensis strains obtained from potato plantations infested with P. vorax were analyzed through crystal morphology, SDS-PAGE, PCR and bioassays. We used site-directed mutagenesis to modify the Cry3Aa protein. Most of the B. thuringiensis isolates had bipyramidal crystal morphology. SDS-PAGE analyses had seven strains groups with δ-endotoxins from 35 to 135 kDa. The genes cry 2 and cry 1 were significantly more frequent in the P. vorax habitat (PCR analyses). Three mutant toxins, 1 (D354E), 2 (R345A, ΔY350, ΔY351), and 3 (Q482A, S484A, R485A), were analyzed to assess their activity against P. vorax larvae. Toxicity was low, or absent, against P. vorax for isolates, wild type cry 3Aa and cry 3Aa mutants. The genetic characterization of the collection provides opportunities for the selection of strains to be tested in bioassays against other insect pests of agricultural importance, and for designing Cry proteins with improved insecticidal toxicity.

Zhao et al. (2011) reported on increased B. thuringiensis δ-endotoxin Cry3Aa toxicity against long horned beetle by fusing to peptide specifically binding to beetle Cx-cellulase. The bioassay results showed that the mortality of larvae fed with the two fused Cry3Aa proteins (PCx-Cry3Aa and Cry3Aa-PCx) was up to three times higher than that
fed with Cry3Aa. Retaining time analysis was performed on excreta that collected at different times after feeding. The result showed that the fused Cry3Aa was concentrated in excreta collected at 6 h, whereas Cry3Aa at 4 h. Meanwhile, the Cry3Aa concentration in midgut juice after fed with fused Cry3Aa was higher than that with Cry3Aa alone. This indicated that the retaining time of fused PCx-Cry3Aa in midgut of larvae is longer compared to that of Cry3Aa alone. In addition, they also analyzed the cellulase activity when bond with fused Cry3Aa or Cry3Aa alone and showed that the fused protein did not affect the activity of cellulase. Therefore, the remaining time of fused Cry3Aa is prolonged after binding with cellulase, thus the enhanced toxicity of fused Cry3Aa is due to the prolonged retaining time.

Oppert and Morgan (2013) developed an improved high-throughput bioassay for Rhizopertha dominica (F.) (Coleoptera: Bostrichidae). R. dominica is an internal feeder during immature stages and presents unique challenges with traditional bioassay methods. The primary goal was to develop a fast method to evaluate larval development on small amounts of material incorporated homogenously in an artificial diet. Herein described a new method that incorporates an artificial diet composed of egg yolk, brewer’s yeast, and amylopectin for evaluating the effect of potential biopesticides on the development of R. dominica larvae. Evaluation is accomplished through visual inspection of digital X-ray images, or weighing containers of infested larvae on diet treatments. They demonstrate the method with aprotinin and Bacillus thuringiensis Cry3Aa protoxin, test materials that retard the development of R. dominica larvae. Different bioassay containers were evaluated, including a single larva assay with pipette tips or 8-strip tubes, or a group bioassay in black 16-well trays. In addition to improvements in time and manual manipulations, larvae can now be obtained for biochemical studies by gently washing away the diet. Discrimination of the effect of test materials by weighing the infested containers at specific time points was the most simplified approach to rapidly screen test compounds.