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Entomovectoring for Precision Biocontrol and Enhanced Pollination of Crops

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Preface

We are happy to present this unique book, not only because of its novelty and interdisciplinary content but also because it provides a different view on bees and how we can employ their pollination behaviour for biodiversity and sustainability on our planet. Yes, the major evolutionary diversification of nectaries in late Cretaceous flowers, about 120 million years ago, also signals the beginning of the mutualism between Hymenoptera and angiosperms. Bees are the best example of this mutualism. When bees go from flower to flower collecting pollen, they also deposit pollen grains onto the flowers, thereby pollinating them.

Indeed, today, the role of insects in pollinating flowers is a commonplace. Pollinating insects by their very activity spread tiny particles (pollen grains) between plants, so why not using them to disseminate other tiny particles, such as microbes, that can serve to suppress plant pests and pathogens? This book is a collection of papers that reviews the concepts and technology that have been developed over the past recent decades and explains some specific applications for crop protection. Chapter 1 introduces some of the newer approaches to using managed pollinators and conserving wild pollinators in agricultural settings. The diversification of approaches to using managed pollination and the roles of wild pollinators in agriculturally dominated landscapes set the stage for the scope of using pollinators for other beneficial roles, including pest management. Chapter 2 places the concepts of "bee vectoring technology" (BVT) as "entomovectoring" or "apivectoring" into the framework of "ecological intensification", a newly coined concept of using and managing biodiversity and ecosystem complexity in agriculture. It also explores, with comprehensive thoroughness, the scope of apivectoring science with respect to the kinds of pollinators that can be used and the kinds of biocontrol agents that can be disseminated by pollinators for suppression of crop pathogens and pest arthropods. It recognizes the potential of using technology in protecting managed pollinators from diseases and parasites and also introduces the multifactorial issues of using the technology in responsible ways from assessing the agents, the diluents, the delivery systems and the possible consequences in the human food chain and environment as they pertain to practical application for food security. Of course, there are regulatory issues to be considered, and they are reviewed in useful detail in

Chap. 14. Moreover, it also recognizes that arthropods used in biocontrol programmes could serve as vectors of other beneficial microbes. Chapters 3 and 15 introduce the value and service of bees as pollinators of crops. Chapter 15 focuses on commercially available managed bumblebees. As such, both chapters serve to cement the links between Chaps. 1 and 2.

Chapter 4 delves deeper into pollinator diversity and focuses on the use and potential of the diversity of solitary bees that are used for crop pollination. As such, Chap. 4 is an important segue, especially for Chap. 6 which explores the diversity of dispensing devices that can be used on the wide diversity of managed pollinator domiciles. These range from the familiar beehive through to domiciles for bumblebees and to the challenges posed by various artificially produced nesting arrangements for solitary bees. Chapter 5, as a specific example, explores the successful use of bumblebees in open field conditions for entomovectoring fungal disease suppressing agents for strawberry crop protection. This chapter is the first in the book that addresses specific examples. Chapter 7 zeroes in on the successful application of the technology in greenhouse vegetable and fruit production. Chapter 8 presents a case study for the utility of the technology in setting the stage for addressing apple storage rot problems at the time of apple pollination. Chapter 9 suggests that an invasive species of pestiferous fruit fly could be suppressed by using pollinators as entomovectors of entomopathogenic microbes. This chapter further expands the potential of entomovectoring against agricultural insect pests as reviewed in Chap. 2. Coffee is globally the most traded and valuable agricultural commodity and benefits from the activities of managed and wild pollinators. Chapter 10 addresses the potential for the use of pollinator entomovectoring by Africanized ("killer" or "assassin") honeybees for the suppression of several coffee diseases and insect pests on the basis of practical research experience in Brazil, Mexico and Ecuador. Chapter 11 explains how bumblebees have been used successfully in Serbia (and in Canada) to suppress sunflower head rot, potentially a very valuable technology for high value-added hybrid seed and confection seed production. In Chap. 12, a comprehensive study from Colombia is reviewed. It explains the successes achieved by using Africanized honeybees as vectors of a biological control agent against fungus diseases on commercially operating strawberry farms. The work is explained from the conceptual base through to the economic advantages to farmers.

In Chap. 13, it is recognized that certain pest insects spread crop diseases but that they could also be used to spread microbes that fight the very diseases the pest insects also carry.

Throughout the book, the authors have made specific mention of the funding agencies that have supported their research and development. Important was the ERA-Net named "Coordination of European Transnational Research in Organic Food and Farming Systems" with the "Bicopoll" project. Special and general thanks are extended to the "International Commission for Plant-Pollinator Relationships" (ICP-PR) and the "International Union of Biological Sciences" (IUBS) for their overarching support on a global level and especially for the sessions at the "XI International Symposium on Pollination" in Berlin (2018) and sponsoring the "International Advanced Course on Using Managed Pollinators for Dissemination

of Biological Control Agents for Suppression of Insect, Fungal & Other Pests of Crops" held in Belgrade, Serbia, 6–10 May 2019.

