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Insect pathogens as biological control agents: Back to the future

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ABSTRACT

The development and use of entomopathogens as classical, conservation and augmentative biological control agents have included a number of successes and some setbacks in the past 15 years. In this forum paper we present current information on development, use and future directions of insect-specific viruses, bacteria, fungi and nematodes as components of integrated pest management strategies for control of arthropod pests of crops, forests, urban habitats, and insects of medical and veterinary importance.

Insect pathogenic viruses are a fruitful source of microbial control agents (MCAs), particularly for the control of lepidopteran pests. Most research is focused on the baculoviruses, important pathogens of some globally important pests for which control has become difficult due to either pesticide resistance or pressure to reduce pesticide residues. Baculoviruses are accepted as safe, readily mass produced, highly pathogenic and easily formulated and applied control agents. New baculovirus products are appearing in many countries and gaining an increased market share. However, the absence of a practical *in vitro* mass production system, generally higher production costs, limited post application persistence, slow rate of kill and high host specificity currently contribute to restricted use in pest control. Overcoming these limitations are key research areas for which progress could open up use of insect viruses to much larger markets.

A small number of entomopathogenic bacteria have been commercially developed for control of insect pests. These include several *Bacillus thuringiensis* sub-species, *Lysinibacillus* (*Bacillus*) *sphaericus*, *Paenibacillus* spp. and *Serratia entomophila*. *B. thuringiensis* sub-species *kurstaki* is the most widely used for control of pest insects of crops and forests, and *B. thuringiensis* sub-species *israelensis* and *L. sphaericus* are the primary pathogens used for control of medically important pests including dipteran vectors. These pathogens combine the advantages of chemical pesticides and MCAs: they are fast acting, easy to produce at a relatively low cost, easy to formulate, have a long shelf life and allow delivery using conventional application equipment and systemics (i.e. in transgenic plants). Unlike broad spectrum chemical pesticides, *B. thuringiensis* toxins are selective and negative environmental impact is very limited. Of the several commercially produced MCAs, *B. thuringiensis* (*Bt*) has more than 50% of market share. Extensive research, particularly on the molecular mode of action of *Bt* toxins, has been conducted over the past two decades. The *Bt* genes used in insect-resistant transgenic crops belong to the Cry and vegetative insecticidal protein families of toxins. *Bt* has been highly efficacious in pest management of corn and cotton, drastically reducing the amount of broad spectrum chemical insecticides used while being safe for consumers and non-target organisms. Despite successes, the adoption of *Bt* crops has not been without controversy. Although there is a lack of scientific evidence regarding their detrimental effects, this controversy has created the widespread perception in some quarters that *Bt* crops are dangerous for the environment. In addition to discovery of more efficacious isolates and toxins, an increase in the use of *Bt* products and transgenes will rely on innovations in formulation, better delivery systems and ultimately, wider public acceptance of transgenic plants expressing insect-specific *Bt* toxins.

Fungi are ubiquitous natural entomopathogens that often cause epizootics in host insects and possess many desirable traits that favor their development as MCAs. Presently, commercialized microbial pesticides based on entomopathogenic fungi largely occupy niche markets. A variety of molecular tools and

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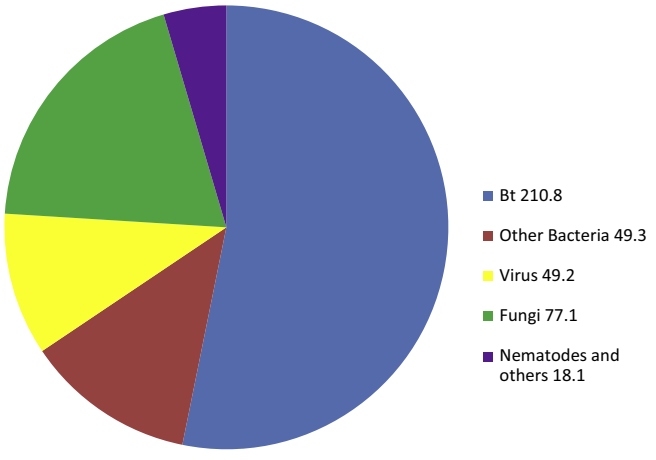
technologies have recently allowed reclassification of numerous species based on phylogeny, as well as matching anamorphs (asexual forms) and teleomorphs (sexual forms) of several entomopathogenic taxa in the Phylum Ascomycota. Although these fungi have been traditionally regarded exclusively as pathogens of arthropods, recent studies have demonstrated that they occupy a great diversity of ecological niches. Entomopathogenic fungi are now known to be plant endophytes, plant disease antagonists, rhizosphere colonizers, and plant growth promoters. These newly understood attributes provide possibilities to use fungi in multiple roles. In addition to arthropod pest control, some fungal species could simultaneously suppress plant pathogens and plant parasitic nematodes as well as promote plant growth. A greater understanding of fungal ecology is needed to define their roles in nature and evaluate their limitations in biological control. More efficient mass production, formulation and delivery systems must be devised to supply an ever increasing market. More testing under field conditions is required to identify effects of biotic and abiotic factors on efficacy and persistence. Lastly, greater attention must be paid to their use within integrated pest management programs; in particular, strategies that incorporate fungi in combination with arthropod predators and parasitoids need to be defined to ensure compatibility and maximize efficacy.

Entomopathogenic nematodes (EPNs) in the genera *Steinernema* and *Heterorhabditis* are potent MCAs. Substantial progress in research and application of EPNs has been made in the past decade. The number of target pests shown to be susceptible to EPNs has continued to increase. Advancements in this regard primarily have been made in soil habitats where EPNs are shielded from environmental extremes, but progress has also been made in use of nematodes in above-ground habitats owing to the development of improved protective formulations. Progress has also resulted from advancements in nematode production technology using both *in vivo* and *in vitro* systems; novel application methods such as distribution of infected host cadavers; and nematode strain improvement via enhancement and stabilization of beneficial traits. Innovative research has also yielded insights into the fundamentals of EPN biology including major advances in genomics, nematode-bacterial symbiont interactions, ecological relationships, and foraging behavior. Additional research is needed to leverage these basic findings toward direct improvements in microbial control.

1. Introduction

Since Lacey et al. (2001) addressed the possible future of microbial control of insects, the development of microbial pesticides and implementation of microbial control has included a number of successes and suffered some setbacks. Entomopathogens are utilized in all three categories of biological control, classical, conservation and augmentative, as defined by Hoy (2008a, 2008b) and McCrevy (2008). Some pathogens that are not commercially produced are currently used as classical biological control agents (Huger, 2005; Hajek, 2007; Hajek et al., 2007, 2008, 2009; Hajek and Delalibera, 2010; Bedding, 2008) or conserved as naturally occurring pathogens in agroecosystems (Hummel et al., 2002; Nielsen et al., 2007; Steinkraus, 2007b; Pell et al., 2010). Augmentative biological control, using inundatively applied microbial control agents (MCAs), is the most common strategy for employing entomopathogens for control of pest arthropods. Over 50 entomopathogenic viruses, bacteria, fungi, and nematodes are now commercially produced and used augmentatively as microbial pesticides (Fig. 1) (Jackson, 2003; Goettel et al., 2005; Grewal et al., 2005a, 2005b; Ekesi and Maniania, 2007; Faria and Wraight, 2007; Kaya and Lacey, 2007; Alves and Lopes, 2008; Copping, 2009; Ravensberg, 2011; Glare et al., 2012; Shapiro-Ilan et al., 2012b; Morales-Ramos et al., 2014). On a global scale, microbial pesticides only account for approximately 1–2% of all pesticides sold (Thakore, 2006; Marrone, 2007; Bailey et al., 2010); however, they have shown long term growth over the past decade in contrast to chemical pesticides, which have consistently declined in the global market (Thakore, 2006; Bailey et al., 2010). Some sources have recently estimated that the growth in microbial pesticides could reach 3% of the pesticide market in 2014 (Glare et al., 2012). A potent driving force for this expansion is the impact of European legislation to restrict residue levels of most synthetic chemical pesticides, and also a forthcoming directive (EC 91/414) to ban many other pesticides including those deemed to be human endocrine disrupters (Ansell, 2008; Bielza et al., 2008; Marx-Stoelting et al., 2011). These regulations are increasingly requiring farmers growing horticultural produce for sale in the European Union (EU) to drastically reduce use of conventional broad spectrum chemical pesticides. Expansion in biopesticide markets in Europe also reflects the effort of biocontrol scientists to rationalize and simplify the EU microbial pesticide registration procedures as part of the Regulation of Biological Control Agents (REBECA) project, and create a more favorable regulatory system that supports efforts of companies to commercialize MCAs (Ehlers, 2007). The global adoption of harmonized and simpler registration protocols would be a valuable step to promote wider MCA commercial availability (Ehlers, 2007; Cherry and Gwynn, 2007; Bailey et al., 2010;



CPL Business Consultants (2010) The 2010 Worldwide Biopesticides Market Summary, (Vol. 1), CAB International Centre. Wallingford.

Fig. 1. Estimated world biopesticide sales by type in 2010 (millions of $US). CPL Business Consultants (2010). The 2010 Worldwide Biopesticides Market Summary, vol. 1. CAB International Centre, Wallingford.

Kabaluk et al., 2010; Meeussen, 2012; Thornström, 2012). The impact of the growing organic sector in horticulture has also played a role in increasing market opportunities for biopesticides (Rohner-Thielen, 2005). Of the several commercially produced MCAs, *Bacillus thuringiensis* has the majority of market share (Glare et al., 2012) (Fig. 1).

Entomopathogens are ready made for use in integrated pest management programs and sustainable agriculture (Berger et al., 2007; Pell, 2007; Alves et al., 2008; Lacey and Shapiro-Ilan, 2008; Birch et al., 2011; Glare et al., 2012). They are safe for applicators, the food supply and environment (Lacey and Siegel, 2000; O.E.C.D., 2002; Akhurst and Smith, 2002; Hokkanen and Hajek, 2003; Lacey and Merritt, 2003; Hajek and Goettel, 2007; O'Callaghan and Brownbridge, 2009; Mudgal et al., 2013), and their specificity minimizes impacts on beneficial and other non-target organisms. This in turn promotes biodiversity and natural control of pest arthropods by parasites and predators. In the following sections we present information on the current status of entomopathogens as MCAs and prospects for their use in the near and distant future. Some of the key questions that we propose to address are: What are the major advances in microbial control that have been made since 2001? How do we expect biological control to change in the next decade or and in the more distant future? What are the major research or implementation barriers that must be overcome to significantly expand the use of MCAs? What are the societal factors that may hinder or promote their use in the near and distant future?

2. Entomopathogenic viruses

2.1. Major advances since 2001

The role of entomopathogenic viruses in global crop protection has grown in the last decade, although steadily and evolutionarily rather than through any major technical advance. Most new virus products are based on species that have been known and studied for at least two decades and represent commercialization based on extant knowledge rather than recent research efforts. Insect viruses appear to be moving out of narrow "niche" biological control products into the mainstream of commercial farming, reflected in the increased availability of commercial viral pesticides over the last few years. Among the different groups of entomopathogenic viruses (Miller and Ball, 1998; Eberle et al., 2012), most product development and research continues to be focused on the Baculoviridae (BV) (Miller, 1997; Moscardi et al., 2011). Of the four genera of baculoviruses, Alpha-, Beta-, Gamma-, and Deltabaculoviruses (Jehle et al., 2006; Eberle et al., 2012; Herniou et al., 2012), only the lepidopteran-specific nucleopolyhedroviruses (NPV; *Alphabaculovirus* spp.) and granuloviruses (GV; *Betabaculovirus* spp.) have been commercially developed to any significant extent (Table 1).

Research on developing non-BV viruses for crop protection has continued but only to a limited extent. Studies include fieldwork on the use of tetraviruses for control of heliothines in Australia (Christian et al., 2005) and *Cypovirus* spp. (Reoviridae) (Belloncik and Mori, 1998) for control of oil palm pests in South America (Zeddam et al., 2003), though none appear to be close to commercialization. The use of *Oryctes* virus (Nudiviridae) for control of rhinoceros beetle on oil palm in Asia is an ongoing program (Ramle et al., 2005) that has evolved to include the use of a pheromone to collect adults that are then infected and used to disseminate the virus (Jackson et al., 2005). This is an interesting application of the "lure and infect" approach, although as yet there are no definitive published data on the success of this research and efficacy in the field.

The dearth of research efforts on these non-BV groups is a significant barrier to further development as crop protection agents, which is surprising in some ways given the importance of some of the potential target pests. Without necessary progress in the fundamental knowledge of viral taxonomy, pathology, ecology and the development of commercially viable mass production systems, non-BV viruses are unlikely to be attractive targets for commercialization by industry in the next decade.

The focus on BV for commercialization can be ascribed to several favorable factors. There is more basic knowledge about BV biology, pathology and ecology than for any other group of invertebrate viruses, and the wealth of data greatly facilitates product development and registration. In addition, there are many scientists with the necessary knowledge to support commercialization initiatives, and established centers of BV research are more geographically widespread, enabling collaborations between academics and local microbial pesticide companies. High levels of *in vivo* replication of most BV that are of commercial interest is also a key factor in making commercial production potentially economically feasible.

The infective stage of BVs is characterized by circular double stranded DNA within rod shaped nucleocapsids that are encased within occlusion bodies (OB) formed of crystalline protein. The details of BV life history, biology and ecology are covered in detail elsewhere and are not discussed here (see Miller, 1997; Fuxa, 2004; Cory and Myers, 2003; Cory and Evans, 2007; Moscardi et al., 2011; Harrison and Hoover, 2012). The robust nature of the OB is a factor facilitating commercial baculovirus product development as it is readily amenable to formulation, application and long-term storage than non-occluded insect viruses. OBs can be visualized using phase contrast light microscopy, facilitating quantification of BV without the need for electron microscopy, which requires expensive equipment that often is not readily available to microbial pesticide companies. In the last decade, there has been a significant expansion in range of commercial BV products (Kabaluk et al., 2010; Gywnn, 2014), notably in the range of BV insecticides available in Europe and North America. Elsewhere the picture is mixed with significant expansion in the production and use of BV microbial pesticides in parts of Asia, Australasia and South America, but as yet little expansion of use in Africa (Cherry and Gwynn, 2007; Kabaluk et al., 2010; Moscardi et al., 2011).

The focus on BV is in large part due to the importance of these pathogens in controlling some globally important lepidopteran pest species such as *Helicoverpa* spp. (Rowley et al., 2011) and *Spodoptera* spp. (Table 1). These pest species have a marked propensity to rapidly develop resistance to conventional chemical insecticides, making their control challenging. These species also are pests on a wide range of crops, providing potential market niches for BV in field crops and in protected crops grown in polytunnels and glasshouses (Grzywacz et al., 2005; Arrizubieta et al., 2014). In China, NPV supply has expanded with nine BV products now commercially available. There are at least 12 Chinese manufacturers of *Helicoverpa armigera* NPV (HearNPV) and several of *Spodoptera litura* NPV (SpltNPV), *Autographa californica* NPV (AucaMNPV), *Plutella xylostella* GV (PlxyGV) and *Spodoptera exigua* NPV (SeMNPV) as well as a number of other BV products (Sun and Peng, 2007; Yang et al., 2012). It is difficult, however, to determine the total use of BV in China. One source estimated that in 2007 around 250 tonnes of formulated material was produced, 80% of which was HearNPV, used on up to 100,000 ha (Sun and Peng, 2007). A more recently published estimate stated that up to 2000 tonnes of formulated BV products may be produced annually, from which it may be inferred that areas treated have expanded significantly from the earlier estimate, and may have reached up to 1 million ha (Yang et al., 2012). In India, many new suppliers of HearNPV and SpltNPV

Table 1
Entomopathogenic viruses that have been used for biological control of insect pests.

Common and species names	Targeted insects	Producer	Selected references
Baculovirus	Principally Lepidoptera, some Hymenoptera and Diptera		Miller (1997), Moscardi (1999, 2007), Theilmann et al. (2005), Szewcyk et al. (2006), Harrison and Hoover (2012)
Corn earworm NPV (HezeSNPV)	*Helicoverpa zea:* corn earworm, tomato fruitworm, tobacco budworm. *Heliothis virescens*	Certis (USA)	Ignoffo (1999), Rowley et al. (2011)
Cotton bollworm NPV (HearNPV)	*Helicoverpa armigera*, cotton bollworm, podborer	Andermatt, (Switzerland), AgBioTech (Australia), Jiyuan Baiyun Industry Company Ltd. (China), BioControl Research Labs (India), Kenya Biologics (Kenya), plus other producers in India, China	Hauxwell et al. (2010), Rabindra and Grzywacz (2010), Rowley et al. (2011), Yang et al. (2012), Gywnn (2014)
Diamond back moth GV (PlxyGV)	*Plutella xylostella*	Jiyuan Baiyun Industry Company Ltd. (China)	Grzywacz et al. (2004), Farrar et al. (2007), Yang et al. (2012)
Unbarred Spodoptera moth (army worm NPV (SdalNPV)	*Spodoptera albula* (*sunia*)	Agricola el Sol (Guatamala)	Moscardi (1999)
Beet armyworm NPV (SpexMNPV)	*Spodoptera exigua*	Andermatt, (Switzerland), Certis (USA), Jiyuan Baiyun Industry Company Ltd.,(China) BioTech (Thailand)	Kolodny-Hirsch et al. (1997), Lasa et al. (2007), Sun and Peng (2007), Gywnn (2014)
Egyptian cotton leafworm NPV (SpliNPV)	*Spodoptera littoralis*	Andermatt (Switzerland)	Jones et al. (1994)
Tobacco armyworm NPV (SpltNPV)	*Spodoptera litura*	Biocontrol Research Lab, Ajay Biotech, Bassarass Biocontrol, Biotech International, BioControl Research Labs (India) Jiyuan Baiyun Industry Company Ltd. (China)	Nakai and Cuc (2005), Department of Biotechnology India (2007), Kunimi (2007), Yang et al. (2012)
Gypsy moth, NPV (LydiMNPV)	*Lymantria dispar*	USDA (USA), Sylvar Technology (Canada), Andermatt (Switzerland)	Podgewaite (1999)
Velvetbean caterpillar, NPV (AngeMNPV)	*Anticarsia gemmatalis*	Coodetec. CNP So, Nova Era Biotechnologica Agricola, Nitral Urbana Laboratorios, Coop Central Milenio Agro Ciencias (Brazil)	Moscardi (2007), Sosa-Gómez et al. (2008), Moscardi et al. (2011), Panazzi (2013)
Red headed pine sawfly NPV (NeleNPV)[1]	*Neodiprion lecontei*	Sylvar Technology (Canada)	Cunningham (1995)
Douglas fir tussock moth NPV (OrpsNPV)	*Orygia pseudotsugata*	Canadian Forest Service	Martignoni (1999)
Balsam fir sawfly NPV (NeabNPV)	*Neodiprion abietis*	Sylvar Technology (Canada)	Lucarotti et al. (2007), Moreau and Lucarotti (2007)
Codling moth GV (CpGV)	*Cydia pomonella*	Certis (USA), BioTepp (Canada), Arysta Lifescience (France), Andermatt (Switzerland), Hoerst (Germany), BioBest (Belgium), Arysta Life Science (France), Agro Roca (Argentine)	Tanada (1964), Cross et al. (1999), Arthurs et al. (2005), Eberle and Jehle (2006), Lacey et al. (2008b)
False codling moth GV (CrleGV)	*Cryptophlebia leucotreta*	Andermatt (Switzerland), River Bioscience (South Africa)	Singh et al. (2003), Moore et al. (2004b)
Potato tubermoth GV (PhopGV)	*Phthorimaea operculella*	Centro Internacional de la Papa (Peru), Proinpa (Bolivia)	Sporleder (2003), Arthurs et al. (2008b), Kroschel and Lacey (2008), Lacey and Kroschel (2009)
Summer fruit totrix GV (AdorGV)	*Adoxophyes orana*	Andermatt (Switzerland)	Blommers (1994), Cross et al. (2005), Nakai (2009)
Tea tortrix (HomaGV)	*Homona magnanima*	Arysta life science (Japan)	Kunimi (2007), Nakai (2009)
Smaller tea tortrix GV (AdhoGV)	*Adoxophyes honmai*	Arysta life science (Japan)	Nakai et al. (2002), Nakai (2009)
Alfalfa looper NPV (AucaMNPV)	Noctuidae	Agricola el Sol (Guatamala)	Vail et al. (1999), Yang et al. (2012)
Cabbage looper (TrniSNPV)[1]	*Trichoplusia ni*	Andermatt (Switzerland)	Vail et al. (1999)
Tea geomotrid EcobNPV	*Extropic obliqua*	Small scale commercial production China[a]	Sun and Peng (2007), Yang et al. (2012)
Tea tussock moth (Eups NPV)	*Euproctis pseudoconspersa*	Small scale commercial production China[a]	Sun and Peng (2007), Yang et al. (2012)
Tea moth (BuzuNPV)	*Buzura suppressaria*	Small scale commercial production China[a]	Sun and Peng (2007), Yang et al. (2012)
Teak defoliator (HypeNPV)	*Hyblea peura*	Kerala Forest Research Institute (India)	Nair et al. (1996)
Imported cabbageworm (PiraGV)	*Artogeia (Pieris) rapae*	Registered in China Small scale commercial production China[a]	Yang et al. (2012)
Oriental armyworm, (LeseNPV)	*Leucania (Mythimna) separata*	Registered in China Small scale commercial production China[a]	Yang et al. (2012)
Reoviridae			
Masson pine moth cypovirus (CPV)	*Dendrolimus punctatus*	Registered in China Small scale commercial production China[a]	Peng et al. (2000), Yang (2007), Yang et al. (2012)
Parvoviridae			
Cockroach densonucleosis virus (DNV)	*Periplaneta fuliginosa*	Registered in China Small scale commercial production China[a]	Bergoin and Tijssen (1998), Yang et al. (2012)
Nudiviruses			
Oryctes virus	*Oryctes rhinoceros*	Not commercially produced but locally produced for autodissemination	Jackson et al. (2005), Huger (2005), Ramle et al. (2005), Jackson (2009)

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have appeared in recent years following the adoption of simplified microbial pesticide registration and in response to the growing problem of synthetic insecticide resistance (Department of Biotechnology India, 2007; Rabindra and Grzywacz, 2010). The total production of BV in India was estimated to be in excess of 50 tonnes in 2004 (Singhal, 2004) with both public and private sector organizations active in manufacturing. Quality control issues remain a concern in India and parts of Southeast Asia (Jenkins and Gryzwacz, 2000; Kambrekar et al., 2007; Grzywacz et al., 2014a). It remains to be seen if truly large-scale market

penetration can be achieved in these regions with the existing generation of products. Australian growers have incorporated BV for management of *H. armigera* in field crops, and importation of *Helicoverpa zea* NPV (HezeSNPV) for *H. armigera* control is now supplemented by local sources of a HearSNPV isolate (Buerger et al., 2007; Hauxwell et al., 2010). A major breakthrough in adoption of BV by producers was bringing together new midge resistant sorghum hybrids with HearSNPV to produce an IPM system that controlled the two major crop pests alongside local production of the BV (Franzmann et al., 2008). HearNPV, SpltNPV and SeMNPV are registered in Thailand and Vietnam, though supply currently appears to depend on imports and public sector suppliers rather than local commercial sources (Nakai and Cuc, 2005; Ratanasatien et al., 2005; Skovmand, 2007). In South America, Brazil leads BV development with a well-established program for production and use of *Anticarsia gemmatalis* NPV (AngeMNPV) for control of velvet bean caterpillar on soy (Moscardi, 1999, 2007; Sosa-Gómez et al., 2008). More recently, production and use of AngeMNPV has begun in Mexico (Williams et al., 2013a). The production of AngeMNPV was initiated in Brazil as a public sector project but commercial producers subsequently were brought in to scale up production. Mass-reared insect production was later introduced in Brazil to supplement the original field-based production system when the treatment areas rose to 2 million ha in 2004 (Moscardi, 2007). However, since the use of no-tillage systems involving the routine prophylactic use of broad spectrum insecticides in place of BV applications have been widely adopted, AngeMNPV is now used on less than 300,000 ha (Moscardi et al., 2011; Panazzi, 2013). The rapid shift in the fortunes of what was a very successful microbial pesticide is an illustration of the dynamic nature of modern commercial agriculture and how continued user acceptance of successful microbial pesticides cannot be taken for granted. Despite the decrease in use, this program remains a model for public sector development of a BV that successfully spawned large-scale commercial use. Development of *Spodoptera frugiperda* NPV (SpfrMNPV) for controlling *S. frugiperda* in maize, *Condylorrhiza vestigialis* NPV (CoveNPV) for pest control on poplar trees (*Populus* spp.) and *Erinnyis ello* GV for cassava pest control (Bellotti et al., 1999; Moscardi et al., 2011) is also underway by research institutes in Brazil, while commercial production of SpfrMNPV and *Autographa californica* MNPV (AcMNPV or AucaMNPV) is also reported in Guatemala, although the scale of use is not clear (Sosa-Gómez et al., 2008). Efforts continue to extend the use of the successful potato tuber moth *Phthorimaea operculella* GV (PhopGV), currently produced in Bolivia by the public or non-government organization (NGO) sector (Sporleder, 2003; Kroschel and Lacey, 2008; Sporleder and Kroschel, 2008; Lacey and Kroschel, 2009) for both field crop (Wraight et al., 2007b; Arthurs et al., 2008c; Sporleder and Kroschel, 2008; Sporleder and Lacey, 2013) and stored product use in North and South America (Arthurs et al., 2008b; Sporleder and Kroschel, 2008; Lacey et al., 2010a; Sporleder and Lacey, 2013). Studies have also focused on the formulation of PhopGV (Sporleder, 2003; Arthurs et al., 2008b) and its propagation *in vivo* (Sporleder et al., 2008) for control of the pest host. In some areas of South America, a new potato pest, *Tecia solanovora*, has supplanted *P. operculella* as the main potato pest, threatening the efficacy of PhopGV in potato stores. The identification of a new strain of PhopGV showing activity against both pests is particularly promising; without such dual activity, farmer use is likely to decline precipitously as *T. solanovora* spreads (Gómez-Bonilla et al., 2011).

One of the most widely used commercially developed viruses is the codling moth, *Cydia pomonella* granulovirus (CpGV). Although CpGV was developed and commercialized for use in Europe in 1987 (Cross et al., 1999; Vincent et al., 2007), it was registered in North America more recently (Vincent et al., 2007; Lacey et al., 2008b) and is now used worldwide (Lacey et al., 2008b; Sosa-Gómez et al., 2008). A comprehensive review of the CpGV literature by Lacey et al. (2008b) concluded that CpGV provides good codling moth population control. Other reasons for its widespread adoption are that no spray interval is required throughout the growing season and before harvest, and it is safe for applicators, the food supply and non-target organisms. Although it is widely used in Europe and in North America, adoption by conventional growers is still limited compared to organic growers. The principal caveat for its use is the relatively low persistence of the virus due to solar degradation, necessitating frequent re-application when codling moth pressure is high. Indeed, given the issue of its low persistence in the field, its relatively successful use by the apple industry is an interesting illustration that even products with less than optimal performance can succeed under the right circumstances. It may well be that if application can be timed to coincide with peak fruit entry by first instar coddling moth larvae and the BV can rapidly infect a high proportion of larvae before significant damage occurs, adequate control can be achieved even in a context where the BV has low persistence (Cherry et al., 2000; Grzywacz et al., 2008). Another factor in CpGV's favor is high virulence and the ease and speed with which it infects (Ballard et al., 2000a). Pest ecology may be another element; in many apple growing systems there are only one or two pest generations per year and growers can target the early larval stages with a high degree of confidence, ensuring that even a short lived virus can achieve acceptable control (Lacey and Shapiro-Ilan, 2008). It must also be noted that CpGV is not a stand-alone product in apple production but a component in a well-developed "soft" IPM system (Lacey et al., 2008b). BVs like other biological control agents (BCA) may perform best as part of a comprehensive IPM system rather than as chemical substitutes (Lacey and Shapiro-Ilan, 2008). The success of soft IPM in apples also may be related to the long duration of tree crop systems that facilitate the successful establishment of natural enemy complexes, a situation less common in annual crops. Another issue may be that the relatively high profile and consumer demand for "organic apples" provides an additional market incentive to enable biological insecticides such as CpGV to capture a significant market niche.

Expansion of BV use is not without potential problems. Following widespread adoption of CpGV in parts of Europe, extremely high levels of resistance have been reported in certain locations where it has been used for 20 years or more (Fritsch et al., 2005; Eberle and Jehle, 2006; Sauphanor et al., 2006; Zichová et al., 2013). Laboratory studies reveal that rapid development of extreme resistance (100,000 resistance ratio) is possible due to sex-linked inheritance of a dominant resistance gene (Asser-Kaiser et al., 2007) and involves a specific mutation affecting an early block on virus replication (Asser-Kaiser et al., 2011). It has been shown that this resistance can be overcome by using BV products containing different CpGV isolates than the original Mexican strain used in all earlier CpGV products (Eberle et al., 2008), and a number of new products incorporating the new CpGV isolates have now been brought to market (Zichová et al., 2013; Andermatt Biocontrol, 2014). However, to ensure future sustainability, an integrated approach that alternates other soft interventions with CpGV products is recommended when the virus is used extensively within a region (Lacey et al., 2008b). An interesting contrast with *C. pomonella* resistance is use of AngeMNPV in Brazil. Despite the ease with which resistance to AngeMNPV can be selected for in laboratory populations of *A. gemmatalis* (Abot et al., 1996) and the extensive use of AngeMNPV over many years, no reports of field resistance to AngeMNPV have been confirmed (Moscardi, 2007). This contrast may indicate that widespread geographical use of a virus is less a factor in selecting resistance than

reliance on a single genetic strain. If so, producers of BV products should plan to incorporate either a wild type mixture of strains in a product or have alternate strains developed and available as part of a product resistance management strategy.

The appearance of a commercial GV product against false codling moth, *Cryptophlebia leucotreta*, in South Africa is an important step as the first commercially available BV produced in Sub-Saharan Africa (Singh et al., 2003; Moore et al., 2004a). Another BV that has been under active development in Africa is the NPV of *Spodoptera exempta* NPV (SpexNPV) for control of the African armyworm, a major migrant pest in Africa (Grzywacz et al., 2008). A pilot production plant was set up in Kenya by a private commercial producer (Van Beek, 2007) and a HearNPV product from this producer was registered in Kenya and Ghana in 2012; however, the scale of use is unclear. Diamond back moth, *Plutella xylostella*, is another global pest that has been a priority target for research of both *P. xylostella* GV (PlxyGV) and *P. xylostella* NPV (PlxyMNPV) (Kariuki and McIntosh, 1999; Grzywacz et al., 2004). A comparison of PlxyGV and PlxyMNPV showed that both had similar pathogenicity on the basis of OB counts but that PlxyGV infections produced many more OBs per unit of host weight (Farrar et al., 2007). Commercial PlxyGV products are available in China though the scale of use is uncertain (Yang et al., 2012).

Turfgrass pest control has also been a focus for pests such as *Agrotis ipsilon* using an NPV (AgipMNPV, Prater et al., 2006). Much of the work involves protecting golf course turf, but while AgipMNPV can give good control of early instars, its persistence is limited by frequent mowing. Additionally, exposure to UV reduces secondary cycling of the virus (Bixby-Brosi and Potter, 2010). BV isolates under development by the public sector (Table 1) have not yet attained product status.

Research on expanding use of other existing BV products continues, including the use of *Spodoptera exigua* NPV (SeMNPV) in glasshouses in southern Europe (Lasa et al., 2007). An interesting development is the commercialization in Japan of a joint formulation of *Adoxophyes orana* GV and *Homona magnanima* GV for controlling two tortrix pests of tea (Kunimi, 2007).

The use of BV in forest insect pest control in North America and Europe, a traditional focus of BV research (Cunningham, 1995; Martignoni, 1999; Podgewaite, 1999), has remained limited. The development of some forest pest BV, such as the gypsy moth NPV has continued (Cadogan et al., 2004; Moreau and Lucarotti, 2007) and commercial production of sawfly *Neodiprion abietis* is now also underway (Lucarotti et al., 2007). The lack of expansion of BV use in forest pest control may reflect the preferential adoption of *Bacillus thuringiensis*-based products, with their ready availability and wider host range (Moreau and Lucarotti, 2007; van Frankenhuyzen et al., 2007), rather than rejection of BV microbial pesticides. In Asia a number of forest pest BV are either in production or use in China, Japan and India; the scale of use remains unclear, although probably limited (Nair et al., 1996; Peng et al., 2000; Kunimi, 2007; Sun and Peng, 2007; Yang et al., 2012). Use of BV in stored products has also been a focus of research, particularly on *Plodia interpunctella* GV (PlinGV) (Vail et al., 1991, 1993). PlinGV has shown promise for control both through direct action and auto-dissemination but as yet has not been commercially developed.

2.2. Research issues that constrain expansion of the use of insect viruses

Mass production of BV at a cost most potential users can bear remains a significant issue. Production of commercial BV insecticides is still dependent on *in vivo* systems utilizing specially reared or wild collected insects (Reid et al., 2014; Grzywacz et al., 2014b). *In vivo* systems for production of BV in live larvae remain the normal production method for commercial companies and for public sector programs (Moscardi, 1999; Van Beek and Davies, 2009; Grzywacz et al., 2014a) but the relatively high cost of producing BV in living insects compared to their chemical insecticide counterparts remains a constraint as farm prices are difficult to reduce below $20 per ha and scaling up in vivo BV production with its demands for high quality disease-free insects is also a challenge (Reid et al., 2014). The use of automation and mechanization in inoculation, rearing, and harvesting has facilitated mass production and made BV a viable commercial option for the current range and usage scale. However, this manufacturing approach remains unattractive to many companies in North America and Europe that are unfamiliar with mass insect culture as a mainstream production technique, and while, the *in vivo* production approach remains capable of meeting the current market needs, the ability to produce the amounts of BV needed for large scale field crop protection is far from certain. It remains to be seen if the recent sharp decline in the use of AgMNPV in Brazil after a major investment in laboratory-based mass production facilities (Moscardi et al., 2011; Panazzi, 2013) will have a significant impact on the willingness to fund a major expansion of *in vivo* BV production.

While most viral pesticides are produced in specialized facilities, field production *in vivo* has been a viable approach for a few commercial BV products such as AgMNPV in developing countries (Hunter-Fujita et al., 1998; Moscardi, 2007; Alves and Lopes, 2008). Field production is planned for SpexNPV in Africa (Grzywacz et al., 2014b), although large scale commercial viable mass production has yet to be successfully established for any BV other than AgMNPV.

Facing future needs for large-scale mass production of BV, in vitro cell culture remains a major approach to overcoming supply and cost constraints that limit BV use (Black et al., 1997; Moscardi et al., 2011). Mass production of hosts to produce viruses has been under development for 30 years but has not yet been successfully scaled up to the levels required to meet commercial acceptability (Granados et al., 2007). While many cell lines capable of supporting BV replication exist, the cells are relatively fragile compared to the bacterial and yeast cells normally used in large scale cell culture systems. Meeting commercial needs for BV production would require bioreactors of >10,000 l that are capable of continuous high efficiency production. (Black et al., 1997; Reid et al., 2014). Successful insect cell production has been reported in a number of different bioreactors but only at volumes of 20–600 l (Reid et al., 2014). Besides developing large-scale reactors suitable for insect cell lines, *in vitro* systems require low cost chemically defined media optimized for insect cell production to be cost effective and this is also not yet available. BV production quality also has been an issue; in particular, low cell yield and the maintenance of acceptable phenotypic qualities are constraints yet to be overcome (Pedrini et al., 2006; Nguyen et al., 2011). Thus, while research to develop cost effective *in vitro* systems continues (Granados et al., 2007; Szewcyk et al., 2006; Moscardi et al., 2011), there are as yet no indications that commercial production will begin in the near future, though technical and commercial "road maps" for such a ventures have been developed (Reid et al., 2014).

The slower killing speed of BV compared with most synthetic insecticides remains a significant barrier to their wider adoption (Copping and Menn, 2000; Szewcyk et al., 2006). Speed of action remains an important factor in selecting strains because faster acting strains would reduce crop damage and would be more attractive to users accustomed to the rapid kills obtained with many, though not all, chemical pesticides. A major focus of applied research to increase speed of action has been genetic modification (GM) of BV to insert or delete genes that quickly initiate cessation of feeding and accelerate death. The inserted genes include insect specific toxins from the scorpions *Androctonus australis*

and *Leiurus quinquestriatus*, the spider *Tengeneria agrestis*, the itch mite *Pyemotes tritici* and juvenile hormone esterases (Burdan et al., 2000; Bonning et al., 2002; Szewcyk et al., 2006). Despite promising field trial results, commercial development of these GM BVs appears to have stalled, perhaps because the recombinants produce poor yields in current *in vivo* systems or because the climate of public opinion and regulatory barriers are not sufficiently favorable to GM products in major potential markets such as the EU (Black et al., 1997; Glare et al., 2012).

The adoption of new natural mutant virus strains such as non-liquefying SfMNPV (Valicente et al., 2008) is another route for improving the cost effectiveness of BV that would not face such perceptual or registration barriers; however, the use of a natural faster-acting strain in practice may not be without drawbacks. A faster killing strain of *S. frugiperda* NPV (SpfrMNPV) was identified, but it was found to produce fewer OBs than the slower killing isolate, an evolutionary trade-off that is probably common and could reduce the impact of secondary cycling (Behle and Popham, 2012). Thus, despite extensive research in genetic modification to overcome some of the recognized BV constraints of restricted host range, slower action, and sensitivity to UV, no BV recombinant products with improved performance have been marketed nor do they seem likely to be in the near future. This is partly due to the technical failure to develop recombinants with the desired characteristics but may also reflect the rising costs of registering and deploying GM technology. In addition, recently published research on the genetic and genomic aspects of BV (with 43 genomes sequenced) has thrown an interesting light on BV relationships and evolution (Jehle et al., 2006; Eberle et al., 2009; Herniou et al., 2012).

It has been hoped that genomic data would assist the development of products with improved efficacy, host range, etc. (Inceoglu et al., 2006), but as yet there has been no commercial impact. While generally OBs are stable, they are sensitive to UV inactivation as well as phytochemical degradation on some plant species (Cory and Hoover, 2006; Cory and Evans, 2007: Behle and Birthisel, 2014). Specific phytochemical mechanisms that interfere with BV infectivity on crops have been identified in cotton (Hoover et al., 1998, 2000) and, more recently, in chickpea (Stevenson et al., 2010). The low persistence of BV on these and other crops is still perceived as a real limitation to the current generation of BV microbial pesticides (Copping and Menn, 2000; Moscardi et al., 2011; Behle and Birthisel, 2014). However, given the relative commercial success of CpGV, which has a short persistence time due mainly to solar inactivation, limited persistence may not be an insurmountable barrier to adoption provided products give a degree of control that meets the users core requirements.

BV can be applied using any commercial spraying system without special formulation (Gan-Mor and Matthews, 2003), although stickers, gustatory stimulants and UV protectants are often routinely incorporated into tank mixes to improve efficacy (Burges and Jones, 1998; Behle and Birthisel, 2014). Effective application rates for field use of NPV species that contain multiple virions vary between $0.5–5 \times 10^{12}$ OBs per ha (Moscardi, 1999), while for the GV with only one virion per occlusion body, rates can be higher (Moscardi, 1999). Research into new technology for applying BV seems to have advanced little in recent years, perhaps in recognition that farmers' decisions on the acquisition and use of sprayers is not likely to be driven to any significant extent by their specific ability to deliver microbial agents such as BV. There is now more interest in using precision application technologies for crop protection. In the next decade, use of minimal or precisely applied inocula in place of the traditional blanket spraying may be one of the most interesting avenues for exploiting BV more successfully and overcoming issues of cost and availability.

In addition to improving speed of kill, efficacy, host range, and persistence, applied research on formulation of BV remains one of the most important routes to BV product improvement (Burges and Jones, 1998; Behle and Birthisel, 2014). However, published research on this issue is very limited, probably due to proprietary issues, so it is unclear if limited publications reflect lack of significant progress. A number of improvements have been reported but it is not clear if advances are likely to appear in products in the near future. Most virus products are produced and sold as suspension concentrates, wettable powders and granules.

A minimum shelf life of 18 months was recommended over 30 years ago (Couch and Ignoffo, 1981) and some products are now available that meet this standard (Burges and Jones, 1998; Lacey et al., 2008b); these usually include adjuvants that stabilize the virus and improve suspension in water. Factors that affect shelf life of viruses (temperature and formulation components) have been reported for the NPV of the celery looper *Anagrapha falcifera* (AnfaMNPV) (Tamez-Guerra et al., 2000; Behle et al., 2003) and CpGV (Lacey et al., 2008a).

Some producers ship virus as frozen product and advise keeping the virus frozen until used, although this may not always be possible under operational conditions. Freezing is not essential to preserve BV, which can remain active in purified suspensions over long periods, even at room temperature. However, refrigeration or freezing does appear to be necessary to prevent the loss of activity related to the proliferation of contaminant bacteria and the oxidation of host derived lipids (Burges and Jones, 1998) and, thus, maintain the infectivity of mass produced suspensions (Lasa et al., 2008). The need for cold storage of BV is less of constraint in glasshouse and protected crops where use of biological control agents such as predators and parasitoids, requiring special storage or immediate use on receipt, has become increasingly common. It does, however, limit adoption in many field crops where biological control agents are less widely utilized.