Thank you for sharing with us this introduction to a complex, yet easily accessible subject of great fascination! This book is intended for people with interest in bees, nature, agriculture and novel technologies to students, teachers, experts and the common man/woman worldwide.

Guelph, ON, Canada — Peter G. Kevan
Ghent, Belgium — Guy Smagghe
17 June 2020

Contents

Agroecosystem Design Supports the Activity of Pollinator Networks

Bettina Maccagnani, Eve Veromann, Roberto Ferrari, Luca Boriani, and Otto Boecking

1 Some Principle Needs of Honey Bees and Wild Bees

Throughout Europe, farmland comprises the major part of land use, namely 48% of the land is agricultural land (European Commission 2016). Traditionally, agricultural land use and biodiversity have been thought to be at opposite extremes, but arable land can be heterogeneous also. Intensively cultivated areas should ideally interchange with non-cultivated and semi-natural elements (green-veins) such as field margins, set asides, woods, hedgerows, brooks, ditches etc. and provide many suitable habitats and resources for the wide range of species common in agricultural landscapes (Bennett et al. 2006; Diekötter et al. 2008; Meek et al. 2002; Tscharntke et al. 2008). These resources include mating and overwintering habitats, food and alternative host resources, shelters and protection from agro-technical activities (Holland et al. 2016). Certainly, the majority of arthropods in agricultural landscapes are reliant on the existence of semi-natural habitats. Thus, semi-natural habitats have the potential to provide and/or support several ecosystem services that are

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the most important for plant producers, such as soil conservation, pest control, and pollination service (Holland et al. 2017). The agro-ecosystem biodiversity is mainly thought to be influenced by the overall habitat heterogeneity in a field's surroundings and also the proportion, quality and spatial arrangement of semi-natural habitats (Fabian et al. 2013; Fahrig et al. 2011; Hendrickx et al. 2007; Schüepp et al. 2011). Fabian et al. (2013) have shown that landscape composition (forest cover and landscape heterogeneity) strongly affects species richness and the abundance of hymenopteran pollinators, wasps and parasitoids.

The importance of pollinators, both managed honey bees and wild pollinators, is hard to overstate – the dependence of global food production on animal pollination has increased over the past decades (Lautenbach et al. 2012). More than 80% of flowering plants depend on animal-mediated pollination for sexual reproduction (for example Kearns et al. 1998; Klein et al. 2007). While there is no doubt on the importance of honey bees as providers of pollination, the value of wild pollinators might have largely been underestimated (Garibaldi et al. 2013). Moreover, facing past and recent colony losses of honey bees, which has decimated up to 53% of European colonies (Neumann and Carreck 2010; Potts et al. 2010), an increasing role of wild pollinators for different crops is expected. Among them, the rich and heterogeneous group of the wild bees consists of species with diverse and sometime specific requirements in terms of nesting habitats and floral nectar resources, so that they can be highly vulnerable to floral diversity and habitat loss and to the habitat degradation caused by intensive agricultural practices. During the last decades, beside the decrease of the honey bees, a strong decline of wild bee populations as well as diversity have also been reported (Biesmeijer et al. 2006). More than 50% of wild bees are rare and listed on national red lists. However, the main crop pollination service is usually provided by the most abundant bee species that are more resilient to landscape changes (Kleijn et al. 2015). In addition to hymenopteran pollinators, hoverflies (*Syrphidae*) are particularly valuable in intensively used agricultural landscapes because they offer two essential ecosystem services, biocontrol and pollination. Hoverflies are generalists and highly mobile therefore they can profit from mass-flowering crops like oilseed rape that provides huge quantities of nectar and protein resources. Similarly to honey bees, alternative pollinators are also threatened by use of agro-chemicals, habitat loss, and landscape fragmentation: in general, by agricultural intensification. Therefore, diversifying the agricultural landscape by including different semi-natural habitats that provide suitable habitats, shelters, overwintering places and food resources can enhance wild pollinators' abundance and species richness and ensure an optimal pollination ecosystem service, which means an increase in fruit and seed quantity and quality.

The agricultural landscape is characterized by a low species diversity of plants with little architectural complexity. In these habitats, plants and animals are short-lived, have high fecundity and a relatively good dispersal capacity but a poor competitive ability. In fact, many agroecosystems are dominated by weeds, insects and pathogens, that are highly adapted for rapid colonization and population increase. In this context, the areas adjacent to crop fields can be extremely important, as they are usually less disturbed and architecturally more complex, with richer and more stable populations of pollinators and beneficial arthropods.