The wider availability of formulations with ambient shelf life comparable to synthetic pesticides (>2 years) would be a substantial stimulus for expansion of BV use. Air-dried, spray-dried, and freeze-dried formulations have been widely studied with promising results for storage stability and activity (Alcázar et al., 1992; Tamez-Guerra et al., 2000, 2002; McGuire et al., 2001; Behle et al., 2003; Arthurs et al., 2008b). Spray drying of AnfaMNPV did not significantly reduce activity of lignin formulations over 6 months storage at 4 °C (Behle et al., 2003). Freeze dried formulations of the PhopGV were comparable in activity to emersion in an aqueous virus suspension (Arthurs et al., 2008b). Freeze dried and microencapsulated formulations of HearSNPV were also found to be as effective in the field as aqueous suspensions when applied on chickpea (Cherry et al., 2000). However, AnfaMNPV spray-dried formulations were reported to have higher residual activity compared with a commercial glycerin-based formulation (Behle et al., 2003). Differences in results may relate to specific crop–pest factors such as chemical inactivation reported on chickpea, so formulations may need to be tailored in some cases to the specific crop (Stevenson et al., 2010). Encapsulation of viral OBs in lignin via spray drying has been developed and tested with MNPV and GV and produced higher mortality and longer persistence than unformulated controls (Tamez-Guerra et al., 2000; McGuire et al., 2001; Behle et al., 2003; Arthurs et al., 2006, 2008a; Behle and Popham, 2012). Castillejos et al. (2002) reported considerably greater persistence with a granular phagostimulant formulation of the SfMNPV than with an aqueous suspension. In contrast, the commercially produced particle films and waxes, marketed as sunburn protectants for fruit are reported as providing no significant additional protection for CpGV (Lacey et al., 2004; Arthurs et al., 2006, 2008a).

A principal concern of growers is the need for frequent reapplication of BV due to rapid inactivation when exposed to sunlight (Behle and Birthisel, 2014). BV are especially sensitive to the ultraviolet spectrum (Ignoffo, 1992; Burges and Jones, 1998; Tamez-Guerra et al., 2000; Lacey and Arthurs, 2005), although specific host plant phytochemical factors can also contribute to low persistence on some crops and tree species (Cory and Hoover, 2006). The relative role of low UV persistence in constraining BV product use varies significantly due to a complex of biotic and abiotic crop specific factors such as UV levels, crop architecture, pest infestation patterns and cropping practices (Stevenson et al., 2010). In tropical crops exposed to high UV, persistence of BV can be less than 24 h; but persistence of other microbial pesticides such as Bt and even chemicals can also be short on these crops due to the combination of high UV and high temperature, which drives inactivation, chemical breakdown and volatization (Cherry et al., 2000).

One issue complicating the evaluation of research on UV persistence is the variability of experimental protocols used by different researchers. Some researchers evaluate natural sunlight exposure, which also has issues of variability, but many studies use various artificial UV sources that may not closely mimic natural sunlight spectra or leaf surface exposure. Exposure distances and duration vary and the choice of substrate can be a confounding issue. For example, direct heating effects may confound the effect of UV exposure when substrate temperatures are not restrained within environmentally valid bounds. Optical brighteners (Tinopal, Blankophor P167, and other stilbene derivatives), with and without titanium dioxide, have been shown to increase the persistence of NPV and GV (Farrar et al., 2003; Monobrullah and Nagata, 2001; Sporleder, 2003). However, Sajap et al. (2009) found that, although adjuvants such as Tinopal gave significantly improved UV protection in laboratory studies of SpltMNPV, in subsequent field trials on brassicas, no clear advantage was conferred over unformulated SpltNPV. A number of other materials that absorb specific wavelengths, including specialized dyes, chemicals and natural substances such as lignin sulfate, polystyrene latex, Congo Red, green tea, antioxidants, iron oxide and others have been tested to improve the residual activity of entomopathogenic viruses (Burges and Jones, 1998; Charmillot et al., 1998; Ballard et al., 2000b; de Morães Lessa and Medugno, 2001; McGuire et al., 2001; Sporleder, 2003; Asano, 2005; Arthurs et al., 2006; Shapiro et al., 2008). Molasses, sucrose and skimmed milk powder have also been reported to slightly improve persistence of CpGV (Charmillot et al., 1998; Ballard et al., 2000b). Alves et al. (2001) demonstrated greater persistence of NPV in an oil emulsion formulation than in a wettable powder for control of *A. gemmatalis*. UV protected petroleum spray oils were also found to be effective with HearSNPV (Mensah et al., 2005). In considering formulations that improve UV stability, it is not only performance that should be taken into account. Some experimentally demonstrated formulation additives have not been adopted for commercial use due to factors such as high cost, phytotoxicity, storage incompatibility, cosmetic unacceptability on fresh produce, or because application at the required concentrations, is impractical due to high viscosity or blocking of spray filters as occurs with some particulate additives.

It has been suggested that the success of HearNPV in Australia is related to very rapid acquisition, mitigating the problem of low BV persistence on crops (Murray et al., 2001), although the use of additives in tank mixes to improve efficacy of HearNPV is also an important factor in its success (Mensah et al., 2005; Hauxwell and Reeson, 2008). Increasing the attractiveness of spray deposits by adding attractants and feeding stimulants to tank mixes has shown promise in accelerating the acquisition of virus; for example, molasses is reported to be one of the most effective feeding stimulants for codling moth larvae (Ballard et al., 2000b). Other phagostimulants with potential for improving efficacy of CpGV include monosodium glutamate (Pszczolkowski et al., 2002) and trans-1-aminocyclobutane-1,3-dicarboxylic acid (trans-ACBD) (Pszczolkowski and Brown, 2004). However, use of high concentrations of additives such as molasses may have unacceptable side effects such as stimulating disfiguring fungal growth such as sooty mold on fresh produce. Schmidt et al. (2008) reported significant improvement of CpGV used in conjunction with the pear ester larval and adult attractant kairomone. However, Arthurs et al. (2007) reported inconsistent results in similar tests on apple and pear, and suggested that more practical improvements in formulation and application strategies were needed. Knight and Witzgall (2013) reported significant increases in larval mortality when combining any one of three yeasts, *Metschnikowia pulcherrima*, *Cryptococcus tephrensis* or *Aureobasidium pullulans*, with CpGV compared with CpGV alone. A field trial confirmed that fruit injury and larval survival were significantly reduced when apple trees were sprayed with CpGV, *M. pulcherrima* and sugar.

Wetting and sticking surfactants are generally recommended to improve mixing, reduce surface tension and increase deposition over plant surfaces (Burges and Jones, 1998). The use of additional stickers with entomopathogenic viruses was reported by Ballard et al. (2000a, 2000b), Tamez-Guerra et al. (2000) and Arthurs et al. (2008a). Optical brighteners have also been shown to enhance the infectivity of a number of NPV species, a response related to effects on the peritrophic membrane (Morales et al., 2001; Murillo et al., 2003; Martinez et al., 2004; Farrar et al., 2005; Toprak et al., 2007). Similarly, Cisneros et al. (2002) demonstrated a synergistic effect of 1% borax on activity of SfMNPV. Formulation research has not yet produced significant impacts on the overall performance of commercial BV products, but the availability of formulations with substantially improved persistence would improve product attractiveness for many crop systems.

The use of other additives to enhance the efficacy of BV infection has been widely explored. The enhancins are a group of viral proteins recognized to increase both NPV and GV viral potency in heterologous hosts and suggest significant potential to expand the host range of specific BV (Slavicek, 2012), although these have not yet been developed for commercial use. Azadaractin and other neem-derived chemicals also have been reported to effectively reduce the BV dosage needed to control pests in bioassays (Zamora-Avilés et al., 2013), and if validated in the field, could prove useful in lowering the cost of product.

The impact of expanded GM crop production on the use of BV remains to be determined. While the adoption of insect resistant GM crops can remove established markets for BV in some crops such as cotton (Buerger et al., 2007), it may also present opportunities for incorporating BV into GM cropping systems to cope with secondary non target pests, or as part of an insect resistance management strategy (Thakore, 2006; Kennedy, 2008). HzNPV significantly improved control of *H. zea* in GM sweet corn, although not as consistently as application of the insecticide spinosad (Farrar et al., 2009). Research on the use of insect virus genes in transgenic plants as a new source of insect resistance may, in the long term, provide the capability to utilize BV in crop protection (Liu et al., 2006).

While BV may be deployed using basic strategies of inoculation, conservation or augmentation, in current practice, BV is applied augmentatively as a microbial pesticide on an "as needed" basis. In the opinion of some researchers, however, pesticidal use is a barrier to realizing the full potential of biological agents and their ability to replicate, persist and spread (Waage, 1997). An alternative to conventional spray application is dissemination of BV formulations via novel lure and contaminate technologies incorporating pheromones (Vega et al., 2007). Adult insects

attracted to BV inoculum become surface contaminated and pass the virus to egg surfaces and subsequently to hatching larvae. This strategy has been recently applied to orchard pests (Cross et al., 2005); other examples are presented by Vega et al. (2007).

Despite the recognized importance of secondary cycling via horizontal and vertical transmission of BV in pest populations, there has been little deliberate exploitation of BV capacity to replicate and cycle in the way that specific inoculation strategies are used for *Oryctes* virus (Jackson et al., 2005) or cropping practices designed to promote BV conservation (Moscardi, 1999; Cory and Evans, 2007). Virus ecology remains a very active field of research for both crop and forest pests (Cory and Myers, 2003; Fuxa, 2004; Harrison and Hoover, 2012), expanding our knowledge of BV epidemiology and virus host population dynamics. Studies have included secondary cycling, horizontal and vertical transmission, and the interaction of BV with other pathogens such as *Wolbachia* (Graham et al., 2010) and offer interesting insights into how BV effectiveness might be enhanced in the field through biotic interactions. Although the research has not yet been exploited in terms of improving our use of BV on most crops, the ecology of host pathogen interactions is envisioned to be a way forward to developing new strategies for novel BV deployment (Waage, 1997).

2.3. Societal factors and their role in determining the adoption of insect viruses

Environmental pressures and consumer health concerns have been increasingly focused on the health and environmental impacts of crop protection products and the well-established safety of BV (O.E.C.D., 2002; Leuschner et al., 2010; Mudgal et al., 2013) is a major advantage. While public surveys have not shown that food safety risks are perceived as a major concern, they are a significant issue for up to 25% of consumers (Food Standards Agency, 2013). The recent controversy over neonicotinoids in the EU has shown that public concerns can drive significant changes in crop protection policy even if the scientific evidence is controversial (Gross, 2013). These concerns in the EU have led to the sustainable use directive (SUD), a policy of reducing reliance on chemical pesticides and mandatory adoption of integrated pest management (IPM) for all crops (Hillocks, 2012). In addition, chemical pesticides must be reregistered, which has led to a reduction in the number of chemical crop protection products allowed from approximately 1000 in 1993 to less than 330 today (European Commission, 2009). These measures are undoubtedly increasing the potential for use of BV; however, increased demand for new BCA elicit serious concerns that the supply of new products remains inadequate to replace the chemical pesticides being withdrawn (Hillocks, 2012).

One barrier to increasing the supply of commercial BV products is registration (Bailey et al., 2010; Ehlers, 2011; Lapointe et al., 2012). Regulatory authorities in many countries and jurisdictions are unable to complete registration of BV products in a timely, economic and transparent manner (Kabaluk et al., 2010; Gywnn, 2014). This may be due to bureaucratic inertia in some cases, but often the absence of the appropriate biological expertise among regulators has been cited as a significant constraint (Bailey et al., 2010). Some regulatory bodies such as the US EPA as appear to be proactive in developing the appropriate expertise and a positive ethos to facilitate the registration of new BV products through effective fast track systems (Bailey et al., 2010) but the EU, although sponsoring active reviews of microbial pesticide registration (Ehlers, 2011), has not yet implemented a specific fast track for microbial pesticides. EU registration has long timelines and higher costs that deter registrations, especially by the small-medium size enterprises (SMEs) that are frequently in the forefront of microbial pesticide innovation and develop 80% of novel microbial pesticide products (Ravensberg, 2011). The use of microbial pesticides has not yet generated serious public concern, although the issue has been mentioned by some authors such as Lapointe et al. (2012), attitudes may change as BV use expands.

2.4. Insect viruses in the next decade

There is a clear need for need for new BV products active against pests that may increase in impact as chemical actives are withdrawn. Most BV products recently commercialised or being brought to market are based on species that are well known and have been studied for at least 30 years. There is a serious concern about the dearth of novel BV species. Given the limited progress since 2001 in identifying new BV, it is far from clear that new crop protection products will emerge without increased funding for research and development of BV against new and emerging threats arising from chemical withdrawal. There is also a need for new technology to mass-produce BV at costs that appropriate for large-scale use of BV in field crops. Although *in vivo* production is an established technology, it is far from certain that it can be expanded to meet the quantum leap in production that is needed to replace chemical pesticides for major field crops. It remains to be seen if *in vitro* systems will overcome the scaling up cost and quality issues that have prevented these from be adopted by commercial producers. The other key need is to develop a better understanding of how BV interact with other BCA to identify synergys that can enhance their overall performance. Many believe that the BV, like other BCA, will never achieve their full potential until they are deployed as components of ecologically based IPM systems rather than substitutes for chemical insecticides.

3. Entomopathogenic bacteria

3.1. Bacillus thuringiensis (Bt)

3.1.1. Background and overall status

An enormous number of bacterial species have been reported from pest and beneficial insects (Jurat-Fuentes and Jackson, 2012) but a relatively small number of entomopathogenic bacteria have been commercially developed for control of insect pests of crops, forests, turf, humans, and livestock. These include several *Bacillus thuringiensis* (Bt) sub-species, *Lysinibacillus (Bacillus) sphaericus*, *Paenibacillus* spp. and *Serratia entomophila* (Table 2). The most widely used bacteria for control of numerous insect pests are Bt subspp. (Glare and O'Callaghan, 2000; Federici, 2005; Bravo et al., 2011; Glare et al., 2012; Jurat-Fuentes and Jackson, 2012).

Highlights of the history and commercial development of Bt are presented by Beegle and Yamamoto (1992), Federici (2005), Sanchis (2011) and Davidson (2012). Bt sub-species represent about 98% of formulated sprayable bacterial microbial pesticides, due in part to the wide host range with activity against Lepidoptera, Diptera (Nematocera), Coleoptera (Chrysomelidae and Scarabaeidae), additional species in other orders of insects and other pest invertebrates (mites and nematodes) (Carneiro et al., 1998; Schnepf et al., 1998; Wei et al., 2003; van Frankenhuyzen, 2009). Three notable examples are Bt strains with activity for scarab larvae (Bt subsp. *japonensis* (Buibui strain), Suzuki et al., 1992); two sawfly species *Diprion pini* and *Pristiphora abietina* (Porcar et al., 2008); and root knot nematodes, *Meloidogyne* spp. (Chen et al., 2000; Li et al., 2008; Khan et al., 2010).

Additional prospection and development will most likely provide *B. thuringiensis* isolates with an even broader spectrum of activity. Crickmore et al. (2014) provide a continually updated list of Bt toxins with links to information on additional host insects and

Table 2
Entomopathogenic bacteria used for control of insect pests of major crops, forest, turf, humans and domesticated animals.

Bacterial species	Major targeted habitat	Examples of major pest orders	Selected references
Bacillus thuringiensis sub-species *kurstaki*[a]	Row crops, forests, orchards	Lepidoptera: numerous families and species	Glare and O'Callaghan (2000), Federici (2005), Huang et al. (2007), Lacey et al. (2007), van Frankenhuyzen (2009), Jurat-Fuentes and Jackson (2012)
B. thuringiensis sub-species *aizawai*[a]	Row crops, orchards	Lepidoptera	Tabashnik et al. (1993), Glare and O'Callaghan (2000), Mashtoly et al. (2011)
B. thuringiensis sub-species *tenebrionis*[a]	Potato	Coleoptera: Chrysomelidae, predominantly *Leptinotarsa decemlineata*	Kreig et al. (1983), Langenbruch et al. (1985), Gelernter (2002)
B. thuringiensis sub-species *israelensis*[a]	Diverse lentic and lotic aquatic habitats	Diptera: Culicidae and Simuliidae	Lacey and Merritt (2003), Lacey (2007), Skovmand et al. (2007), Despres et al. (2011)
B. thuringiensis sub-species *japonensis* strain Buibui	Lawn and turf	Coleoptera: Scarabaeidae	Alm et al. (1997), Klein et al. (2007), Mashtoly et al. (2010)
Lysinibacillus sphaericus[a]	Lentic aquatic habitats	Diptera: Culicidae	Charles et al. (2000), Lacey (2007), Skovmand et al. (2007)
Paenibacillus popilliae	Lawn and turf	Coleoptera: Scarabaeidae: *Popillia japonica*	Klein et al. (2007), Koppenhöfer et al. (2012)
Serratia entomophila[a]	Pasture	Coleoptera: Scarabaeidae: *Costelytra zealandica*	Jackson et al. (1992, 2001), Jackson (2003), Jackson and Klein (2006)

[a] Commercially produced.

other organisms that are susceptible to them. There are currently no less than 73 families of crystal (CRY) toxins comprising a total of 732 toxins, 3 families of cytotoxic (Cyt) proteins including 38 different toxins and 125 Vegetative Insecticidal Proteins (VIPs) belonging to 4 different families (Crickmore et al., 2014).

The primary reason for the utilization of Bt is that it combines advantages of chemical pesticides and microbial pesticides. Like chemical pesticides, Bt is fast acting, easy to produce at low cost, easy to formulate, and has a long shelf life. It also can be applied using conventional application equipment and systemics (i.e. in transgenic plants). Unlike broad spectrum chemical pesticides, *B. thuringiensis* toxins are selective and negative environmental impact is very limited (Glare and O'Callaghan, 2000; Lacey and Siegel, 2000; Hokkanen and Hajek, 2003; Lacey and Merritt, 2003; Birch et al., 2011).

3.1.2. Control of pest insects with B. thuringiensis microbial pesticide products

3.1.2.1. Crops and orchards. Bt has no pre-harvest spray interval and can be applied until harvest begins. It has minimal or no impact on beneficial organisms in these agroecosystems; however, although efficacious, it is sensitive to solar degradation and requires frequent application.

B. thuringiensis subsp. *kurstaki* (Btk, Dipel) and to a lesser extent *B. thuringiensis* subsp. *aizawai* (Xentari) are used for control of lepidopteran pests in orchards and in vegetable production (Glare and O'Callaghan, 2000; Lacey and Shapiro-Ilan, 2008; Glare et al., 2012). It is used extensively in organic vegetable production and is increasingly being utilized by conventional growers. Control of a plethora of pest Lepidoptera is common in row crops including crucifers, solanaceous vegetables, cucurbits, corn, legumes, soybeans, cotton, and others. The implementation of Btk for control of orchard pests, particularly leafrollers and other defoliators, was described by Lacey et al. (2007) and Lacey and Shapiro-Ilan (2008).

A multitude of papers on applied research and use of Bt-based products for protection against lepidopteran pests of vegetables and tree fruit have been published since 2000 and many are referenced by Glare and O'Callaghan (2000), Metz (2003), Lacey and Kaya (2007), Jurat-Fuentes and Jackson (2012). Kabaluk and Gazdik (2005) provide a directory of biopesticides that includes producers of several commercial Bt products for control of Lepidoptera.

Control of coleopteran pests in crops using commercially produced *B. thuringiensis* is limited to beetles in the family Chrysomelidae, principally the Colorado potato beetle, *Leptinotarsa decemlineata* (Wraight et al., 2007b, 2009). The beetle-active toxin (Cry 3Aa) is produced by *B. thuringiensis* subsp. *tenebrionis* (Btt). It can provide an effective means of control, especially when applied at regular intervals against early instars. Btt was rapidly developed as a microbial pesticide in the late 1980s and early 1990s (Gelernter and Trumble, 1999; Gelernter, 2002). However, several factors, most notably competition with neonicotinyl insecticides, resulted in its near disappearance from the marketplace (Gelernter, 2002). The Cry3Aa toxin expressed in transgenic potato provides complete protection from *L. decemlineata* but current public opposition to transgenes in food has resulted in removal of transgenic potato from the market in North America and Europe. Transgenic 'Spunta' potato lines with the *cry1Ia1* gene were completely resistant to potato tuberworm in laboratory and field tests (Douches et al., 2002, 2011).

3.1.2.2. Stored product pests. Several pest insects attack stored grain, fruit, nuts, potatoes and other stored food products. Btk products have been used to control several of these pests (Lord et al., 2007; Shapiro-Ilan et al., 2007; Kroschel and Lacey, 2008). Good efficacy of Btk has been demonstrated and protocols have been published for the evaluation of Btk control against *Plodia interpunctella* and other lepidopteran pests of stored grain (Lord et al., 2007). Despite the massive volume of grain in grain silos, only the top 10 cm of grain require treatment (Lord et al., 2007). Kroschel and Lacey (2008) and Lacey and Kroschel (2009) described examples of large-scale implementation of Btk in several countries for control of the potato tuber moth, *P. operculella* in rustic stores of potato tubers.

3.1.2.3. Forests. Btk is the principal non-chemical means of control for lepidopteran pests of forests. Its development and use in the 1970s and 1980s facilitated broader commercial development in the 1980s and 1990s (van Frankenhuyzen et al., 2007). Btk has been used extensively against the spruce budworm, (*Choristoneura fumiferana*) and gypsy moth (*Lymantria dispar*) (Bauce et al., 2004; van Frankenhuyzen et al., 2007). Protocols for the evaluation of Btk and other isolates of Bt for control of *C. fumiferana* and *L. dispar* are presented by van Frankenhuyzen et al. (2007). Btk has also been

used for control of other lepidopteran forest defoliators across North America and Europe including *Thaumetopoea processionea*, *T. pityocampa*, *Lymantria monacha*, *Dendrolimus* sp., *Bupalus piniaria*, *Panolis flammea*, *Tortrix viridana*, *Operophtera brumata*, *Dioryctria abietella*, *Lambdina fiscellaria fiscellaria*, *Choristoneura occidentalis*, *C. pinus pinus*, *Orgyia leucostigmata*, *O. pseudotsugata*, and others (Fuxa et al., 1998; van Frankenhuyzen, 2000).

The only non-lepidopteran forest pest insects that are susceptible to Bt are in the coleopteran family Chrysomelidae. Bauer (1992) bioassayed Btt for larvicidal activity against the imported willow leaf beetle, *Plagiodera versicolora*, reared on poplar (*Populus*) or willow (*Salix*). Good larvicidal activity of the bacterium was only observed on the larvae reared on poplar. Genissel et al. (2003) reported on the deleterious effects of feeding *Chrysomela tremulae* larvae and adults on leaves from transgenic poplar expressing the *cry3Aa* gene from Btt. No large scale field trials have yet been conducted with Btt for control of chrysomelids in forests.

3.1.2.4. Lawn and turf. Klein et al. (2007) and Koppenhöfer et al. (2012) provide overviews of the use of Bt subspp. for control of turf pests. Btk and Bt subsp. *aizawai* are registered for control of sod webworms and armyworm, *Mythimna* (*Pseudaletia*) *unipuncta*, in turf. Although not widely used for control of these pests, Bt strains provide some control if used when early instars are present and applications are made during the early evening to avoid as much UV degradation as possible. Oestergaard et al. (2006) demonstrated control of the European crane fly, *Tipula paladosa*, with *B. thuringiensis* subsp. *israelensis* (Bti) applied against early instars; however, there are no reports in the literature of routine use of Bti for crane fly control in turf. The Bt subsp. *japonensis* (Buibui strain) is insecticidal for the Japanese beetle, *Popillia japonica*, and other scarab species that are turf pests (Suzuki et al., 1992; Alm et al., 1997; Koppenhöfer et al., 1999, 2012; Bixby et al., 2007). Koppenhöfer et al. (1999) observed an additive and synergistic interaction between entomopathogenic nematodes (*Steinernema* spp. and *Heterorhabditis bacteriophora*) and Bt subsp. *japonensis* (Buibui strain) for control of the grub *Cyclocephala* spp. An advantage of Bt subsp. *japonensis* over *Paenibacillus popilliae*, another bacterium used for *P. japonica* control, is that it can be grown on artificial media and has a broader host range within the Scarabaeidae.

3.1.2.5. Medically important insects. Several species of mosquitoes (Culicidae) are widespread pests, many of which transmit disease causing agents such as *Plasmodium* spp. (malaria), filaroid nematodes (elephantiasis, Mansonellosis) and viruses (yellow fever, dengue, and several that cause encephalitis) (Foster and Walker, 2009). The aquatic habitats in which Bti is used for mosquito control are extremely diverse in terms of location (salt marsh, tree holes, wetlands, containers, and a variety of other habitats) and water quality (Skovmand et al., 2007). Black flies (Simuliidae) are always found in lotic habitats (rivers, streams, creeks) (Adler et al., 2004; Adler and McCreadie, 2009) and, in addition to their highly pestiferous activity, some species transmit the filaroid nematodes that cause human and bovine onchocerciasis (Adler and McCreadie, 2009). Bti is the only Bt subsp. that is commercially produced for control of vector and pestiferous Diptera in both the Culicidae (Lacey, 2007; Despres et al., 2011) and Simuliidae (Adler et al., 2004; Skovmand et al., 2007). Although Bti is very efficacious, its persistence in the environment, especially those with high organic content, is short lived and requires frequent reapplication. Dense foliar canopy and rapid settling of toxin in deeper lentic habitats decrease the amount of inoculum reaching the habitat and decrease the duration of larval exposure. Toxin is carried shorter distances in shallow streams with large substrate to water volume ratios (wide and shallow). Large rivers can result in effective carry of the toxin up to 30 km. Further improvements in formulations and delivery systems are expected to increase efficacy in mosquito and black fly habitats.

3.1.3. Production of B. thuringiensis

The nutrient media and conditions under which Bt and *L. sphaericus* are produced can markedly influence larvicidal activity. Guidelines and typical media ingredients for shake flask, stir tank and deep tank fermentation are presented by Beegle et al. (1991), Lisansky et al. (1993), and Couch (2000). Although there is continued improvement in fermentation technology for *B. thuringiensis*, information on any specific changes in methods and media by industry nearly always is proprietary (Couch, 2000). However, there have been developments in small scale production using unique media components such as local raw ingredients of plant and animal origin and bi-products (such as whey) which provide inexpensive nitrogen and carbon sources for the production of Bt and *L. sphaericus* (Aranda et al., 2000; Lacey, 2007).

3.1.4. Transgenic crops or Bt-crops

The largest market progress over the last two decades was associated with the development of a Bt product different from the microbial pesticides, the Bt transgenic crops. The Cry toxins and VIPs are the only toxins currently used in commercial insecticidal transgenic crops. VIP toxins are only found in transgenics but several Cry toxins produced by Bt-crops are the same as those produced for Bt microbial pesticides such as Dipel or Xentari. GM crops have been the most rapidly adopted production technology in agriculture (Brookes and Barfoot, 2013; James, 2013). Although implementation has not been without controversy, wide acceptance is due to specificity to insects and high efficacy of *B. thuringiensis* Cry toxins, and safety for consumers and non-target organisms (Shelton et al., 2002; Bravo et al., 2011). A large diversity of toxin genes that are relatively simple to clone and express are found in different *B. thuringiensis* strains. The toxin genes are distributed into families that are easy to characterize and the toxins are organized into clearly distinguishable functional domains (Bravo et al., 2007). These traits not only make the mode of action (MOA) of the toxins easier to elucidate but also make both toxins and toxin genes good models for genetic engineering. Early in the 1980s, *B. thuringiensis* was already a commercially successful product. *B. thuringiensis* insecticidal proteins were some of the only gene products meeting the technical and ethical requirements for plant biotechnology. Subsequently, *B. thuringiensis* toxins became the most promising source for development of insect-resistant transgenic plants (Kennedy, 2008).

Global Bt-crop acreage has increased enormously in the last two decades, reaching 175 million ha in 2013 (Choudhary and Gaur, 2010; Brookes and Barfoot, 2013; James, 2013). The adoption rate was 100% or near 100% in 2013 for all major transgenic crops in the primary producing countries. Stabilization of adoption rate and area planted are therefore expected in the coming years (James, 2013).

The increased use of Bt-cotton and Bt-corn has resulted in a significant decrease in the use of chemical insecticides (Phipps and Park, 2002; Brookes and Barfoot, 2013), particularly in cotton (Huanga et al., 2003; Edwards and Poppy, 2009; Krishna and Qaim, 2012). However, transgenic technologies also compete with sprayable formulations of Bt due to the similarity of toxins used and result in a lower commercial share left to Bt microbial pesticides. Furthermore, while reducing the overall market for chemical insecticides, widespread adoption of Bt crops can increase the market for herbicides as new generations of transgenic plants expressing stacked Bt and herbicide-resistance genes are now on the market (James, 2013). Given the widespread environmental concerns over broad-spectrum chemical pesticides, it is possible that

GM crops deploying pest specific safe gene products such as Bt toxins may finally be considered a more environmentally acceptable solution for pest control than the development and widespread application of newer chemical pesticides.

Given the high cost of developing and deploying a new transgenic crop, currently estimated as $136 million (McDougall, 2013; Mumm, 2013), it will not be economically viable to develop GM varieties for all crops, including many minor use or locally important crops, or to control all specific pests and diseases (Shelton, 2012). Non-GM crop diversity and local varieties must be maintained for many reasons ranging from differing climates and specific cultural food practices to the need for a diverse genetic base for disease tolerance. Because not all crops and varieties will be transgenic, other conventional but still environmentally friendly means of control must be retained and developed. Among these should be new Bt-based microbial pesticides, as well as other entomopathogen-based pesticides. However, niches for microbial pesticides must address new issues to avoid competition with, for example, focusing on a mosaic of secondary pest problems. Microbial pesticides for forestry and vector control may be an exception to treatment of row crops because competition with trangenics does not exist. We predict that microbial pesticides, such as sprayable and other Bt formulations, will continue to have a successful future in the coming decades.

3.1.5. Controversy around *Bacillus thuringiensis* toxins in GM crops

In this section we address the biological aspects of the controversy over the use of Bt crops and focus on safety and environmental concerns. Divisive socio-economic and political issues will not be covered and should be the subject of a separate forum discussion. The Bt toxins are essential in the deployment of a number of major insect resistant GM crops and, therefore, *B. thuringiensis* microbial pesticides were also involved in the extensive controversy around the safety and efficacy of GM crops. A notable example has been concern about effects of Bt toxins on the monarch butterfly, *Danaus plexippus*. Pollen from Bt-maize dusted onto milkweed under laboratory conditions was reported to produce mortality in *D. plexippus* larvae (Losey et al., 1999). Follow up research determined that the deleterious results were related to a specific maize variety (Bt176, no longer in commercial use) and that there was no negative impact to monarchs under field conditions (Hellmich et al., 2001; Minorsky, 2001; Pleasants et al., 2001; Sears et al., 2001; Stanley-Horn et al., 2001; Tschenn et al., 2001; Zangerl et al., 2001; Dively et al., 2004; Anderson et al., 2005). Nevertheless, the controversy generated a widespread perception that Bt-engineered crops are dangerous for the environment. This issue was revived 10 years later when France and Germany banned the Bt-maize variety MON810 on the basis of a threat to *D. plexippus*, despite the facts that MON810 was found to be harmless to monarch larvae and *D. plexippus* is not found in Europe (Ricroch et al., 2010).

A second example relating to the health impact of Bt crops is the "StarLink case" (Bucchini and Goldman, 2002; Bernstein et al., 2003). In this instance, the StarLink product, a feed-registered insect-resistant Bt-maize engineered with the Cry9Ca toxin, was found in human food. This was followed by reports of allergic shock in consumers, although follow-up studies by the Centers for Disease Control failed to confirm any link to the Cry9Ca toxin (CDC, 2001). Nevertheless, problems were confirmed in the management and control of feed-registered BT-corn products that allowed them to be comingled with food for human consumption (Bucchini and Goldman, 2002). The controversy subsequently led to a serious loss of market share for U.S. corn growers (Schmitz et al., 2005). An additional consequence of the controversy has resulted in stories implicating Bt crops either in health scares or as contributors to disastrous crop failures (Tirado, 2010; Coalition for GM free India, 2012). Several of these stories subsequently were shown to be untrue (Gruere et al., 2008; Brookes and Barfoot, 2013).

Bt microbial pesticides, while accepted in pest control, organic agriculture and vector control, also have become subjects of debate in the crop biotechnology arena and have been represented by some as a threat to human or environmental health. For example, Poulin et al. (2010) and Poulin (2012) demonstrated the negative trophic effect of Bti treatment for mosquito control on non-target fauna. The reduction of mosquitoes and chironomids and consequently their predators as prey of breeding house martins, *Delichon urbicum*, resulted in reduced clutch size and fledgling survival. Among other measures, Poulin et al. (2010) recommended suspension of mosquito control in certain habitats. We believe that such measures should take into account the effect of mosquito reduction on quality of life for humans and domestic animals, but most importantly the interruption of disease transmission.

A positive aspect of the debates on the safety of Bt products is that they have prompted renewed studies on actual health and environmental effects of Bt toxins. These have shown that commercially approved Bt products and Bt genes are safe and can have positive benefits for the environment, mostly through the reduced use of chemical pesticides and lack of effects on non-target organisms (Saxena and Stotsky, 2001; Phipps and Park, 2002; Shelton et al., 2002, 2007; Lacey and Merritt, 2003; O'Callaghan et al., 2005; Wu et al., 2005; Romeis et al., 2006; Marvier et al., 2007; Roh et al., 2007; Chen et al., 2008; Kumar et al., 2008; Wolfenbarger et al., 2008).

3.1.6. Insect resistance and mode of action of Bt toxins

One of the most important aspects to address with Bt-based products and Bt crops is resistance management. *B. thuringiensis* shares with chemical pesticides the negative trait of producing resistance to the toxic effects in target insect populations. Resistance is the interruption of the mode of action (MOA) of any pesticide, and understanding insect resistance and proposed insect resistance strategies requires first summarizing the MOA. This section is intended to underline the sequential nature of the MOA of *B. thuringiensis* insecticidal proteins and its susceptibility to resistance. Resistance can result from the interruption of any of the steps described in this section and, indeed, several mechanisms of resistance have been described. The MOA is well understood for a limited number of Bt toxins, including the Cry and Cyt families used in microbial pesticides, and the Cry and VIP in transgenic crops.

The MOA of Cry proteins is by far the best known (Whalon and Wingerd, 2003; Bravo et al., 2007, 2011; Pigott and Ellar, 2007; Vachon et al., 2012). Pathogenesis begins with the ingestion of the Bt crystal. The crystal, which contains protoxins, is then solubilized by the alkaline pH of the insect midgut and the soluble protoxins are activated by midgut serine proteases releasing the active toxin. The structure of these activated toxins has been determined for several families. In the Cry1 family, three functional domains have been identified (Li et al., 1991; Grochulski et al., 1995; Galitsky et al., 2001; Morse et al., 2001; Boonserm et al., 2005, 2006) (Fig. 2). Domain I consists of 7 alpha helices organized in a barrel-like structure and is involved in pore formation. Domains II and III are comprised of layers of beta sheets that recognize specific binding sites at the surface of the midgut brush border (Pigott and Ellar, 2007). These binding sites have been identified mostly as aminopeptidase N-like proteins (APN) and cadherin-like proteins, although other putative receptors such as alkaline phosphatases (ALP), glycolipids or a 270-kda glycoconjugate (Pigott and Ellar, 2007) have been identified. Following specific binding, the toxin undergoes a change of conformation and inserts into the midgut membrane to form an ionic channel or pore

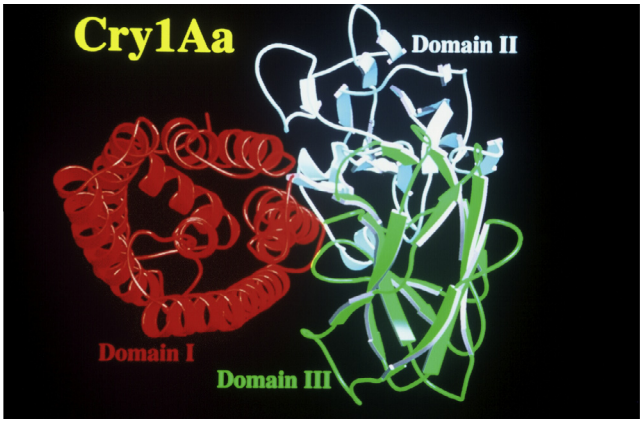

Fig. 2. Apical ribbon view representing the 3-D structure of the *Bacillus thuringiensis* Cry1Aa toxin.

(Knowles and Ellar, 1987; Vié et al., 2001; Bravo et al., 2004; Vachon et al., 2012) transporting ions with their free charged-amino acids (Masson et al., 1999; Vachon et al., 2002, 2004; Girard et al., 2009; Lebel et al., 2009). Ion transport triggers a physiological imbalance leading to the death of the cell, destruction of the midgut and ultimately death of the insect. This process of ionic imbalance, originally described as colloid-osmotic lysis (Knowles and Ellar, 1987; Bravo et al., 2004), is probably not the only mechanism involved in cell death. Signaling pathways that follow receptor binding recently have been described (Zhang et al., 2006). These pathways are triggered upon receptor activation by protein binding and initiate cell death mechanisms. However, these two mechanisms are not exclusive and could both contribute to the overall toxicity of Cry toxins as suggested by Jurat-Fuentes and Adang (2006) and discussed by Vachon et al. (2012).

Resistance to Bt toxins was first reported in *Plodia interpunctella*, an insect pest of stored grain, by McGaughey (1985, 1994). Field resistance has since been reported in diamond back moth, *Plutella xylostella*, and cabbage looper, *Trichoplusia ni*, and several major insect pests under laboratory selection (Tabashnik, 1994; Moar et al., 1995; Rahman et al., 2004; Shelton et al., 2007; Furlong et al., 2013). In common with Bt microbial pesticides, Bt crops also are susceptible to resistance problems and a number of cases have been reported, particularly with first generation single gene constructs (Rahman et al., 2004; Shelton et al., 2007; Tabashnik, 2008; Tabashnik et al., 2008a, 2008b, 2009, 2013). Modification of the Bt binding sites is the most commonly reported resistance mechanism, however other mechanisms affecting different steps of the MOA have been described and can potentially develop (Frutos et al., 1999; Griffitts and Aroian, 2005; Heckel et al., 2007). A key point is that resistance affects both microbial pesticides and transgenic crops in the same way, and cross-resistance to other similar toxins used in both modes of delivery could occur.

3.1.7. Future directions

Since *B. thuringiensis* remains the primary sprayable microbial pesticide, the increasing demand for organic products should encourage the development of additional Bt products. Demand would also be driven partly by safety legislation requiring reduction of the number of chemical pesticides. The future sustainability of Bt crops will rely on a combination of multistacked toxin genes and refugia to delay resistance (Caprio and Sumerford, 2007; Tabashnik, 2008; Head and Greenplate, 2012; Storer et al., 2012). Addressing resistance and resistance management will depend on detailed knowledge of the MOA of Bt toxins (Griffitts and Aroian, 2005; Shelton et al., 2007). Multiple-gene constructs targeting different binding sites is the basis for the gene pyramiding that underlies the development of novel generations of Bt crops (Shelton et al., 2002). In addition to discovery of more efficacious isolates and toxins, an increase in the use of Bt products and transgenes will rely on innovations in formulation, better delivery systems and ultimately, wider public acceptance of transgenic plants expressing Bt toxins.

3.2. Lysinibacillus (Bacillus) sphaericus

Although less commonly used than Bti for control of mosquitoes, *L. sphaericus* offers some advantages that Bti does not. Only the IIA sub-group includes isolates with larvicidal activity for mosquitoes (Charles et al., 1996). The moiety responsible for mosquito larvicidal activity in serovar 5a5b isolates of *L. sphaericus* is a binary toxin (Charles et al., 1996) with both proteins required for full toxicity. The individual roles of the toxin components were elucidated by Charles et al. (1997) and Schwartz et al. (2001). As with Bti, ingested toxins are solubilized in the alkaline midgut and cleaved to the active moiety by proteases. The two component proteins of the toxin, BinA (42 kDa) and BinB (51 kDa) bind to specific receptors on the brush border of epithelial cells of the gastric caecum and midgut and cause pore formation resulting in disruption of osmotic balance, lysis of the cells, and ultimately death of the insect (Charles et al., 1996). *L. sphaericus* binary toxin is more specific and narrower in range than the Bti toxins; it is principally active against *Culex* mosquitoes. Several *Aedes* species in the *Stegomyia* group (such as *Aedes aegypti*) are not susceptible to *L. sphaericus* formulations.

Protocols for the short-term evaluation of *L. sphaericus* formulations in the field are similar to that of Bti (Skovmand et al., 2007). Biotic and abiotic factors that influence the larvicidal activity of Bti and *L. sphaericus* include the species of mosquito and their respective feeding strategies, rate of ingestion, age and density of larvae, habitat factors (temperature, solar radiation, depth of water, turbidity, tannin and organic content, presence of vegetation, etc.), formulation factors (type of formulation, toxin content, how effectively the material reaches the target, and settling rate), storage conditions, production factors, and means of application and frequency of treatments (Lacey, 2007). *L. sphaericus* formulations have been utilized predominantly in organically enriched habitats, but they are also active against numerous species, and across several genera in habitats with low organic enrichment. The bacterium has been shown to persist longer than Bti in polluted habitats and can recycle in larval cadavers (Lacey, 2007). A disadvantage of *L. sphaericus* is the development of resistance in certain populations of *Culex quinquefasciatus* and *Cx. pipiens*. Low to extremely high levels of resistance to the *L. sphaericus* binary toxin have been reported in populations of *Cx. quinquefasciatus* in India, Brazil, China, Thailand, Tunisia and France (Charles et al., 1996; Lacey, 2007). The combination of *L. sphaericus* and toxin genes from Bti increases the host range of the bacterium and could offer a means of combatting resistance (Federici et al., 2007).