The mixed cultivation systems and, more generally, the botanical species' diversification favor an increase of biodiversity: the practical benefit is the increase in the buffering capacity of the agroecosystem and, consequently, in its greater self-regulation capacity. For this reason, sustainable agriculture tends to go toward this direction.

Boecking and Kubersky (2007) recorded 18 different bee species beside honey bees and one oligolectic bee (*Andrena lapponica*) as potential pollinators during the main blooming period in an organic highbush blueberry orchard in Lower Saxony, Germany. Nine different bumblebee species were the most abundant (36%) within this pollinator species community. However, at that season of the year only bumblebee queens are collecting pollen and nectar, while most of the different species present at that early time of the year are still in the initial stage of colony development. Being the blooming period of the highbush blueberries relatively short in comparison to the long-lasting demands of a developing bumblebee colony, the shortly blooming mass flowering crop cannot obviously provide enough resources (pollen and nectar) for the needs of the bumblebees. This is particularly true while highlighting the need to produce new queens, which is essential to guarantee an efficient pollination service during the crop blooming period in the following year (see Fig. 1). Pollination by bumblebees (*Bombus terrestris*) under cage conditions revealed a four-fold higher blueberry crop (variety 'Patriot') compared to the control cages, where pollinators had been excluded (Boecking and Kubersky 2007). Thus, a positive pollination effect can be postulated for bumblebees, as they are able to buzz-pollinate and all *Vaccinium* species hide their pollen within special pollen tubes. Records along a line transect within the blueberry orchard showed that

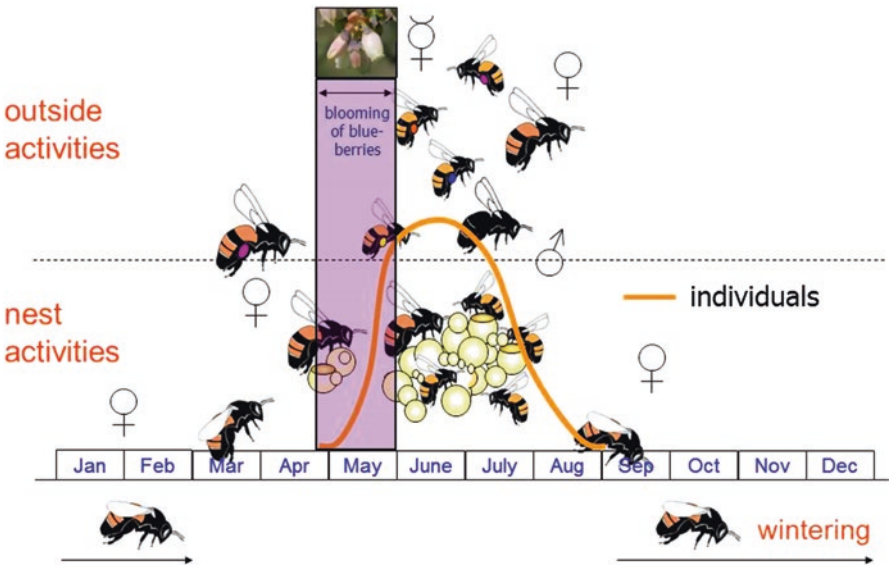

Fig. 1 Schematic drawing of a bumblebee colony development during the course of the year

bumblebee queens are as abundant in numbers as honey bees, whose hives had been placed into the field during the blooming period (see Fig. 2). Since the bumblebee queens recorded along the transect built up their colonies within or nearby the highbush blueberry orchard, it should be of economic interest of the farmers to support this cost-free pollination service in order to optimize the crop yield and its quality.

Blaauw and Isaacs (2014) tested whether wildflower plantings established adjacent to highbush blueberry fields can increase the abundance of wild pollinators (wild bees and hoverflies) during crop blooming and enhance the pollination service and thereby the yield. These plantings included a mix of 15 perennial wildflower species that provided a season-long flower-conveyer. In the four-year study, the authors showed that the crop pollination parameters, including the proportion of fruit set, berry weight and mature seeds per berry, were significantly greater in the blueberry fields adjacent to wildflower plantings, leading to the higher crop yields than in the control. Moreover, the associated revenue exceeded the costs of wildflower establishment and maintenance.

These studies demonstrated that the improvement of yield quality and quantity can be achieved through a finalized landscape management that proved to be, at the same time, economically and environmentally sustainable in terms of pollinators' species conservation. The benefits achievable through a finalized landscape management can increase over time, and, in addition, they can sustain the populations and the heath of the managed pollinators, too.