3.3. Paenibacillus species

Paenibacillus spp. are spore-forming obligate pathogens of larval coleopterans in the family Scarabaeidae (Klein, 1992; Klein et al., 2007; Koppenhöfer et al., 2012). The disease caused by these bacteria is known as milky disease due to the milky appearance of the hemolymph in infected larvae. Spores of the bacterium must be ingested in order to invade the hemocoel and produce an infection. Natural epizootics have been observed in *P. japonica*, but variable results have been obtained after application of spore powders. In

some cases, epizootics have been induced following applications (Klein, 1992), in others, little or no activity was observed (Klein, 1992; Lacey et al., 1994). The spores have been known to persist for several years in the soil (Klein, 1992). *P. popilliae* was the first microbial pesticide registered in North America (1948) for control of *P. japonica* (Klein, 1992), but large-scale commercial development has been limited due to the requirement for *in vivo* production and the narrow host range within the Scarabaeidae. A breakthrough in *in vitro* production of *P. popilliae* and development of strains effective against other important scarab species (e.g., *Cyclocephala* spp., *R. majalis*, *A. orientalis*, and *Melolontha melolontha*) would significantly improve the marketability of these bacteria.

3.4. Serratia entomophila

The endemic non-sporeforming bacterium *Serratia entomophila* (Enterobacteriaceae) was discovered and developed in New Zealand, and is used for control of the New Zealand grass grub, *Costelytra zealandia* (Jackson et al., 1992; Jackson, 2007). Cultivation of pastures for cropping and re-sowing generally kills grass grubs and eliminates pathogenic strains of bacteria, leaving new pastures vulnerable to pest attack. This provides an opportunity for augmentative biological control, where *S. entomophila* is applied to *C. zealandia* populations to promote epizootics and prevent the occurrence of pasture damage.

Strains of the *Serratia* spp. cause amber disease in *C. zealandia* (Jackson et al., 2001). The bacterium must be ingested for toxin production to be initiated and disease progression is accompanied by a cessation of feeding, clearance of the gut and a halt in the synthesis of digestive enzymes. Infected larvae take on a distinctive amber coloration prior to death (Jackson et al., 2001). *Serratia entomophila* is now commercialized as a stabilized dry granular product Bioshield™ (Jackson et al., 1992; Johnson et al., 2001). The formulation is stable under ambient conditions for several months and is applied using a conventional seed drill, which has enhanced adoption of this microbial pesticide by the pastoral sector in New Zealand (Jackson, 2007). Recycling of the disease through grass grub larvae produces an endemic population of pathogenic bacteria preventing recurrent damaging outbreaks of the pest. The technology for stabilization of this non-spore forming bacterium could be useful in the future for other non-spore forming entomopathogenic species of bacteria.

3.5. Chromobacterium subtsugae

Martin et al. (2007a, 2007b) isolated *Chromobacterium subtsugae*, a new species and genus of a motile, Gram-negative bacterium, with *per os* toxicity to larval Colorado potato beetle, *Leptinotarsa decemlineata*, adults of the corn rootworms, *Diabrotica* spp., and the southern green stinkbug, *Nezara viridula*. Encouragingly, live bacteria were not needed for toxicity to *N. viridula* adults (Martin et al., 2007b). Marrone Bio Innovations (MBI) has registered a biological insecticide/miticide (Grandevo®) containing *C. subtsugae* strain PRAA4-1T and spent fermentation medium for use on edible crops, ornamental plants and turf against defoliating caterpillars and certain Coleoptera (EPA Reg. No.: 84059-17-87865). MBI also reported the formulation to have multiple effects such as reduced fecundity and oviposition, reduced feeding and activity as a stomach poison on aphids, psyllids, whiteflies, *Lygus*, mealybugs, thrips and phytophagous mites. Genes encoding toxins and VIPs of this bacterium could conceivably be candidates for incorporation into GM crops for targeting a broad pest host range.

4. Entomopathogenic fungi

4.1. Background and overall status

Fungi are the predominant natural pathogens in arthropod populations. Observations of epizootics among insect populations are common, indicating the great potential of these microbes for regulation of pestiferous species. Entomopathogenic fungi infect their hosts through the external cuticle and are pathogenic to both soft- and hard-bodied insects, as well as a range of other arthropods including Acari (ticks, mites). Cuticular invasion also enables fungi to infect sucking insects such as aphids, whiteflies, psyllids and scales (Burges, 2007; McCoy et al., 2009; Lacey et al., 2011). Consequently, fungi have been widely evaluated as control agents for a diverse variety of noxious arthropods of agricultural (including forestry and livestock) and horticultural importance (Chandler et al., 2000; Shah and Pell, 2003; Brownbridge, 2006; Abolins et al., 2007; Charnley and Collins, 2007; Jaronski, 2007; Maniania et al., 2007; Wraight et al., 2007a; Zimmermann, 2007a, 2007b, 2008; Alves et al., 2008; Kaufman et al., 2008; James, 2009; Glare et al., 2010; Goettel et al., 2010). Recent discoveries of the effects of entomopathogenic fungi on adult mosquitoes, including the prevention of development of vectored human pathogens within fungal infected mosquitoes, has resulted in an upsurge of research on their potential for control of mosquito-borne diseases such a malaria (Blanford et al., 2005, 2009; Scholte et al., 2003, 2004, 2005; Kikankie et al., 2010). Although entomopathogenic fungi traditionally have been regarded exclusively as pathogens of arthropods, recent studies suggest that they play additional roles in nature. Many are now known to be plant endophytes, plant disease antagonists, rhizosphere colonizers, and plant growth promoters (Elliot et al., 2000; Vega et al., 2009; Behie et al., 2012; Jaber and Salem, 2014).

Several hypocrealean entomopathogenic fungi are important constituents of natural- and agro-ecosystems and appear to be ubiquitous inhabitants of soils worldwide. They have been recovered from a diverse array of geographic, climatic, and agro-ecological zones (Bidochka et al., 2001, 2002; Shimazu et al., 2002; Keller et al., 2003; Shapiro-Ilan et al., 2003a; Meyling and Eilenberg, 2006a, 2006b, 2007; Jaronski, 2007; Queseda-Moraga et al., 2007; Zimmermann, 2007a, 2007b, 2008; Inglis et al., 2008, 2012; Reay et al., 2008; Meyling et al., 2009; Scheepmaker and Butt, 2010). Fungi such as *Beauveria bassiana s.l.* and *Metarhizium anisopliae s.l.* are commonly found in both cultivated and undisturbed soils, although their natural distribution appears to be linked to habitat (Bidochka et al., 2001; Keller et al., 2003; Meyling and Eilenberg, 2006a; Meyling et al., 2009), and soil populations are influenced by agricultural practices (Hummel et al., 2002; Jaronski, 2007, 2010; Meyling and Eilenberg, 2007).

Fungi have many desirable traits that favor their development as biological control agents. They pose minimal risk to beneficial non-target organisms such as bees, earthworms and Collembola, which are key ecosystem service-providers, and arthropod natural enemies such as parasitic wasps and predatory beetles (Goettel et al., 2001; Traugott et al., 2005; Brownbridge and Glare, 2007; O'Callaghan and Brownbridge, 2009). This enhances their potential role in IPM; the preservation of natural enemies allows them to make a greater contribution to the overall regulation of pests, and maintenance of biodiversity is increasingly recognized as being critical to the long-term productivity of our farms and forests. Their newly found attributes also provide the possibility of their use in multiple roles, for instance in addition to arthropod pest control, simultaneous suppression of plant pathogens and plant parasitic nematodes (Goettel et al., 2008; Kim et al., 2009; Koike et al., 2011) or biofertilizers (Kabaluk and Ericsson, 2007; Behie et al., 2012).

Chandler et al. (2008) considered the development of anamorphic fungi, e.g., *B. bassiana*, *M. anisopliae*, to have followed an 'industrial' pathway; mass-production systems have been devised to provide large quantities of inoculum which can then be formulated and repeatedly applied as sprays, granules, etc. (Shah and Pell, 2003; Brownbridge, 2006; Charnley and Collins, 2007). Conversely, pest control strategies using entomophthoralean fungi have relied more on 'ecological' approaches; accompanying research has focused on understanding conditions that promote natural epizootics, e.g. manipulating environmental conditions to enhance disease incidence and spread, use of inoculative releases to establish the disease within a pest population to achieve long-term suppression, or conservation of natural epizootics (Steinkraus et al., 2002; Steinkraus, 2006, 2007a, 2007b; Nielsen et al., 2007; Pell, 2007; Hajek, 2009; Solter and Hajek, 2009; Pell et al., 2010).

Commercial products based on some of the pathogenic fungi – mycoinsecticides and mycoacaricides – are primarily based on *Beauveria* spp., *Metarhizium* spp., *Isaria fumosorosea* (formerly *Paecilomyces fumosoroseus*), and *Lecanicillium* spp. (Inglis et al., 2001; Faria and Wraight, 2007; Wraight et al., 2007a, 2007b; Alves et al., 2008). Table 3 provides examples of fungi used for the microbial control of several insect and mite pests. Fungal products largely occupy niche markets, often within individual countries or geographically linked regions. In most cases, fungi are actively applied as microbial pesticides to regulate pest populations, and pathways toward their development and regulation have generally mirrored those of synthetic pesticides. Despite these positive developments, fungi remain an under-utilized resource for pest management. How far has the field progressed since Lacey et al.'s 2001 publication to move us closer to realizing this biological control potential? Here, we will highlight some of the recent developments that may promote opportunities to use entomopathogenic fungi and identify some of the critical factors that still need to be addressed to enable their wider utilization.

4.2. Mode of action

All fungi have the same basic mode of action. Excellent reviews of the mechanical, molecular and biochemical processes involved in insect infection are available and consequently will not be covered here in detail (e.g., see Hajek and St. Leger, 1994; Hajek, 1997; Inglis et al., 2001; Charnley, 2003; Charnley and Collins, 2007; Ortiz-Urquiza and Keyhani, 2013). Insect control by entomopathogenic fungi is achieved when sufficient infective propagules (generally conidia) contact a susceptible host and conditions are suitable for a lethal mycosis to develop. Fungi have been applied for soil pest control by direct incorporation of conidia, mycelial pellets, microslerotia or inert or nutrient-based granules containing fungal propagules (conidia or mycelia) (Ansari et al., 2006b, 2008a, 2008b; Brownbridge, 2006; Charnley and Collins, 2007; Jaronski, 2007; Jaronski and Jackson, 2008), whereas foliar-feeding pests have typically been targeted by sprays of formulated conidia (Jaronski, 2010).

Fungal isolate virulence toward different arthropod hosts varies. Virulence generally decreases with repeated sub-culture on artificial media, and can often be regained through host passage (e.g. Nahar et al., 2008). Virulent isolates generally express an abundance of spore-bound proteases, efficiently produce and release exoenzymes during cuticular penetration, and generate toxins as the fungus colonizes the host (Vey et al., 2001; Freimoser et al., 2005; Shah et al., 2005; Qazi and Khachatourians, 2007; Zimmermann, 2007a, 2007b, 2008; Khan et al., 2012). Selecting superior strains exhibiting these characteristics, or manipulating isolates to promote these traits, has been seen as a way of overcoming what is often considered a significant impediment to their wider use, i.e., fungi kill their hosts too slowly. Fungal virulence can also be improved through directed genetic manipulation whereby specific genes are inserted into the fungal genome to promote expression of toxins that increase the virulence of the parent organisms, e.g., insertion of scorpion toxin genes into *M. anisopliae* and *B. bassiana* (Wang and St. Leger,

Table 3
An overview of the entomopathogenic fungi that have been developed for microbial control of insect pests.[a]

Species names	Targeted insects	Produced in	Selected references
Aschersonia aleyrodis	Hemiptera (Aleyrodidae)	Russia	Fransen (1990), Meekers et al. (2002), Lacey et al. (2008a, 2008b), McCoy et al. (2009)
Beauveria bassiana sensu lato	Acari, Coleoptera, Diplopoda, Diptera, Hemiptera, Hymenoptera, Isoptera, Lepidoptera, Orthoptera, Siphonoptera, Thysanoptera,	Africa, Asia, Australia, Europe, South & North America	de la Rosa et al. (2000), Wraight et al. (2000, 2007b), Brownbridge et al. (2001, 2006), Chandler et al. (2005), Wekesa et al. (2005), Brownbridge et al. (2006), Labbé et al. (2009)
Beauveria brongniartii	Coleoptera (Scarabaeidae)	Europe, Colombia, Reunion Island	Zimmermann (1992), Keller (2000), Keller et al. (2003), Dolci et al. (2006), Townsend et al. (2010)
Conidiobolus thromboides	Acari Hemiptera, Thysanoptera	Colombia, India, South Africa	Papierok and Hajek (1997), Nielsen and Hajek (2005), Hajek et al. (2012)
Hirsutella thompsonii	Acari	India	McCoy (1981), Chandler et al. (2000, 2005), McCoy et al. (2009)
Isaria fumosorosea	Acari, Diptera, Coleoptera, Hemiptera, Thysanoptera,	Belgium, Colombia, Mexico, USA, Venezuela	Wraight et al. (2000, 2007a, 2007b), Lacey et al. (2008a, 2008b, 2011), Zimmermann (2008)
Lagenidium giganteum	Diptera (Culicidae)	USA	Kerwin and Petersen (1997), Skovmand et al. (2007)
Lecanicillium longisporum	Hemiptera	Brazil, Netherlands	Bird et al. (2004), Down et al. (2009), Kim et al. (2009)
Lecanicillium muscarium	Acari, Hemiptera, Thysanoptera	Netherlands, Russia	Chandler et al. (2005), Cuthbertson and Walters (2005), Burges (2007), Goettel et al. (2008)
Metarhizium anisopliae sensu lato	Acari, Blattoidea, Coleoptera, Diptera, Hemiptera, Isoptera, Lepidoptera, Orthoptera	Africa, Asia, Australia, Europe, South, Central & North America	de la Rosa et al. (2000), Chandler et al. (2005), Wekesa et al. (2005), Jaronski and Jackson (2012), Lacey et al. (2011)
Metarhizium acridum	Orthoptera	Australia, South Africa, USA	Lomer et al. (1999, 2001), Thomas (2000)
Nomuraea rileyi	Lepidoptera	Columbia, India	Moscardi and Sosa-Gomez (2007), Thakre et al. (2011)

[a] Condensed and modified from de Faria and Wraight (2007). For up to date information on products registered in the OECD Countries, visit https://www5.agr.gc.ca/MPDD-CPM/search-recherche.do?lang=eng. For information on the production and successful use of entomopathogenic fungi as microbial pesticides in Latin America see Alves et al. (2008).

2007; Pava-Ripoll et al., 2008; St Leger et al., 2011). In both cases, the recombinant strains exhibited dramatically increased virulence. This approach has the potential to improve insect kill and reduce the amount of inoculum needed to regulate a pest population. In addition, protoplast fusion can be used to enhance virulence and increase host range. For instance, protoplast fusion was used with several strains and species of *Lecanicillium* to develop hybrid strains with multiple effects (toxic and parasitic) against plant parasitic nematodes, plant pathogens and aphids, with plant competency (as root colonizers and endophytes), making these strains promising for development as broad-spectrum microbial pesticides targeting plant pathogens, insects, and plant parasitic nematodes (Goettel et al., 2008; Koike et al., 2011).

Entomophthoralean fungi actively eject spores when conditions are favorable (high humidity) that can rapidly infect a susceptible insect, even when these conditions only prevail for short periods (Steinkraus, 2006). This trait gives these pathogens great epizootic potential, and in many groups of insects, they are among the most important natural mortality factors. In contrast, spores of the hypocrealean fungi *Beauveria* and *Metarhizium* spp. tend to be dispersed passively, via wind currents or rain splash, although transmission can also occur when susceptible insects contact infected individuals, or conidia can be distributed on the bodies of other arthropods (Rath, 2002; Wraight and Ramos, 2002; Meyling and Eilenberg, 2006b; Meyling et al., 2006; Roy et al., 2007; Vega et al., 2007). Both hypocrealean and entomphthoralean fungi can survive repeated intervals of low humidity, recommencing development (infection) when favorable conditions return. This can result in spectacular epizootics such as those observed in whitefly infestations on cotton when the canopy closes and creates a humid microclimate that favors host infection and spread of the disease within the population (Lacey et al., 1996). These fungi can, though, infect insects even under conditions of low ambient humidity; attachment of the small conidia at infection sites within inter-segmental folds or under elytra where humidity levels are high may account for this, and the localized microclimate that exists around an insect or at the insect–leaf interface may have a more significant impact on the infection process than ambient conditions (Inglis et al., 2001; Vidal et al., 2003; Vidal and Fargues, 2007; Jaronski, 2010).

Fungi can persist in the soil for several years with new 'flushes' of inoculum provided following the successful infection and colonization of a susceptible host. This leads to localized high concentrations of infective conidia and greater opportunities for insect infection to occur (Enkerli et al., 2001; Keller et al., 2000, 2003; Rath, 2002; Milner et al., 2003; Meyling and Eilenberg, 2007). Long-term survival of entomopathogenic fungi within an environment appeared to be reliant upon access to susceptible hosts, though, as they were generally considered weak saprophytes (Keller et al., 2003; Hummel et al., 2002; Roberts and St. Leger, 2004; Jaronski, 2007). However, the recent discoveries of their roles as endophytes or rhizosphere competent organisms require further investigations in this regard. For those species with relatively narrow host-spectra, lack of hosts can limit their natural occurrence and longevity (Keller et al., 2003; Meyling and Eilenberg, 2007).

4.3. The changing face of fungi

A variety of molecular tools and systems now augment more traditional fungal classification schemes, allowing examination of evolutionary (phylogenetic) relationships between isolates as well as matching anamorphs and teleomorphs (Driver et al., 2000; Rehner and Buckley, 2005; Hibbett et al., 2007; Humber, 2008; Bischoff et al., 2009; Blackwell, 2010). Furthermore, they aid in the differentiation and identification of fungi in environmental samples, enable definition of potential associations (habitat, host), and may provide valuable insights that enable strain improvements or selection of isolates with specific traits (Nielsen et al., 2001, 2005; Ranjard et al., 2001; Sung et al., 2001, 2007; Bidochka et al., 2002; Enkerli et al., 2005; Huang et al., 2005; McGuire et al., 2006; Rehner et al., 2006; Hibbett et al., 2007; Inglis et al., 2007; Meyling et al., 2009; Enkerli and Widmer, 2010). These techniques are changing the way we observe fungi in the environment, and potentially alter pathways toward their development as MCAs.

4.4. The importance of selecting the appropriate fungal isolate and other considerations

The literature is replete with examples of fungi that have performed well in laboratory trials and shown "great potential" (Vega et al., 2012) only to fail once they were tested in the field. This has often led to a search for 'new and better' isolates rather than investigating underlying factors impacting performance in the environment. Without diminishing the implicit value of looking for new organisms (in general there is no shortage of excellent candidates) more research emphasis is instead needed to address critical factors to turn 'potential' into viable 'product'.

Isolates must be ecologically competent to function and persist in the environment of the target pest, and selection of candidates must not be solely based on performance in an optimized bioassay system. Bioassays need to be carried out under discriminatory conditions that attempt to replicate conditions where the pathogen will be used (Butt and Goettel, 2000). Environmental and insect behavioral factors all influence pathogen activity, so their incorporation into a testing scheme will enable robust isolates to be identified prior to downstream development activities.

Fungi and arthropods have evolved complex relationships, and some soil-dwelling arthropods show adaptive behavioral responses that prevent their coming into contact with fungal inoculum (Villani et al., 2002; Thompson and Brandenburg, 2005; Baverstock et al., 2010). There also appears to be variation in the level of response to different fungal isolates or fungal growth stages, i.e. vegetative stage vs conidia (Thompson and Brandenburg, 2005), and in some instances, insects may be attracted or repelled by fungal volatiles or metabolites which could enhance or deter activity (Villani et al., 1994; Engler and Gold, 2004; Kepler and Bruck, 2006; Meyling and Pell, 2006; Rohles and Churchill, 2011). Such behavioral responses should be taken into consideration when selecting appropriate strains for insect pest management, and the type of inoculum used in a pest management program. Similarly, our ability to manipulate insect behavior through the use of a variety of compounds may provide new opportunities to enhance pathogen efficacy (Roy et al., 2007).

4.5. Ecological considerations

Entomopathogenic fungi are natural components of most terrestrial ecosystems.. Greater understanding of the fundamental ecology of these organisms in the natural environment and post-application would be of immense value in the development of more ecologically sound control approaches (Wraight and Hajek, 2009; Vega et al., 2009; Roy et al., 2010a, 2010b). The lack of field data is due, in part, to the complexity of the environment and the intricate interactions between different environmental and biological factors that can confound observations around cause and effect (Jaronski, 2007). Likewise, interactions among biotic and abiotic factors, e.g., sunlight, humidity, and microbial activity on the phylloplane, affect efficacy and persistence of fungal treatments applied against foliar pests (Jaronski, 2010). While in vitro testing can provide valuable insights into fungal responses to

specific inputs, they rarely yield data that can be directly extrapolated to predict field responses. More effort needs to be invested in the evaluation of effects of agricultural practices (e.g., Klingen et al., 2002a, 2002b; Hummel et al., 2002; Townsend et al., 2003) on persistence and particularly efficacy under field conditions.

Production of good ecological data has also been impeded by a historic lack of tools to examine and quantify fungal populations. Traditionally, studies have relied on time-consuming isolation and plating techniques. Similarly, risk assessments have tended to focus on interactions with macroorganisms; monitoring of interactions with other microbes has been limited and biased by our inability to culture all soil and foliar microorganisms. However, new tools and increasingly powerful molecular methods are becoming available to examine fungal communities and may be applied to the study of entomopathogens. For example, use of nuclear ITS and EF1-alpha sequences have enabled isolates to be differentiated and phylogenetic relationships within species to be determined, enabling links to geographic and host origins to be defined (Driver et al., 2000; Bidochka et al., 2001, 2002; Rehner and Buckley, 2005; Rehner et al., 2006; Inglis et al., 2008, 2012; Meyling et al., 2009). The ability to transform fungi to express the green fluorescent protein (GFP) allows GFP-mutants to be observed in-situ, and expression of the protein may be tied to specific events during infection or growth through choice of an appropriate promoter (Lorang et al., 2001; Hu and St. Leger, 2002; Skadsen and Hohn, 2004; Wu et al., 2008). A variety of other molecular techniques such as RFLP, T-RFLP, AFLP and strain-specific microsatellite markers have been used as diagnostic tools allowing fungi to be tracked in the environment (Enkerli et al., 2001, 2004, 2005; Castrillo et al., 2003; Rehner and Buckley, 2003; Schwarzenbach et al., 2007a, 2007b; Inglis et al., 2008, 2012; Enkerli and Widmer, 2010). Advances in the use of PCR techniques provide highly specific methods of monitoring fungal populations in 'real time' and in a quantitative manner, in soils, insects, and in plants (Ownley et al., 2004; Wang et al., 2004; Entz et al., 2005; Castrillo et al., 2008; Meyling et al., 2009; Enkerli and Widmer, 2010; Inglis et al., 2012). Use of qPCR with automated ribosomal intergenic spacer analysis (ARISA) allow soil microbial communities to be profiled and responses to specific events to be monitored; these techniques are likely to be increasingly applied to the study of entomopathogens to assess the fate of biological control species and their impacts on microbial community structure (Ranjard et al., 2001; Hartmann et al., 2005; Shah et al., 2009; Torzilli et al., 2006; Martin, 2007; Enkerli et al., 2008; Enkerli and Widmer, 2010; Inglis et al., 2012).

All biotic factors in soils are influenced by prevailing environmental conditions, soil types, nutrient status, agricultural practices and inputs in the form of pesticides and soil amendments. Intricate interactions between abiotic and biotic factors make it extremely difficult to quantify the specific effects of each of these on the dynamics of entomopathogenic fungi in soil (e.g. Queseda-Moraga et al., 2007). However, we can identify three principle biotic components that have a major influence on fungal persistence and efficacy. These are: soil microorganisms, plants, and invertebrates.

Generally speaking, entomopathogenic fungi are considered weak saprophytes in the competitive soil environment, and introduced inoculum levels will decline in the absence of an arthropod host (Inglis et al., 2001; Roberts and St. Leger, 2004; Längle et al., 2005). Metabolites produced by other soil microbes can adversely affect germination and growth, or be directly toxic, leading to reduced infectivity or multiplication; consequently, survival and efficacy of entomopathogens is commonly superior in sterilized vs non-sterilized soils (Jaronski, 2007). Even so, in native soils conidia will infect a susceptible host when they contact the insect cuticle; *Metarhizium* and *Beauveria* will germinate, grow, and conidiate when applied to soil and amendment of soil with nutrients can overcome (apparent) fungistasis (Keller, 2000; Milner et al., 2003; Bruck, 2005; Chandler and Davidson, 2005; Brownbridge, 2006; Jaronski, 2007; Jaronski and Jackson, 2008). This suggests that fungistasis alone is not the sole reason for the low germination in soil and fungi may require additional host- or nutrient-derived cues to initiate development. Antibiosis also occurs between entomopathogenic fungi and other microorganisms, a phenomenon that has implications for protection of crop plants from pathogens (Ownley et al., 2004, 2010). Very few attempts have been made to evaluate effects of phylloplane microorganisms on persistence and infectivity of fungi applied to foliage, in spite of the fact that plant surfaces are occupied by a diverse range of microfauna (Jaronski, 2010).

Crop plant species and tillage practices affect the prevalence and persistence of fungi (Hummel et al., 2002; Klingen et al., 2002b; Jaronski, 2007). Fungal entomopathogens could be affected by plant surface chemistry and volatiles (Cory and Ericsson, 2010). Some entomopathogens, particularly *M. anisopliae*, are more commonly associated with agricultural (tilled) soils than natural habitats, although fungal prevalence and diversity is normally greater in undisturbed soils (Bidochka et al., 2001, 2002; Inglis et al., 2008; Meyling and Eilenberg, 2007; Meyling et al., 2009). Plant root exudates contain many nutrients that support the development of microbial populations in the rhizosphere; in vitro tests demonstrated that carbohydrates and nitrogen compounds stimulate germination and growth of *M. anisopliae* conidia, while organic acids may inhibit germination (Li and Holdom, 1993). Some *M. anisopliae* isolates are rhizosphere-competent, a trait that enhances persistence in the root zone (Hu and St. Leger, 2002; Bruck, 2005; St. Leger, 2008). The physiological adaptation of the fungus to function as a pathogen or saprophyte involves expression of different gene products, demonstrating that the fungus appears to have evolved various mechanisms that enhance survival in different environments (Wang et al., 2005; Wang and St. Leger, 2007; Bruck, 2010; St. Leger et al., 2011).

Endophytes may be broadly defined as microbes that live in healthy plant tissue (Hyde and Soytong, 2008). Commonly, these are bacteria and fungi that have either no effect or have a beneficial relationship with their host, including the ability to naturally confer resistance to pests and diseases (Backman and Sikora, 2008). Recently, *B. bassiana* has been recognized as an endophyte that occurs naturally in, or has been successfully introduced into a diverse range of plant species (Vega et al., 2008; Parsa et al., 2013). In several instances, colonization of plant tissues by the fungus has provided protection against insect damage or has inhibited insect development and establishment, such as the banana weevil, *Cosmoplites sordidus* (Akello et al., 2007), stem borer, *Sesamia calamistis* (Cherry et al., 2004), and the cyniprid, *Iraella luteipes* (Queseda-Moraga et al., 2009), probably as a result of *in planta* production of insecticidal metabolites by triggering host–plant defenses, or as a result of feeding deterrence/antibiosis. Some isolates have also demonstrated anti-microbial activity and can provide protection against infection by plant pathogens (Ownley et al., 2004, 2010) including most recently, the zucchini yellow mosaic virus in curcurbits (Jaber and Salem, 2014). As endophytes, the fungi are in a protected environment where they are not exposed to abiotic and biotic factors that can limit efficacy when fungi are applied to foliage or the soil, and may offer protection against cryptic species, e.g., stem borers, that would otherwise be difficult to control (Brownbridge, 2006; Jaronski, 2007, 2010).

Foliar topography and chemistry can affect fungal activity and persistence (Jaronski, 2010). While the specific physical traits or compounds responsible for these observed differences are often unknown, the work of several authors indicate that both factors can significantly impact insect infection due to reduced rates of

conidial acquisition (Kouassi et al., 2003; Ugine et al., 2007a, 2007b) and the toxic effects of chemicals produced (as exudates or volatiles) at the leaf surface (Inyang et al., 1998) or consumed by the host (Olleka et al., 2009). Efficacy may be further compromised by the use of inefficient application practices and different spray parameters on crops at different stages of development, which has been clearly shown to affect insect infection rates (Ugine et al., 2007a, 2007b). Clearly, we need to develop a better understanding of the complex interactions between a range of factors, e.g., crop type and physiology, age, fungal strain, pest biology, method of application, etc., to devise efficient use practices.

Invertebrates have many effects on entomopathogen levels in soil. Some, such as Collembola, mites and earthworms, ingest conidia and play a role in their dispersion within and removal from soil (Broza et al., 2001; Dromph, 2003; Milner et al., 2003; Brownbridge and Glare, 2007; Shapiro-Ilan and Brown, 2013). Insect hosts are critical to the long-term survival of many species of entomopathogenic fungi. Access to and successful infection of a host is the only way in which some species can significantly multiply. Fungal prevalence over time may thus be closely correlated with the presence of susceptible insect populations (Meyling and Eilenberg, 2007), although the extent that they reproduce endophytically or epiphytically remains to be determined. Use of insecticides may contribute to the decline of fungal populations by reducing the availability of suitable hosts rather than having direct negative effects on fungal survival (Klingen and Haukeland, 2006). Unfortunately, most studies on effects of chemical pesticides on viability of entomopathogenic fungi have been carried out using in vitro techniques that bear little resemblance to the agricultural system in which the pathogen will encounter the chemical. This is an area of research that could be highly beneficial. Knowledge of positive or negative interactions could allow IPM practices to be adjusted to favor insect infection.

An avoidance response to conidia of both *M. anisopliae* and *B. bassiana* has been observed in mole crickets, which may lead to inconsistent performance of these fungi in the field (Villani et al., 2002; Thompson and Brandenburg, 2005). However, there appears to be variation in the level of response to different isolates (Thompson and Brandenburg, 2005). Insects may also be attracted to fungi. Engler and Gold (2004) showed that termites were attracted to mycelial preparations and volatile extracts of *M. anisopliae*, and *P. japonica* females preferentially oviposited in soils treated with mycelia (Villani et al., 1994). This recruitment effect was also seen with black vine weevil (BVW) *Otiorhynchus sulcatus* larvae, which responded positively to *M. anisopliae*-treated media (Kepler and Bruck, 2006). Such behavioral responses should be taken into consideration when selecting appropriate strains for insect pest management and may be useful in the development of more effective biological control strategies.

4.6. Production and formulation

Following the traditional model, mass production systems have been devised to maximize inoculum yield at the lowest possible cost for use in inundative applications (Wraight et al., 2001; Cliquet and Jackson, 2005; Jackson et al., 2010; Jaronski, 2010; Jaronski and Jackson, 2012). Research emphasis has been placed on optimization of biomass production, stability, and ease of handling for application (Charnley and Collins, 2007). The general assumption has been that control could be achieved if sufficient inoculum could be produced cheaply enough and applied at sufficiently high rates (Brownbridge et al., 2008; Jaronski, 2010). The role of the environment and its impact on fungal activity has not necessarily been a primary consideration driving the development of production and formulation techniques (Jackson et al., 2010). However, there is considerable scope to modify production media and techniques to provide more ecologically competent infective material that is better suited to use in specific environments. Greater knowledge of prevailing ecological factors in the pest's habitat will allow potential constraints to fungal survival and/or infection to be identified, and will provide leads for research to overcome these constraints. When combined with development of alternative delivery mechanisms, it is likely that more efficacious microbial control products will become available.

Efficacy against soil-inhabiting pests is influenced by many biotic and abiotic factors. Consequently environmental factors are critical to performance, and maintenance of bioactivity must be a primary consideration when developing production media (Kiewnick, 2004; Tarocco et al., 2005; Brownbridge, 2006; Jaronski, 2007, 2010). Formulation can enhance characteristics or render fungal preparations easier to apply, but their performance is ultimately reliant upon inclusion of robust biological material that is "fit for purpose" (Jackson, 1999; Brownbridge et al., 2008). The production method selected will depend upon the nature of the inoculum required, and isolates may have different growth characteristics on different production media (Shah et al., 2005; Charnley and Collins, 2007; Jaronski and Jackson, 2012). An excellent overview of ecological considerations in the production and formulation of entomopathogenic fungi was recently published by Jackson et al. (2010), and readers are referred to it for a more complete review of these factors.

Solid substrates have been widely used to produce aerial conidia of entomopathogenic and other beneficial fungi (Kiewnick, 2001; Wraight et al., 2001; Krishna, 2005; Charnley and Collins, 2007; Jaronski and Jackson, 2012). Temperature, pH, aeration and substrate components all influence conidial yield, viability, stability and virulence (Jaffee and Zasoski, 2001; Shah and Butt, 2005; Shah et al., 2005; Rangel, 2006; Jackson et al., 2010). Although these parameters are more difficult to regulate in a solid-substrate system, this remains the predominant method used for commercial products due, in part, to the flexibility of a system that lends itself to the cottage-industry production scale used in many parts of the world. Solid-state fermentation bioreactors yielding up to 3×10^{13} conidia per kg of substrate have been developed (Jenkins and Gryzwacz, 2000; Wraight et al., 2001; Kiewnick, 2004; Kang et al., 2005; Kiewnick and Sikora, 2006; Jaronski and Jackson, 2012).

The economies of large-scale liquid fermentation processes for microorganisms is well established and has provided the paradigm for the mass production of microbes with pharmaceutical (e.g., production of insulin) or nutraceutical (e.g., probiotics) applications. Large-scale liquid fermentation systems are successfully used for agriculturally important bacteria (e.g., *B. thuringiensis*, *S. entomophila*). In submerged culture, fungi generally produce vegetative propagules – mycelia or yeast-like blastospores; culture conditions and media composition will have a primary influence on the type and amount of inoculum produced (Jackson et al., 2003; Vega et al., 2003; Cliquet and Jackson, 2005; Charnley and Collins, 2007; Jaronski and Jackson, 2012). Production systems have been designed with high yield as a primary goal but again, the infectivity of the resulting biomass and its ecological competence and stability are key factors that must be considered during process development. Culture conditions and media can be manipulated to impart specific traits to the resulting biomass, including enhanced infectivity (potency) and stability during drying and in storage (Vega et al., 2003; Cliquet and Jackson, 2005; Liu and Chen, 2005; Leland et al., 2005a, 2005b; Jackson et al., 2006; Jaronski and Jackson, 2008, 2012). Jaronski and Jackson (2008, 2012) and Jackson et al. (2010) recently described methods to induce production of microsclerotia by *M. anisopliae* in liquid media. The aggregates were readily air-dried, stable at room temperature, and showed superior efficacy against sugarbeet root

maggot in soil assays compared with conventional corn-grit granules. The material sporulated profusely in non-sterile soils and was active at low soil moisture levels (Jaronski, 2007; Jaronski and Jackson, 2008). Such production/formulation techniques overcome some of the biotic and abiotic constraints to fungal efficacy and may increase opportunities to utilize these biocontrol agents against soil pests.

Advances in formulation technologies now permit stabilization of environmentally sensitive microbes and have applications to a diverse variety of beneficial organisms. Formulations can improve the handling characteristics and safety of a microorganism (e.g., by eliminating spore dust during preparation of a spray mixture), enhance stability pre- and post-application, improve persistence, promote efficacy, and facilitate easy delivery to the target pest (Wraight et al., 2001; Brownbridge, 2006; Brownbridge et al., 2006; Jackson et al., 2006; Thompson et al., 2006; Charnley and Collins, 2007; Jaronski, 2007, 2010; Jaronski and Jackson, 2008; Jackson et al., 2010). Critical, however, is maintenance of viability, ideally even when storage conditions are sub-optimal (Jackson et al., 2010). Effective formulation is integral to the wider utility of microbial pesticides in agricultural production systems, and microbes can fail if formulated poorly. Formulations may be tailored to suit the environment in which the microbial will be used, the delivery system envisioned, and the nature of the inoculum being used. Like production systems, they must be rationally developed to ensure retention of key characteristics that are critical to microbial efficacy, in both foliar and soil environments (Jaronski, 2010). For example, an oil formulation of *M. anisopliae* var. *acridum* was developed to overcome the limitations of dry habitats for the control of locusts and grasshoppers (Lomer et al., 1999, 2001; Bateman, 2004; Moore, 2008).

4.7. Improving delivery

While mass production systems can be refined to overcome particular environmental constraints, strategies for more efficient use also need to be investigated to capture the full potential of these microbes, as well as to reduce the amount of inoculum required to achieve satisfactory control because there is a physical and economical limit to the amount of material that can be applied. Some circumstances may require repeated pesticidal application of fungal biocontrol agents where simple sprays are not appropriate or effective. Control of cryptic insects, for example, cannot be achieved using conventional sprays. We thus need to look to application techniques that are not only more efficient, but use less material. As with other development criteria, consideration of the pest's biology is paramount to devising novel delivery techniques.

The pollen beetle *Meligethes aeneus*, is a widespread pest of oilseed rape and other important cruciferous crops in Europe. Adults and larvae feed on pollen in buds and open flowers, affecting seed set and hence yield. The beetles are very difficult to reach with regular sprays in this protected environment. Honey-bees (*Apis mellifera*), frequent visitors to oilseed crop flowers to forage for nectar and pollen, were successfully used to vector dry *M. anisopliae* conidia to flowers of oilseed rape, leading to subsequent high levels of pollen beetle mortality and mycosis (Butt et al., 1998). Honey bees have subsequently been used to disseminate *B. bassiana* to canola flowers for control of tarnished plant bug, *Lygus lineolaris* (Al-mazra'awi et al., 2006a) and can vector dry conidia to a range of agriculturally important crops, demonstrating additional opportunities to use bees to deliver these control agents (Al-mazra'awi et al., 2007). Bumble bees are used to pollinate many greenhouse crops, and can also be employed to vector *B. bassiana* and other microbial inoculants to control thrips, tarnished plant bug and grey mold in greenhouse tomato and sweet pepper (Al-mazra'awi et al., 2006b). In all cases, fungal delivery was efficiently targeted to the portion of a crop where pest damage was occurring, and relatively small amounts of conidia were needed to effect control (Kapongo et al., 2008a, 2008b; Kevan et al., 2008).

Fungi can be delivered into the soil environment via seed coatings. This technique has traditionally been used to protect seeds and developing seedlings from soil-borne diseases and subterranean pests with persistent broad-spectrum fungicides and insecticides. With the advent of new polymers that can be used to coat materials onto seeds without heat, seed-coating with microbes has become possible. Seed coating with fungal inoculants can be used to establish fungi such as *Trichoderma* spp. in the rhizosphere and prevent losses to root diseases. Rhizo-competent entomopathogens such as *M. anisopliae* may establish on the developing roots of seedlings, mitigating insect damage, and endophytic entomopathogens such as *B. bassiana* may colonize the plant providing resistance to plant pathogens. Although the biological control effectiveness of these approaches needs to be validated, targeted suppression of a pest with reduced amounts of inoculum could be provided.

Efficiencies may also be realized using auto-dissemination devices. Several insect pests have been effectively regulated using this approach (Vega et al., 2007; Baverstock et al., 2010). Tsetse flies, *Glossina* spp., are major impediments to rural development in many African countries. Previous control attempts have focused on habitat manipulation and widespread application of insecticides. The long-term efficacy of these approaches is poor and the high cost and environmental risks posed by widespread insecticide applications have provided the impetus to develop alternative management approaches. Area-wide spray applications of fungi are impractical due to issues of cost, targeting, and poor field persistence, creating an ideal scenario for development of an auto-inoculation device. Various traps have been devised that are highly attractive to tsetse, e.g., bi-conical traps baited with cow urine (Dransfield et al., 1990); by combining this technology with an inexpensive trap-and-release inoculation device, an efficient and economical method of delivering lethal doses of *M. anisopliae* conidia to adult tsetse was developed in Kenya (Maniania, 2002). A similar approach was taken to the development of an auto-dissemination device for control of adult fruit flies (Dimbi et al., 2003; Ekesi et al., 2007). The potential for horizontal transmission among inoculated individuals further enhances the likelihood that these pests can be controlled using fungi in an auto-inoculation device (Quesada-Moraga et al., 2008; Thaochan and Ngampongsai, 2015).