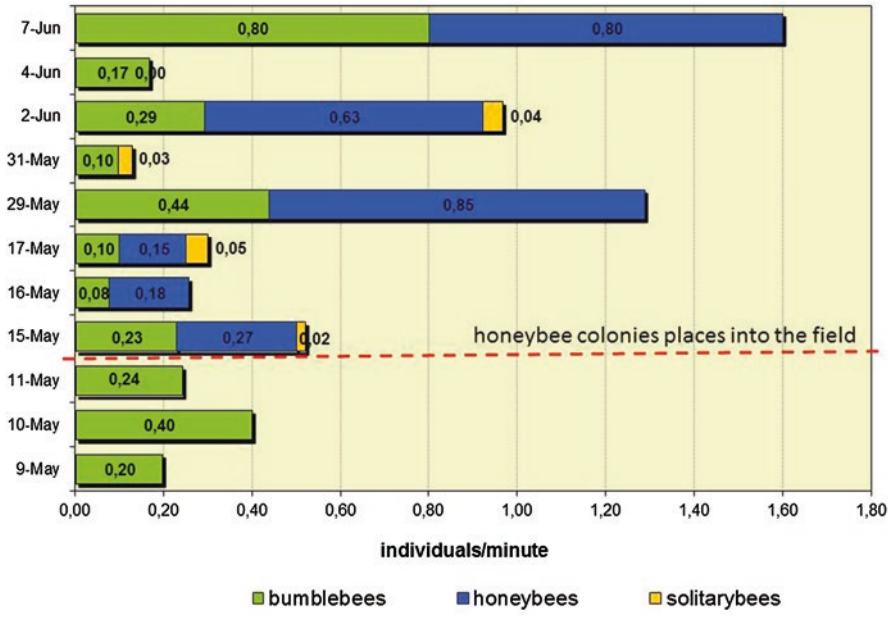

Fig. 2 Flower visits and activity of bumble bees and other wild bees compared to honey bees along a line transect within a high bush blueberry orchard. All recorded wild bees were naturally abundant compared to the honey bees, which had placed with their hives in the orchard

2 Ecological Compensation Areas to Enhance Pollination Service

The maintenance and management of ecological infrastructures such as ecological compensation areas, which can be defined as all natural vegetation and non-crop plants within the rural landscape (semi-natural habitats, hayfields managed at low intensity) are crucial in enhancing functional biodiversity for the conservation of pollinators and pest suppression (Burgio et al. 2004; Burgio 2007; Burgio et al. 2006; Rossing et al. 2003). A recent study revealed that ecological compensation is the most relevant measure to promote farmland biodiversity, as it contributes to species richness and abundance of both flora and fauna (Stoeckli et al. 2017). The beneficial effects depended on the quality, design and spatial arrangement of the ecological compensation areas. Several kinds of intervention can be operated to the aim of providing essential resources for maximize the ecosystem services offered by pollinators and beneficial arthropods.

2.1 Resource Orientated Scheme

One specific landscape management measure is to promote the cultivation of certain mass-flowering crops according to the pollinator group that need to be supported. For example, Rollin et al. (2013) have shown that alfalfa is evenly used by honey bees, bumble bees, and wild bees, whereas crops like oilseed rape and sunflower benefit mainly honey bees and, to a lesser extent, bumble bees, while wild bees are relatively rare in those crops. However, other studies have found that the role of wild pollinators as oilseed rape pollinators may be underestimated (Garibaldi et al. 2011; Riedinger et al. 2015; Stanley et al. 2013). Mass-flowering fields like oilseed rape and bean fields can strongly increase the visitation rate of bumble bees and syrphid flies on flowering wild plants in semi-natural habitats adjacent to these mass-flowering crops (Haenke et al. 2014; Hanley et al. 2011). Riedinger et al. (2015) found the inter-annual long-term effect of mass-flowering crops such as oilseed rape on wild bees density. They showed that the cultivation of oilseed rape increased the solitary bees' productivity (i.e. bee density in the next year) by six times in comparison to non-enriched cultivated landscape. However, not only the presence of a mass-flowering crop but also its proportion in the landscape matters. The positive effect of mass-flowering crops to adjacent semi-natural habitats occurs only if the proportion of mass-flowering crops in the landscape is low (Haenke et al. 2014). Haenke et al. (2014) found that in landscapes with high proportions of oilseed rape fields, the abundance of syrphids recorded in semi-natural habitats was surprisingly low. This finding indicates that in the case of high oilseed rape proportions, the abundance of pollinators in semi-natural habitats is depressed due to their landscape-wide dilution among easily available oilseed rape fields. In addition, in case farmers add honey bee hives nearby the mass-flowering crops, e.g. oilseed rape, then the

abundance and species richness of wild pollinators will be depressed (Lindström et al. 2016). Lindström et al. (2016) demonstrated that honeybee hives' presence depressed the densities of several guilds of wild pollinators including bumble bees, solitary bees, hoverflies and other flower-visiting insects. Interestingly, surrounding landscape complexity had no impact on the depressing effect. Additionally, overusing of mass-flowering crops, especially oilseed rape, may cause negative effects such as increased competition between crop and wild plants for pollination and thus decrease wild plant pollination rates (Diekötter et al. 2010; Holzschuh et al. 2011; Kovacs-Hostyanszki et al. 2013). Moreover, it is important to bear in mind that pest management in oilseed rape relies heavily on insecticides and some of them are applied during crop flowering and therefore can have detrimental effect on pollinators.