Auto-dissemination devices show promise for use against pests of field vegetable and fruit crops, and in forested areas, where widespread conventional applications of fungal pathogens are impractical. A common behavioral phenomenon among many beetles is that they overwinter *en-masse*, providing opportunities to target a fungal treatment to a compact population (Dowd and Vega, 2003). Overwintering sap beetles, *Carpophilus luqubris*, were contaminated and infected with a virulent strain of *B. bassiana* using an auto-inoculative device baited with pheromones. Insects were targeted as they left harvested cornfields in the fall; the disease spread within the population by horizontal transmission and established in the overwintering population (Dowd and Vega, 2003). Autoinoculative devices were also successfully used to introduce *B. bassiana* into a population of spruce bark beetle, *Ips typographus* (Kreutz et al., 2004). Transmission of the pathogen occurred between treated and non-treated individuals and significantly reduced adult beetle damage to spruce trees and numbers of beetle larvae under spruce bark. The capacity to control other insects of agricultural importance using this technology has been reviewed by Vega et al. (2007). This includes pests with cryptic habits such as leafminers, which are very difficult to control with microbial or conventional pesticides (Migiro et al., 2010).

Knowledge of pest biology is essential to the development of these novel yet simple technologies, which have excellent potential to provide selective and cost-efficient means of control.

Insect behavior may be manipulated with a variety of allelochemicals and other compounds in ways that may improve the efficiency of pathogen-based pest control strategies (Pell et al., 2007; Baverstock et al., 2010). For example, a variety of thrips allelochemicals will attract, arrest or repel these insects, raising the possibility of using these materials to concentrate thrips into specific areas of a crop (Tsao et al., 2005; Teulon et al., 2007a, 2007b; Davidson et al., 2007, 2008). Use of attractants with repellent compounds allows us to consider development of a "push–pull" approach in greenhouse crops (van Tol et al., 2007). By concentrating infestations in a limited area, control efforts can be focused there, rather than blanket-spraying an entire crop.

The differential attraction of some insect pests to particular plant varieties or species offers another way in which pest behavior can be modified to enhance the efficacy of fungal biocontrol agents. For example, western flower thrips are more strongly attracted to some varieties of chrysanthemum, which can be used as 'trap plants' within a production system (Buitenhuis and Shipp, 2006). Trap plants can be arranged as "islands" within a crop and fungal biocontrol agents applied to the islands within a wider cropping area. Despite a wide host range, the black vine weevil has distinct preferences for feeding and oviposition. Adults are differentially attracted to plant volatiles (van Tol et al., 2002), and insect feeding damage on *Taxus* and *Euonymous* spp. invokes the production of odors that are highly attractive to other beetles (Van Tol et al., 2002, 2004). These and other attractive plants can be used as trap crops to limit weevil distribution and egg-laying to specific areas, allowing control efforts such as *M. anisopliae* (Bruck, 2005; Shah et al., 2007) to be focused on the trap plants. Furthermore, some fungi appear to attract the weevils, which may further improve efficacy (Kepler and Bruck, 2006). By defining more efficient use practices for insect pathogens, such controls become more cost-effective.

Synergistic interactions have often been observed when fungal pathogens have been co-applied with sub-lethal doses of insecticides. Synergism is thought to occur due to the action of the insecticide on the insect's behavior, either stimulating movement through treated media in an attempt to escape to a less toxic environment and, in the process, leading to the acquisition of more fungal inoculum, or adversely affecting movement and grooming behavior, leading to greater retention of inoculum on the body of an insect (Quintela and McCoy, 1998; Jaramillo et al., 2005; Shah et al., 2007, 2008; Ansari et al., 2007). Synergism leading to improved efficacy and control may also occur when different species or strains of fungi are applied concurrently. For example, combined application of *B. bassiana* and *M. acridum* (identified as *M. flavoviride*) could be used to overcome some of the constraints of temperature in thermoregulating pests such as grasshoppers, especially where temperatures fluctuate or are high for a significant period of time (Inglis et al., 1997). Application of entomopathogenic fungi can also be practiced in combination with other insect pathogens, including nematodes and Bt (Ansari et al., 2008a, 2008b, 2010; Wraight et al., 2009). Combined applications may render the insect host more susceptible by way of compromising health, prolonging developmental stages, or simply by the combined action of two microbes on different components of the pest population. Similar effects can be obtained by using entomopathogens in combination with predators or parasitoids (Roy and Pell, 2000; Wraight, 2003). For example, Labbé et al. (2009) demonstrated that applications of *B. bassiana* for control of greenhouse whiteflies (*Trialeurodes vaporariorum*) was compatible with concurrent use of the parasitoid, *Encarsia formosa*, and the generalist predator, *Dicyphus hesperus*.

Clearly, opportunities exist to use a variety of mechanisms to improve the efficiency of fungal biocontrol strategies. Such approaches can reduce the amount of inoculum needed to control a pest and provide protection against environmental factors that would otherwise rapidly degrade the organism post application, while improving efficacy and cost-effectiveness. This area needs to be explored further rather than remaining focused on the pesticide paradigm.

4.8. Conservation biological control

Contrary to the inoculative or augmentative approaches discussed above, conservation biocontrol relies on the modification of habitats or of crop management techniques to promote the impact of ecosystem service providers, specifically the natural activity of biocontrol agents within a crop system (Steinkraus, 2007a, 2007b; Pell et al., 2010). The successful use of this approach relies on a thorough understanding of the biology and ecology of the pest and the natural enemy complex and, in the case of fungi, conditions that promote the development of epizootics (Pell et al., 2010; Meyling and Hajek, 2010). Although conservation biocontrol may be considered to be in its infancy for entomopathogens, this tactic has been successfully used on a large scale. For example, predictive systems have been devised to inform farmers when conditions favor the development of natural epizootics of *Neozyygites fresenii* in cotton aphids, reducing the need for other mitigation strategies (Steinkraus et al., 2002; Steinkraus, 2007a, 2007b). There are opportunities to create a new norm around the 'use' of these natural enemies. They do not necessarily create commercial opportunities for sale of bioinsecticides, however development of systems whereby environmental conditions can be manipulated to promote the natural incidence and efficacy of fungi can provide an environmentally friendly and efficacious method for pest management. Both entomphthoralean and hypocrealean entomopathogenic fungi can make a significant contribution to pest reduction and can form the foundation of an integrated crop management program (Meyling and Eilenberg, 2007; Pell, 2007; Pell et al., 2010).

Greater adoption of fungal controls in agriculture will rely on achieving greater efficacy, cost reduction, and an ability to broaden the range of pest species that may be targeted. Many of these potential approaches go beyond the use of fungi as microbial pesticides, and require a more ecological approach to their application.

There are several key areas where we must continue to derive new knowledge to advance the development and use of fungal controls. Detailed knowledge of fungal ecology is needed to better understand their role in nature and limitations in biological control. More efficient mass production, formulation, and delivery systems are needed to supply a larger market; most fungi are mass-produced using solid substrates and there are obvious physical limitations to the amount of inoculum that can be produced using these processes. More testing under field conditions is required to identify effects of biotic and abiotic factors and their interactions on efficacy, persistence, and potential limitations to the use of these biocontrol agents in certain crops or locations; and greater investment in the optimization of use practices is needed. There are great opportunities to use fungi in classical and conservation biological control approaches that can improve environmental stability, efficacy and the cost effectiveness.

5. Entomopathogenic nematodes

5.1. Background and overall status

Although there are numerous nematode taxa that have shown potential in biological control, the entomopathogenic nematodes

(EPN), Rhabditida: Steinernematidae and Heterorhabditidae, have been most successful and have received the most attention (Grewal et al., 2005a), and therefore constitute the focus in this article. We include only a brief description of EPN basic biology and life cycles; more detailed aspects may be found elsewhere (e.g., Kaya and Gaugler, 1993; Gaugler, 2002; Grewal et al., 2005a, 2005b).

EPNs kill arthropod hosts via a mutualistic symbiosis with bacteria, *Xenorhabdus* spp. and *Photorhabdus* spp. for steinernematids and heterorhabditids, respectively (Poinar, 1990). Infective juveniles (IJs), the only free-living stage, enter hosts through natural openings (mouth, anus, and spiracles), or in some cases, through the cuticle. After entering the host's hemocoel, nematodes release their bacterial symbionts, which are primarily responsible for killing the host within 24–48 h, defending against secondary invaders, and providing the nematodes with nutrition (Dowds and Peters, 2002). The nematodes molt and complete up to three generations within the host, after which IJs exit the cadaver to find new hosts (Kaya and Gaugler, 1993).

EPNs possess many positive attributes as biological control agents (Shapiro-Ilan and Grewal, 2008). They are safe to humans and are generally safe to other nontarget organisms and the environment (Akhurst and Smith, 2002; Ehlers, 2005), which has led to a lack of pesticide registration requirements in many countries such as the United States and nations in the European Union (Ehlers, 2005). With few exceptions, e.g., *Steinernema scarabaei* (Koppenhöfer and Fuzy, 2003), entomopathogenic nematodes have a wide host range. Some nematode species have been reported to infect dozens of insect species across five or more orders (Poinar, 1979; Klein, 1990), and certain nematode species are used commercially against 12 or more insect species (see Table 4). Entomopathogenic nematodes are amenable to mass production using *in vivo* (infected insects) or *in vitro* (solid or liquid fermentation) methods (Shapiro-Ilan and Gaugler, 2002; Shapiro-Ilan et al., 2014a).

A number of biotic and abiotic factors affect EPN pest control efficacy (Kaya and Gaugler, 1993; Shapiro-Ilan et al., 2002a, 2006a). Biotic factors such as choice of nematode species and rate of application (generally a minimum of 25 IJs per cm^2 is required) are critical (Shapiro-Ilan et al., 2002a). Environmental factors are also critical in determining efficacy of EPN applications (Shapiro-Ilan et al., 2006a, 2012b). For example, the nematodes are highly sensitive to desiccation and ultraviolet light, thus applications made to soil or other cryptic habitats, and made during the early morning or evening, tend to be most successful. EPNs have been developed as biocontrol agents on a commercial level. They are currently being produced by at least 12 companies in Asia, Europe, and North America (Kaya et al., 2006), and, to date, at least 13 different species have reached commercial development, application, and sales: *Heterorhabditis bacteriophora*, *H. indica*, *H. marelata*, *H. megidis*, *H. zealandica*, *Steinernema carpocapsae*, *S. feltiae*, *S. glaseri*, *S. kushidai*, *S. kraussei*, *S. longicaudum*, *S. riobrave*, and *S. scapterisci* (Lacey et al., 2001; Georgis et al., 2006; Kaya et al., 2006: Shapiro-Ilan et al., 2014a). Commercial application extends to a considerable variety of economically important pests in various commodities (Table 4) (Shapiro-Ilan and Gaugler, 2002; Georgis et al., 2006). Significant advances have increased the biocontrol utility of EPNs since 2001; new pests have been targeted, production and application technologies have been improved, and our fundamental knowledge of ecology and genetics has greatly expanded. The following is an update in research progress relative to EPN application since 2001.

5.2. Novel EPN targets

The quest to develop EPNs for new target pests has remained active. High levels of efficacy have been demonstrated against previously untested (or insufficiently tested) insect pests. Most of the new targets are soil pests because the environment is favorable for EPNs. For example, EPNs have caused substantial field suppression (75–100%) in two root-boring pests of stone fruits, the Mediterranean flat-headed rootborer, *Capnodis tenebrionis* (L.) (Morton and Garcia-del-Pino, 2008; Martinez del Altube et al., 2008) and the peachtree borer, *Synanthedon exitiosa* (Cottrell and Shapiro-Ilan, 2006; Shapiro-Ilan et al., 2009a). In addition to root-borers, advances have been made in effectively controlling soil-dwelling stages of other insect pests, such as the filbertworm, *Cydia latiferreana* (Bruck and Walton, 2007; Chambers et al., 2010), guava weevil, *Conotrachelus psidii* (Dolinski et al., 2006), large pine weevil, *Hylobius abietis* L. (Dillon et al., 2007; Williams et al., 2013b), navel orangeworm, *Amyelois transitella* (Siegel et al., 2006), pecan weevil, *Curculio caryae* (Shapiro-Ilan et al., 2006b), plum curculio, *Conotrachelus nenuphar* (Shapiro-Ilan et al., 2004a, 2008a, 2013; Alston et al., 2005; Pereault et al., 2009), oriental fruit moth, *Grapholita molesta*, (Riga et al., 2006; De Carvalho Barbosa Negrisoli et al., 2013), and small hive beetle, *Aethina tumida* (Ellis et al., 2010; Shapiro-Ilan et al., 2010a).

New developments in EPN usage have also taken place in non-soil habitats. Because nematodes are sensitive to adverse environmental conditions, a major barrier to expanded use of EPNs has been difficulties encountered with application to aboveground targets. Nevertheless, some significant progress has been made in that arena over the past several years, including the application of *S. feltiae* for control of the sweetpotato whitefly, *Bemisia tabaci*, in the greenhouses (>80% control) (Cuthbertson et al., 2007) and application of *S. carpocapsae* for control of *P. xylostella*, which is enhanced by a novel surfactant–polymer formulation (Schroer and Ehlers, 2005; Schroer et al., 2005). Furthermore, *S. carpocapsae* treatments for control of the lesser peachtree borer, *Synanthedon pictipes*, were greatly enhanced by a follow-up application of a sprayable gel that is commonly used for protecting structures from fire (Shapiro-Ilan et al., 2010b), and *S. carpocapsae* treatments resulted in high levels of suppression of the red palm weevil, *Rhynchophorus ferrugineus* when applied in a chitosan formulation (Llàcer et al., 2009). Applications of EPNs to apple tree trunks for control of codling moth, *C. pomonella*, were improved when the treatments included the sprayable fire-gel or wood flour foam as a protective agent (Lacey et al., 2010a, 2010b). Additionally, some promise has been demonstrated for using EPNs for control of stored product pests (Mbata and Shapiro-Ilan, 2005; Ramos-Rodríguez et al., 2006; Athanassiou et al., 2008).

In addition to developing new targets for EPNs, significant expansion and improvements have been made in the control of a number of "traditional" target pests, i.e., those that have been considered commercial targets, or potential commercial targets, for over a decade. A case in point is the use of EPNs for control of white grubs (Coleoptera: Scarabaeidae). Advances in white grub control have been made based on the discovery of new highly virulent steinernematid and heterorhabditid species or strains, as well as an in-depth analysis of nematode–host specificity and elucidation of the mechanisms behind that specificity (e.g., differences in infection routes and optimum soil parameters) (Koppenhöfer and Fuzy, 2003, 2007; An and Grewal, 2007; Grewal et al., 2004; Koppenhöfer et al., 2006, 2007).

A new discovery of particular promise is the recently discovered *S. scarabaei*, which is highly virulent against a variety of white grubs and exhibits long-term persistence in the soil environment (Stock and Koppenhöfer, 2003; Koppenhöfer and Fuzy, 2003; Koppenhöfer et al., 2009). Additionally, enhanced control of codling moth, *C. pomonella* was observed based on use of optimum application equipment, addition of adjuvants, and mulching (Unruh and Lacey, 2001; Lacey et al., 2006a, 2006b). A novel control approach for codling moth is to add EPNs to the water in apple dump tanks, thereby targeting the overwintering insects that are

Table 4
Efficacy and commercialization of entomopathogenic nematodes for suppression of some major insect pests.

Pest common name	Pest scientific name	Key crop(s) targeted	⩾75% Efficacy observed[a]	Targeted commercially[c]
Artichoke plume moth	*Platyptilia carduidactyla*	Artichoke	Yes (Sc)	Yes
Armyworms	Lepidoptera: Noctuidae[b]	Vegetables	Yes (Sc, Sf, Sr)	Yes
Banana moth	*Opogona sachari*	Ornamentals	Yes (Hb, Sc)	Yes
Banana root borer	*Cosmopolites sordidus*	Banana	Yes (Sc, Sf, Sg)	Yes
Billbug	*Sphenophorus* spp. (Coleoptera: Curculionidae)	Turf	Yes (Hb, Sc)	Yes
Black cutworm	*Agrotis ipsilon*	Turf, vegetables	Yes (Sc)	Yes
Black vine weevil	*Otiorhynchus sulcatus*	Berries, ornamentals	Yes (Hb, Hd, Hm, Hmeg, Sc, Sg)	Yes
Borers	*Synanthedon* spp. and other sesiids	Fruit trees & ornamentals	Yes (Hb, Sc, Sf)	Yes
Cat flea	*Ctenocephalides felis*	Home yard, turf	No	Yes
Chinch bugs	Hemiptera: Blissidae	Turf	No	Yes
Citrus root weevil	*Pachnaeus* spp. (Coleoptera: Curculionidae	Citrus, ornamentals	Yes (Sr, Hb)	Yes
Codling moth	*Cydia pomonella*	Pome fruit	Yes (Sc, Sf)	Yes
Corn earworm	*Helicoverpa zea*	Vegetables	Yes (Sc, Sf, Sr)	Yes
Corn rootworm	*Diabrotica* spp.	Vegetables	Yes (Hb, Sc)	Yes
Cranberry girdler	*Chrysoteuchia topiaria*	Cranberries	Yes (Sc)	Yes
Crane fly	Diptera: Tipulidae	Turf	Yes (Sc)	Yes
Diamondback moth	*Plutella xylostella*	Vegetables	No	Yes
Diaprepes root weevil	*Diaprepes abbreviatus*	Citrus, ornamentals	Yes (Hb, Sr)	Yes
Fungus gnats	Diptera: Sciaridae	Mushrooms, greenhouse	Yes (Sf, Hb)	Yes
German cockroach	*Blattella germanica*	Household	No	Yes
Grape root borer	*Vitacea polistiformis*	Grapes	Yes (Hz)	No
Iris borer	*Macronoctua onusta*	Iris	Yes (Hb, Sc)	Yes
Large pine weevil	*Hylobius albietis*	Forest plantings	Yes (Hd, Sc)	Yes
Leafminers	*Liriomyza* spp. (Diptera: Agromyzidae)	Vegetables, ornamentals	Yes (Sc, Sf)	Yes
Mint flea beetle	*Longitartsus waterhousei*	Mint	No	Yes
Mint root borer	*Fumibotys fumalis*	Mint	No	Yes
Mole crickets	*Scapteriscus* spp.	Turf	Yes (Sc, Sr, Scap)	Yes
Navel orangeworm	*Amyelois transitella*	Nut and fruit trees	Yes (Sc)	Yes
Oriental fruit moth	*Grapholita molesta*	Fruit trees	Yes (Sf)	No
Pecan weevil	*Curculio caryae*	Pecan	Yes (Sc)	Yes
Plum curculio	*Conotrachelus nenuphar*	Fruit trees	Yes (Sr)	Yes
Scarab grubs	Coleoptera: Scarabaeidae	Turf, ornamentals	Yes (Hb, Sc, Sg, Ss, Hz)[b]	Yes
Shore flies	*Scatella* spp.	Ornamentals	Yes (Sc, Sf)	Yes
Sod webworms	Lepidoptera: Pyralidae	Turf	No	Yes
Strawberry root weevil	*Otiorhynchus ovatus*	Berries	Yes (Hm)	Yes
Sugarbeet weevil	*Temnorhinus mendicus*	Sugar beets	Yes (Hb, Sc)	No
Sweetpotato weevil	*Cylas formicarius*	Sweet potato	Yes (Hb, Sc, Sf)	Yes
Wireworms	Coleoptera: Elateridae	Vegetables	No	Yes

[a] At least one scientific paper reported ⩾75% suppression of these pests in the field or greenhouse. Hb = *Heterorhabditis bacteriophora*, Hd = *H. downesi*, Hm = *H. marelatus*, Hmeg = *H. megidis*, Hz = *H. zealandica*, Sc = *Steinernema carpocapsae*, Sf = *S. feltiae*, Sg = *S. glaseri*, Sk = *S. kushidai*, Sr = *S. riobrave*, Sscap = *S. scapterisci*, Ss = *S. scarabaei*.
[b] Efficacy against various pest species within this group varies among nematode species.
[c] http://www.biocontrol.entomology.cornell.edu/pathogens/nematodes.php.

harbored in infested fruit bins (Lacey et al., 2005). Advances in suppression have been made for other established target pests including fungus gnats (Diptera: Sciaridae) (optimized substrate media and timing of applications) (Cloyd and Zaborski, 2004; Jagdale et al., 2004, 2007), the diaprepes root weevil, *Diaprepes abbreviatus* (expansion of control to other host plants) (Jenkins et al., 2008), grape root borer, *Vitacea polistiformis*, (Williams et al., 2010), and the western corn rootworm, *Diabrotica virgifera virgifera*, in Europe (Toepfer et al., 2008).

Research has progressed significantly beyond direct application of EPNs as single control agents for suppression of insect pests. Studies on combining EPNs with other control tactics have increased substantially since 2001. Positive/synergistic interactions have been observed among various novel combinations with chemicals (Koppenhöfer and Fuzy, 2002, 2008; Polavarapu et al., 2007; Reis-Menini and Prata, 2008), microbial agents (e.g., *M. anisopliae s.l.*) (Ansari et al., 2004, 2006a; Acevedo et al., 2007) and arthropod predators (Premachandra et al., 2003). However, neutral or negative interactions with these agents may also be observed depending on the specific pathogens, hosts, or application parameters (Koppenhöfer and Fuzy, 2002; Shapiro-Ilan et al., 2004b). Interestingly, entomopathogenic nematodes have also been reported as synergists in conjunction with GM crops (i.e., Bt-corn) (Gassmann et al., 2008).