Wildflower strips are aimed to provide pollinators (and biological control agents) with nectar, honeydew and pollen as food resources to maintain high species abundance and richness close to the agricultural fields. The benefits provided by the availability of food sources can potentially also improve biological control by parasitoids (Burgio et al. 2004). Sown wildflower strips are a useful, effective and simple measure, which has been shown to promote pollination service and support various insect groups via increased flower abundance and plant diversity, as well as via improved vegetation structure (Fabian et al. 2013; Haaland and Bersier 2011; Scheper et al. 2013). In fact, plant species composition in wildflower strips has a crucial value: they have to consist of species that offer continuous flower conveyer all over the vegetation period and provide nectar, extra-floral nectar and pollen. Carvell et al. (2007), Smitley et al. (2016) found that pollination service was clearly and rapidly enhanced via improved availability of floral resources, especially if *Centaurea* species were included in wild-flower strips. As plants have dense interspecific competition, it is important to re-sow at regular intervals to compensate for the decrease of flowers in long-term strips.

Bumblebee abundance is effectively enhanced both by narrow strips (Potts et al. 2009; Pywell et al. 2006) and by larger set-aside fields (Alanen et al. 2011) sown with suitable nectar and pollen plants. Flower patches are able to promote the actual pollination success of crops in the landscape (Carvalheiro et al. 2012).

Field margins are a key feature of agricultural landscapes, widespread and easily managed. Removing arable field margins from the cropping system can provide increased foraging resources for bumble bees as well as a greater diversity of habitats for other invertebrates (Meek et al. 2002). Allowing natural regeneration on uncultivated field margins is a simple and inexpensive management option that could be easily achieved by farmers. Kells et al. (2001) have shown that naturally regenerated field margins provide an improved habitat for foraging bees compared with conservation headland. However, in that case some weeds which are highly attractive for bees (e.g. *Cirsium* species) can create some agronomic problems. Therefore, sowing a mixture of perennial grass, wildflower and legume species has obvious advantages for farmers whose field productivity will be improved thanks to the better pollination service assured. The most important factors that influence the abundance and diversity of bumble bees in the field margins are the composition and

seasonal flowering patterns of seed mixtures (Carvell et al. 2007). Sowing a diverse mixture of native wildflowers and non-aggressive grasses on arable margins seems to be best solution to enhance the diversity of different wild pollinators.

2.2 Habitat Oriented Scheme

Forest edges have been shown as ample providers of pollinators. They present a complex vertical structure and an undisturbed soil offering shelters for all bees and a wide range of nesting sites for both cavity and ground-nesting bees. In addition, they provide a diversity of floral resources throughout the bees' activity period. Bailey et al. (2014) have shown a negative effect of distance from forest edge on bee abundance and richness. Distance also affected species composition of bee pollinators (Bailey et al. 2014). Providing nesting sites as close as possible to the arable land enhances the pollination service supplied by wild bees, because native pollinator visitation rate has been shown to drop to 50% of the maximum at a location placed 668 m away from natural habitats (Ricketts et al. 2008). Also hedgerows, that are common linear semi-natural agricultural landscape elements all over the world, provide valuable ecological niches and food resources for pollinators as well as for biocontrol agents (Amy et al. 2015; Ponisio et al. 2016; Garratt et al. 2017). Hedgerows can be a key component within agricultural landscape, for functionally important taxa, but it is clear that the configuration, quality and location of these elements influence the range of ecosystem service delivery (Garratt et al. 2017). Promoting semi-natural woody habitats benefits wild bees which are found more frequently and in greater abundance in semi-natural habitats, especially woody habitats in spring and herbaceous habitats in summer and they are substantially less numerous in mass-flowering crops than honey bees (Rollin et al. 2013). The reason why wild bees prefer to forage in semi-natural habitats is probably because those habitats harbor more diverse food resources and offer suitable nesting opportunities (Potts et al. 2003; Steffan-Dewenter and Tscharntke 2001). Hedgerows, little woods and rows of trees made up of a layer of very dense low bushes, some tall bushes, trees and herbaceous vegetation on the ground can be an area of restoration and refuge, both for pollinators and natural enemies. A well-diversified and developed herbaceous layer also ensures the presence of abundant and prolonged blooms for most of the year, thus providing food for a variety of well-known pollinator groups (honey bees, wild bees, diurnal Lepidoptera, etc.), that can offer their pollination service to surrounding crops, if sufficiently developed and surrounded by a buffer zone. The presence of buffer zones maintained permanently with spontaneous herbaceous species along waterways are a necessary measure to mitigate the effect of human activities and ensure effective environmental sustainability.

To further help pollinators, in these spaces the grass should be cut in late summer, after the flowering of most species, even if the creation of completely uncut refuges on a relatively small fraction of a grassland/hay meadow can quickly and efficiently support pollinating insects (such as wild bees) productivity and their

abundance during the following year (Buri et al. 2014). In fact, the widely used measure of delaying mowing of hay meadows has relatively smaller effects on bees compared to uncut refuges (Buri et al. 2014). Delaying mowing attracts bees to concentrate on the few patches with flowering plants that remain in farmland that otherwise become hostile for pollinators after late spring mowing operations. What is more, creating of uncut refuges does not disturb hay production to the same extent as delayed mowing, because only certain fraction of the meadow remains unsown. A diligent implementation of uncut refuges within extensive hay meadows across the agricultural landscapes could efficiently amplify wild bee populations. Similar to wildflower-sown margins, uncut refuges could be integrated in the toolkit for promoting pollinators within farmland and it would widely improve the pollination services in the agricultural landscape (Buri et al. 2014; Pywell et al. 2011).

3 Interactions Among Neighboring Plants: Favoring Pollination Service or Competition

Highly attractive plant species density and spatial configuration affect the foraging choices of pollinators. For example, the presence of a conspicuous species may increase the number of pollinators attracted to its vicinity, indirectly increasing visitation rates also to neighboring plants. Because pollinator choices are frequently density dependent, the presence of a conspicuous species at high densities may also increase competition for pollination services (Seifan et al. 2014). Highly conspicuous species strongly contributed to the attractiveness of its local patch and thus benefited its neighbors. Because of the strong density effect, the conspicuous species changed its role and became a competitor for the pollinators (Seifan et al. 2014). When the introduced conspicuous species was regularly distributed among other plants in the patch, it increased the visitation rate and the seed set to its neighbors (Seifan et al. 2014).

4 Not Only Pollinators: Ecological Compensation Areas as a Natural Biofactory of Beneficials

Numerous studies carried out in Italy (Emilia-Romagna region) have highlighted the nature of the complex relationship between natural areas (hedges, woods, grassy borders, etc.), beneficial insects, and cultivated fields. There is a continuous exchange between crops and semi-natural areas throughout spring and summer, simultaneously with the movement of beneficial organisms between different crops, and their role is particularly important during critical periods:

- scarce food on the crop (also depending on the seasonal trend),
- non-selective pesticide treatments,

- drought and high temperatures,
- harvesting of the crop, mowing and/or landfill.

Under these conditions, natural enemies and wild pollinators may remain nearby the fields and at the return of favorable conditions in the crop they can quickly re-establish in it. The studies carried out in the Po river's plain (Northern Italy) on uncultivated areas of different botanical structure and composition have highlighted the role of the different plant species as refuges/food source/reproduction sites for beneficial insects. Hedgerows, little woods and rows of trees can be restoration and refuge areas for natural enemies that can become a sort of "biofactory". These areas contain many spontaneous plant species and provide shelter and food for a large number of beneficial organisms (not only predator and parasitoid insects, but also spiders and mites; not only to arthropods, but to several species of vertebrates: amphibians, small reptiles and mammals, and birds). Predators and parasitoids can feed, reproduce and sometimes conclude their life cycle on the ecological infrastructures during the initial and unsuitable stages of crops' development in spring, and may later migrate to the crops to control pests.

Some species among trees and shrubs are particularly rich in beneficial insects: *Populus* sp. (poplar), *Prunus avium* (cherrywood), *Ulmus minor* (Elm), *Acer campestre* (field maple), *Robinia pseudoacacia* (false acacia) and *Pyrus pyraster* (wild pear). Among shrubs, the best results are provided by *Prunus spinosa* (blackthorn), *Prunus cerasifera* (myrobalan), *Cornus sanguinea* (dogwood), *Evonymus europaeus* (spindle-tree) and *Corylus avellana* (hazel). All these plants are authocthonous species and are among the most representative of the typical rural landscape of the main plains in Northern Italy (Boriani et al. 1998). They can feed pollinators and host many species of phytophagous insects of no agricultural interest, which can allow the multiplication of predators and parasitoids. Also, other wild species like *Crataegus monogyna* (hawthorn) represent an excellent food source for pollinators and refuge for beneficial insects. In mature hedgerows, the number of beneficials living in the herbaceous layer was comparable or even superior to that found on shrubs and trees, both as number of species and of individuals, especially in recently planted hedges. Hoverflies, in particular, take advantage of the presence of plants belonging to the Brassicaceae and Apiaceae families. On these plants, they feed on nectar and pollen, which are important carbohydrate and protein sources for the nourishment of adults and egg's maturation for females. Many ladybird (Coccinellidae) species, in the absence of prey, feed on the flower pollen. In this case, usually Apiaceae and mainly *Daucus carota* and *Oenanthe silaifolia*, can be of crucial importance to provide protein-rich food.