EPN research has expanded beyond the targeting of insects pests to include such pests as plant-parasitic nematodes; efficacy in control of plant parasitic nematodes using EPNs has varied based on a number of factors such as target species and the cropping system (Lewis et al., 2001; Fallon et al., 2002, 2004; Jagdale et al., 2002, 2009; Nyczepir et al., 2004; Perez and Lewis, 2004; Lewis and Grewal, 2005; Shapiro-Ilan et al., 2006c). Finally, research has included utilization of nematode symbiotic bacteria partners (separate from the nematodes) or byproducts thereof, as control mechanisms for arthropods (Mohan et al., 2003; Jung and Kim, 2006; Bussaman et al., 2006; ffrench-Constant et al., 2007; Abdel-Razek, 2010; Da Silva et al., 2013) or plant pathogens (Isaacson and Webster, 2002; Ji et al., 2004; Böszörmènyi et al., 2009; Shapiro-Ilan et al., 2009b).

5.3. Advances in basic research

Fundamental research on EPNs expands utility of the organisms in biological control efforts. Basic research in ecology of EPNs has progressed substantially in the past several years. For example, a number of advances in understanding the dynamics of host attraction and infection have been made. Novel cues eliciting EPN responses have been discovered including vibration (Torr et al., 2004), electromagnetic stimuli (Shapiro-Ilan et al., 2009c, 2012a; Ilan et al., 2013), and attraction to plant roots in response to chemical "distress calls" triggered by pest attack (van Tol et al., 2001; Rasmann et al., 2005; Ali et al., 2013). Plant roots were also found to enhance nematode infection by providing routes for nematode

movement (Ennis et al., 2010). Infection and foraging behaviors such as jumping response (Campbell and Kaya, 1999, 2002), response to host exudates (Kunkel et al., 2006), differential response to infected vs. uninfected hosts (Christen et al., 2007; Ramos-Rodríguez et al., 2007), chemical signaling (Kaplan et al., 2012) and olfactory response (Dillman et al., 2012), and competition within the host (male fighting) (Zenner et al., 2014) have been elucidated. Additionally, broad models of host–parasite infection dynamics have been developed and/or tested, such as the phased infectivity hypothesis (Campbell et al., 1999; Dempsey and Griffin, 2002; Ryder and Griffin, 2003), optimal infection strategies based on trade-offs (Fenton and Rands, 2004), risk-sensitive infection and "follow the leader" behavior (Fushing et al., 2009), and aggregative group movement/foraging behavior (Shapiro-Ilan et al., 2014b). These discoveries greatly expand our knowledge of factors that drive foraging and infection strategies (e.g., the discovery of aggregative movement suggests that nematodes may move together in the soil in groups, akin to a pack of wolves).

Fundamental research has also progressed in the realm of soil ecology. Insight has been gained into interactions with other biotic agents such as phoretic associations (Campos-Herrera et al., 2006), an alternative role for EPNs as scavengers rather than parasites (San-Blas and Gowen, 2008), food web response and competition among entomopathogenic or non-entomopathogenic nematode species (Millar and Barbercheck, 2001; Somasekhar et al., 2002; Duncan et al., 2003a, 2003b, 2007; Hodson et al., 2012), and deterrence or susceptibility to antagonists (Zhou et al., 2002; El-Borai et al., 2009). Some of these relationships, e.g., phoretic associations causing enhanced EPN dispersal, have direct impacts toward improved biocontrol efficacy (Shapiro-Ilan and Brown, 2013). Additionally, advances were made in elucidating the impact of soil habitat complexity in reference to EPN spatial dynamics and trophic cascade theory (Efron et al., 2001; Spiridonov et al., 2007; Denno et al., 2008; Hoy et al., 2008; Jabbour and Barbercheck, 2008; Ram et al., 2008). Research focused on soil dynamics, such as the studies cited above, elucidate biotic and abiotic factors that impact nematode distribution and persistence and therefore directly impacts our ability to enhance efficacy of short-term inundative applications, and also serves as foundation for development of inoculative, classical, or conservation approaches (Loya and Hower, 2002; Preisser et al., 2005; Adjei et al., 2006; Barbara and Buss, 2006; Stuart et al., 2008).

Expansion of basic research in entomopathogenic nematology has also been made through extensive progress in fundamental genetic studies including molecular genetics and genomics. Of particular note, the entire genomes of entomopathogenic nematodes and their symbionts have been sequenced (e.g., Duchaud et al., 2003; Bai and Grewal, 2007; Ciche, 2007; Bai et al., 2009, 2013; Schwartz et al., 2011). Additional tools (i.e., RNAi) for evaluating functional genomics of the sequence as it becomes available have been developed (Ciche and Sternberg, 2007), and analyses of certain EPN genes and their expression have already been reported including genes related to stress, involvement in host colonization, and the host–pathogen relationship (Chen et al., 2006; Sandhu et al., 2006; Bai and Grewal, 2007; Tyson et al., 2007; Cowles and Goodrich-Blair, 2008; Hao et al., 2008, 2012; Somvanshi et al., 2008; Bai et al., 2009; Easom et al., 2010). Given the unique characters of EPN biology and the progress made in genetic studies, the entomopathogenic nematode–bacterium complex is being developed and recognized as model system for understanding pathogenicity and symbiosis (Goodrich-Blair, 2007; Clarke, 2008; Hussa and Goodrich-Blair, 2013).

Although the outcomes may not be immediately apparent, advancements in molecular genetics and genomics will cultivate the development of new tools for enhancing biocontrol with EPNs. Additionally, significant progress has been made in applied genetic studies that may have more near-term benefits to EPN utility. For example, new EPN strains with enhanced traits (e.g., environmental tolerance) have been developed through genetic improvement methods of selection and or hybridization (Strauch et al., 2004; Ehlers et al., 2005; Shapiro-Ilan et al., 2005; Nimkingrat et al., 2013). Beneficial trait deterioration is a significant problem that can occur during repeated EPN culturing; for example, virulence, environmental tolerance and reproductive capacity can decline after several passages *in vivo* (Bai et al., 2005; Bilgrami et al., 2006). Insights into the nature of beneficial trait deterioration (Bai et al., 2005; Bilgrami et al., 2006; Wang et al., 2007) as well as the discovery of methodologies to overcome the problem, e.g., through the creation of homozygous inbred lines (Bai et al., 2005; Anbesse et al., 2013), and insight into the specific genes that change (Adhikari et al., 2009) will foster maintenance of strain stability and biocontrol performance.

5.4. Production and application technology

Considerable advances in EPN production and application technology have been made, including liquid culture media improvement (Gil et al., 2002; Islas-López et al., 2005; Chavarria-Hernandez et al., 2006) and increased understanding of the EPN biology, population dynamics, and physical parameters within the bioreactor (Chavarria-Hernandez and de la Torre, 2001; Han and Ehlers, 2001; Neves et al., 2001; Johnigk et al., 2004; Chavarria-Hernandez et al., 2008; Hirao and Ehlers, 2010; Hirao et al., 2010; Belur et al., 2013). Detailed microbiological and molecular aspects of the EPN life-cycle have also been elucidated (Chaston et al., 2013; Moshayov et al., 2013). In vivo production of EPNs has been enhanced through the development of mechanized equipment (Gaugler et al., 2002) and improved inoculation procedures (Shapiro-Ilan et al., 2002b, 2008b; Brown et al., 2006).

Aqueous application has benefited from advanced understanding the impacts of various types of application equipment on the EPNs (Fife et al., 2003, 2004, 2006; Brusselman et al., 2012). Additionally, in terms of application technology, substantial interest in the approach of using infected host cadavers as a vehicle for EPN distribution has been garnered. In this approach, nematode infected hosts are applied to the target area and pest suppression is achieved by the progeny IJs that emerge from the insect cadavers. Over the past several years, a number of different pests have been targeted using the infected host application method (Bruck et al., 2005; Dillon et al., 2007; Del Valle et al., 2008; Jagdale and Grewal, 2008). Research has confirmed that, relative to application in aqueous suspension, infected host application can be superior in EPN infectivity, survival, dispersal, and pest control efficacy (Perez et al., 2003; Shapiro-Ilan et al., 2003b; Fujimoto et al., 2007). Moreover, studies indicate that the approach can be facilitated by formulating the infected hosts in coatings (Shapiro-Ilan et al., 2001, 2010a; Ansari et al., 2009; Del Valle et al., 2009) using hard-bodied insects as the host (Shapiro-Ilan et al., 2008c) and development of equipment to distribute the cadavers (Zhu et al., 2011). Nonetheless, the cadaver application method has thus far only been used commercially on a very small scale relative to conventional methods.

5.5. The future for entomopathogenic nematodes

EPNs have been cultured commercially for more than 25 years. Substantial progress has been made in terms of the number of insect pests that are targeted as well as the number of different nematode species produced. Nonetheless, commercial level application has not reached expectations. In the 1980s and 1990s, companies projected sales of well over $100 million, yet currently the market is closer to only 10% of those projections (Gaugler and

Han, 2002; Georgis, 2002). A number of barriers exist that have hindered further expansion of EPN markets including cost of product, efficacy, and shelf life. These barriers may be overcome through a variety of endeavors as outlined below.

One approach to improving efficacy and expanding the list of target pests to which EPNs can be marketed is to improve the EPNs themselves. Methods to improve and expand the use of EPNs include discovery of more effective strains or species and genetic improvement via selection, hybridization or molecular manipulation (Gaugler, 1987; Burnell, 2002; Grewal et al., 2005b). Discovery of new strains and species is a straightforward approach that can quickly lead to enhanced efficacy based on innate differences in nematode virulence, environmental tolerance, or other properties. For example, in the 1990s, the discovery and subsequent commercialization of *S. scapterisci* for control of mole crickets and *S. riobrave* for *Diaprepes* root weevils and other insects made a considerable impact on EPN markets (Shapiro-Ilan et al., 2002a). The rate of EPN species discovery has been increasing dramatically (Poinar, 1990; Adams and Nguyen, 2002; Stock and Hunt, 2005). Of the more than 100 EPN species reported to-date (e.g., in the last nine decades) more than 40% have been described in the last decade (after 2001). Additionally, the numerous new strains of existing species that are being discovered can also offer enhanced virulence or other properties (e.g., Stuart et al., 2004). Certainly the number of new strains and species will continue to rise, adding more potential options for biocontrol development. However, in order to leverage the advantages that strain/species discoveries offer, biocontrol characterization of these new organisms must keep pace with the survey/discovery research. Currently, less than 20% of the >35 species discovered since 2001 have been tested for biocontrol efficacy in the laboratory, greenhouse, or field; clearly there is significant untapped potential. In addition to expanded utility derived from discovery, we can also expect the upcoming advances in genomics (Bai and Grewal, 2007; Ciche, 2007; Bai et al., 2009, 2013) to offer substantial opportunities for directed strain improvement through genetic methods.

Improved production, formulation and application technology will lead to improved efficacy. Production efficiency and reduced costs are expected with the recent significant increase in number of laboratories or companies that are researching liquid culture methodology as well as the renewed interest in developing efficient automated in vivo systems (de la Torre, 2003; Ehlers and Shapiro-Ilan, 2005; Shapiro-Ilan et al., 2014a). Additionally, fruitful advancements are expected through implementation of novel approaches to application such as distribution of infected hosts, attract and kill methodologies, slow-release teabags, habitat manipulation, and prophylactic plant dips as well as advanced research on the impact of application equipment (Wright et al., 2005; Hiltpold et al., 2012; Nielsen and Lewis, 2012; Duncan et al., 2013). In contrast to production technology, with a few exceptions, activity in development of improved formulation has lagged, and shelf life (particularly at room temperature) continues to be a barrier to expansion of EPN markets. Thus, creative solutions to developing superior formulations are needed; alternatively, new approaches to marketing e.g., "fresh" marketing, where shelf life is not a substantial issue, may be an option.

Commercial use will also expand as the list of target pests deemed suitable for application increases. As indicated above, research toward increasing the use of EPNs to control new or existing targets has been an active area of research over the past decade and we can expect that such efforts will continue. Expansion of target pests and markets depends largely on establishment of field efficacy. At a certain point, if innate virulence is too low then there is little chance for success (Shapiro-Ilan et al., 2002a). Thus, substantial research efforts have been devoted to determining field efficacy, and a large body of literature has demonstrated high levels (e.g., $\geqslant$75%) of control against numerous economically important pests (Klein, 1990; Shapiro-Ilan et al., 2002a; Grewal et al., 2005a) (Table 4). Note that some pests listed in Table 4 have never become significant commercial targets despite the fact that high levels of efficacy can be demonstrated under field conditions. Thus it is clear that efficacy is not the sole factor for establishing market success.

It also should be noted that some of the commercial targets pests are not necessarily strongly supported by high levels of field efficacy (e.g., $\geqslant$75%) reported in several refereed papers. Possibly, some of these pests are not actually suitable for control with EPNs, but are listed as targets by some commercial companies nonetheless. In some of these cases however, it may be that substantial "in-house" research by EPN producers led to the existing markets. Alternatively, it may be that for some target pests, high levels of efficacy, similar to that expected for chemical pesticides, may not be necessary for EPN success.

6. Commercialization

Although research into the use of entomopathogens as MCAs has been conducted for over 150 years (Davidson, 2012) much of the effort has failed to lead to commercially successful microbial pesticide products. While some of the issues are related to biological constraints, a major factor is the absence of a clearly understood model for the commercialization of MCAs. A variety of factors contribute to the potential for market success, which is essentially a measure of cost and benefits including expected protection of the crop and crop value, and efficiency of competing products (Black et al., 1997; Shapiro-Ilan et al., 2002a, 2012b; Ravensberg, 2011; Glare et al., 2012). The development of MCAs is an extremely complex business, which many scientists fail to appreciate properly (Lisansky, 1997).

The publication of the book *A Roadmap to the Successful Development and Commercialization of Microbial Pest Control Products* by Ravensberg (2011) is the first comprehensive attempt to analyze and communicate in a publically available single volume the entire process of developing products from entomopathogens. It is of particular value that examples were drawn from real product development projects and the author explains the regulatory and commercial challenges that may be unfamiliar to research scientists who are focused on biological studies, but that need to be addressed in developing research programs that will facilitate eventual commercialization.

Registration is often identified as the biggest barrier to commercialization of MCAs (Montesinos, 2003; Chandler et al., 2008; Ravensberg, 2011; Sundh et al., 2012a). The issues around registration of MCAs have been discussed extensively in three recent books that addressed ways to simplify registration and reduce the costs for MCA development (Bailey et al., 2010; Ehlers, 2011; Sundh et al., 2012b). MCAs must be regarded as living entities within an ecosystem rather than simply as replacements for chemical pesticides (Sundh and Goettel, 2013). Kabaluk et al. (2010) compared in detail many registration systems used worldwide. The particular issues of developing successful MCA products for Africa have also been explored in some detail (Cherry and Gwynn, 2007; Grzywacz et al., 2009).

7. Conclusions

Globally, pests annually consume the amount of food estimated to feed an additional one billion people (Birch et al., 2011). The human population is expected to grow from 6 billion today to 9 billion in 2050 and the amount of food produced must increase commensurately. Increased crop production will mean increased

amounts of food available for pests, with pest population increases and higher pest pressure as a consequence.

The higher cost associated with the current generation of microbial pesticide products in comparison to most chemical insecticides is still considered a major limiting factor in many promising markets, especially in Asia and developing countries (Skovmand, 2007). The expanding global impact of Maximum Residue Limit regulations in removing older cheaper broad spectrum chemicals is expected to lower this barrier somewhat, although the ready availability of cheap "off patent" pesticides in many markets still constitutes a serious challenge to microbial pesticides.

Glare et al. (2012) contend that MCAs have not yet reached their full potential, even though all predictions suggest microbial pesticides will outperform other pest control options in terms of market share increases in the near future. While the outlook for most microbial products is more positive than it has been for many years, there are a number of generic issues that will determine how much their use expands in the near to long term future.

Most MCAs are arthropod-specific, and most crops are likely to be affected by a suite of pests, therefore MCAs will need to be successfully integrated with other microbial products or pest management strategies in order to provide the comprehensive pest control that farmers require. Several studies have been carried out to assess interactions of insect pathogens with chemical pesticides and fungicides. In general, few deleterious effects have been observed under field conditions and adverse effects observed in vitro were often not reliable predictors of antagonism under natural conditions. We cannot assume that all biocontrol agents, simply because they are living organisms, are compatible or interact positively, yet few studies have documented interactions among MCAs. The importance of such studies is evident, and clearly more research is needed to provide integrated, compatible, cost-effective and reliable bio-based pest control strategies for cropping systems, not only for individual crop pests. For example, synergistic virulence to the scarab, *Cyclocephala* spp., was observed for combinations of EPNs with *P. popilliae* (Thurston et al., 1993, 1994) or with *B. thuringiensis* subspecies *japonensis* (Koppenhöfer and Kaya, 1997; Koppenhöfer et al., 1999). However, interactions between entomopathogenic nematodes and other entomopathogens can also be antagonistic (Baur et al., 1998; Brinkman and Gardner, 2000; Koppenhöfer and Kaya, 1997; Shapiro-Ilan et al., 2004b). Advances in our understanding of infection processes, combined with the availability of new molecular tools that aid our ability to monitor the fate of entomopathogens in the environment and quantify effects of environmental factors on efficacy and persistence, continue to provide new insights that will support the rational development of these technologies.

Legislation to increasingly restrict the residues of chemical pesticides in agricultural produce (including flowers and non-food products), is providing a major thrust for farmers to adopt non-chemical controls in place of chemical pesticides. Consumer awareness and demand is also driving major produce retailers to force growers to implement more sustainable pest and disease management techniques. This is creating new market opportunities for microbials and resulting in the expansion of the range of microbial products available to farmers. There seems little doubt that over the next decade major new opportunities to expand the use of microbials in agriculture will occur.

However, while legislators are reducing the number of chemical pesticides and restricting their use, the regulatory agencies continue to operate in a regulatory framework for chemicals, which restricts progress by regulating microbial pesticides similarly to chemical insecticides. While there are moves to change regulations to create an easier pathway for the registration of biologicals, the current system remains a major impediment to the wider availability of microbial pesticides and their expanded use. Greater harmonization of registration practices across international boundaries, and acceptance of 'generic' safety data will help to streamline the registration process, and reduce the time and cost of bringing new microbial products to market.

Microbial products, even when effective, must be able to compete successfully with other non-chemical technologies such as cultural controls, predators and parasitoids, on both cost and ease of use. This requires that research focuses on improving production techniques to lower costs and on formulation to improve storage and use, as well as on persistence to reduce the need for frequent application. A major task is to ensure that quality products are available and that farmers are equipped with the knowledge to apply them. By focusing resources on transitional research to devise robust practices, microbial pesticides can become important components of integrated crop production systems.

8. Recommendations

Clear efforts must be made to engage stakeholders along the entire marketing chain including producers, regulators, farmers, retailers and consumers, to ensure acceptance and support of biocontrol approaches and the incorporation of MCAs in IPM strategies. Outreach and demonstration programs that promote understanding of what growers can (or cannot) expect from these control agents, coupled with appropriate training on their use, will further enhance their successful integration into agricultural production systems. Even though the climate for microbial pesticides is becoming more positive, significant research is still needed to overcome the limitations of current microbial products and expand the range of products available if they are to play a significantly greater role in the next generation of farming and pest control. Our recommendations to address these needs include:

1. Continue the search for new entomopathogens. Given the withdrawal of chemical pesticides, new and diverse host-specific and multi-host entomopathogens are urgently needed. Pathogens can provide new efficacious MCAs and also the genetic diversity needed for adaptation to a wider range of habitats and climates. New entomopathogens can also serve as sources of novel genes for insect resistance and other advantageous traits that can be incorporated into the genomes of other microorganisms or plants.
2. Continue development of production, formulations and application methods that will improve the efficacy, user acceptability and cost efficiency of MCAs for a variety of crops and climates.
3. Focus on strategic selections of target pests and markets to meet the challenge of developing non-chemical control of global pests, including disease vectors. Control of vectors of human, animal and plant diseases is a growing global public priority and MCA research needs to address these targets.
4. Continue development of transgenic plants using MCA genes for additional major crops. Develop objective and evidence-based knowledge to increase public understanding of transgenic crops.
5. Adopt streamlined registration procedures for MCAs and harmonize global registration systems.
6. Conduct further studies on the ecology of insect pathogens and their role in the environment, which will increase their potential for efficient and sustainable use in pest management.

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