Numerous hymenopteran (especially Ichneumonidae, Braconidae and Aphelinidae) and dipteran (mainly Tachinidae) parasitoid species showed a significant increase in parasitic efficiency after the consumption of nectar and pollen from flowers of seeded plants. In particular, among different nectar-providing plant species *Phacelia tanacetifolia* has recently aroused some interest, an annual North-American plant used together with other species to form borders on the edge of the grassy fields in order to attract hoverflies and other wild pollinators.

Even the honeydew produced by aphids can be an important source of alternative food for parasitoids as well as for the true pollinator species, and can sustain their populations and provide continuity in the availability of carbohydrates during the periods of scarce availability of flower species, as very often occurs after the end of the blooming period of the main crops.

5 Ecological Corridors: Experiences in Italy

Building up connections among hedgerows, field margins, mass flowering patches, grassland and meadows represent pathways of spread of pollinators. The presence of ecological corridors decreases landscape fragmentation (a major cause of biodiversity loss) and increases biodiversity on a large scale. There is an increasing body of evidence suggesting that connectivity and quality of habitats have a significant effect on survival of plant and animal species in agricultural landscapes. Roadsides, ditches, hedgerows and other non-crop elements can be seen as reservoirs and corridors of biodiversity in rural landscapes. In farms, ecological compensation areas favor small-scale movements of beneficial insects, while on a larger scale and in complex ecological networks, act as real ecological corridors for the insect (but not only limited to insect) fauna. If sometimes the ecological compensation areas can also host insect pests, the biological balances in which they are established prevent very often that the infrastructure can cause outbreaks of infestation for crops, while supporting wild pollinator populations.

Judicious management of ecological compensation areas is important in order to prevent the damage of arthropod pests on crops (Andow 1991; Altieri and Letourneau 1982; Landis et al. 2000; Wratten et al. 2003) and particular attention is given to ladybirds (Coleoptera: Coccinellidae) for their importance in controlling aphid populations on many crops grown in northern Italy. In a study conducted in Italy, among the many wild species *Sinapis arvensis, Coriandrum sativum, Fagopyrum esculentum, Phacelia tanacetifolia*, are widely used, in combinations that may include leguminous plants (*Medicago sativa, Trifolium pratense, Vicia faba, Vicia sativa*) (Burgio et al. 2004; 2006).

Recent studies aimed particularly to investigate how to sustain wild pollinators in the intensively cultivated Po Valley plain in 2013 and 2014. Trials were conducted in two farms, whose characteristics are summarized in Table 1. In Table 2, the most visited herbaceous plant during the summer season are reported. Data showed the importance of herbaceous perennial plants with flowering periods from spring to late summer, spontaneously present at the field borders, if they are not eliminated by herbicides or repeated cuttings. Their support to pollinators is crucial from June and later, as the flowering of the main crops is concluded (cultivated Brassicaceae, Fabaceae, rosaceous fruit crops).

Samplings performed on categorized group of pollinators (honey bees, bumble bees, solitary bees, hoverflies, butterflies) showed that each botanical species sustains differently the different pollinator groups according to their requirements in

Table 1 Main characteristics of the study farms

	Azienda 1	Azienda 2
Name	Maccaferri	Le Terremare
Location	Sant'Agata Bolognese	Anzola dell'Emilia
Coordinates	44° 41′ 29″ N – 11° 08′ 25″ E	44° 34′ 47″ N – 11° 11′ 20″ E
Main crops	Extensive crops	Fruit crops
Ecological complexity	Scarce	High
Ecological compensation areas	Scarce	High
Crop management type	Integrated Pest management	Organic

terms of need of carbohydrates or pollen, and according to the morphology of the flower and or the collecting device of the pollinators. For example, the numerous species of the Asteraceae family were particularly attractive to honey bees and wild bees. Brassicaceae family were visited mainly by honey bees and syrphid flies, but significantly less by solitary and bumble bees. Bumble bees were numerous on Leguminosae and on some species of the Asteraceae family like *Cirsium* spp.

In 2014, in the same farms trials were run to compare the presence of pollinators between natural meadows and flower strips sowed with the seed mix named Operation Pollinator by Syngenta, with the following composition:

- 1/3 rape (*Brassica napus*) – flowering period: March–April
- 2/3 Fabaceae mix flowering from spring to May: lucerne/alfalfa (*Medicago sativa*), common bird's-foot trefoil (*Lotus corniculatus*), sainfoin (*Onobrychis viciifolia*), French honeysuckle (*Hedysarum coronarium*), red clover (*Trifolium pratense*) and white clover (*Trifolium repens*).

Results per pollinator group are summarized in Table 3. In both of the farms the flower strips presented:

- Higher number of pollinator species
- Higher number of individuals that means that flower strips were highly attractive. Differences were found between flower strips and the natural meadows regard to the density of hymenopterans and dipterans, but not for butterflies.
- High attractiveness also towards the most specialized pollinators, like bumble bees and solitary bees.
- Increased availability of food and refuges also to other beneficials and occasional pollinators like coleopteran species, mainly Coccinellidae.
- Chance of implement the floral biodiversity by colonization of non-sown spontaneous herbaceous plants.
- Very limited management needs, as only one cut was needed at the end of June.

Differences between the natural meadow and the flower strips were observed all along the period of study, except in the first week of July, when the flower strips were cut to renew the flowerings, and in the second half of September, when most of the species were near to the end of the flowering and also most of pollinator

Table 2 Main plant species visited in the flower strips and in the natural meadows (excluding those introduced in the flower strips)

Plant species	Family
Silene alba (white campion)	Caryophyllaceae
Papaver rhoeas (common poppy)	Papaveraceae
Ranunculus spp. (buttercup)	Ranunculaceae
Calepina irregularis	Brassicaceae
Raphanus sp. (wild radish)	Brassicaceae
Potentilla reptans (cinquefoil)	Rosaceae
Medicago lupulina (black medick)	Fabaceae
Melilotus officinalis (ribbed melilot)	Fabaceae
Trifolium campestre (hop trefoil)	Fabaceae
Vicia sativa (common vetch)	Fabaceae
Geranium spp. (crane's-bill)	Geraniaceae
Malva sylvestris (common mallow)	Malvaceae
Daucus carota (wild carrot)	Apiaceae
Convolvulus arvense (field bindweed)	Convolvulaceae
Myosotis arvensis (field forget-me-not)	Boraginaceae
Lamium purpureum (red dead nettle)	Lamiaceae
Melissa officinalis (balm)	Lamiaceae
Mentha sp. (mint)	Lamiaceae
Verbena officinalis (vervain)	Verbenaceae
Veronica persica (common field speedwell)	Scrophulariaceae
Plantago lanceolata (ribwort plantain)	Plantaginaceae
Knautia arvensis (field scabious)	Dipsacaceae
Achillea millefolium (yarrow)	Asteraceae
Bellis perennis (daisy)	Asteraceae
Centaurea x pratensis (knapweed)	Asteraceae
Cichorium intybus (chicory)	Asteraceae
Cirsium arvense (creeping thistle)	Asteraceae
Cirsium vulgare (spear thistle)	Asteraceae
Crepis spp. (hawk's – beard)	Asteraceae
Lactuca serriola (prickly lettuce)	Asteraceae
Picris echioides (bristly oxtongue)	Asteraceae
Senecio erucifolius (hoary ragwort)	Asteraceae
Sonchus asper (prickly sowthistle)	Asteraceae
Taraxacum officinale (dandelion)	Asteraceae

species were near to the end of their life cycle. The main differences in the presence and abundance of pollinators between the two farms with different crop management and different level of complexity or the agroecosystem, was particularly evident for the most specialized pollinators, which were more abundant in the farm that presented a more complex ecological infrastructure. The most relevant species were the lepidopteran *Papilio machaon* and *Iphiclides podalirius*, the hymenopteran *Anthophora* sp., *Anthidium* sp., *Megachile* sp. and *Xylocopa violacea*, whose

Table 3 Pollinator species sampled in the course of the study

Pollinator group	Species name
Bumble bees	*Bombus terrestris*
	Bombus hortorum
	Bombus pascuorum
	Bombus sylvarum
Wild solitary bees	*Andrena* sp. – different species
	Anthidium sp.
	Anthophora sp.
	Ceratina sp.
	Dasypoda sp.
	Eucera longicornis
	Halictus sp. – different species
	Macropis sp.
	Megachile sp.
	Panurgus sp.
	Xylocopa violacea
Butterflies	*Ochlodes venatus*
	Papilio machaon
	Iphiclides podalirius
	Pieris brassicae
	Pieris rapae
	Colias crocea
	Lycaena phlaeas
	Leptotes pirithous
	Polyommatus icarus
	Melitea didyma
	Vanessa io
	Vanessa atalanta
	Vanessa cardui
	Lasiommata megera
	Coenonympha pamphilus
	Pyrgus malvoides
	Erynnis tages
Syrphid flies	*Episyrphus balteatus*
	Eristalis arbustorum
	Eristalis tenax
	Helophilus pendulus
	Melanostoma sp.
	Metasyrphus sp.
	Myathropa florea
	Scaeva pyrastri
	Sphaerophoria scripta
	Syrphus sp.

presence was related to the presence of mature trees or dead trees where nests can be prepared. On the contrary, in the farm with the lower ecological complexity, the presence of borders increased the number of pollinators with respect to the natural meadow but without differences between the pollinator groups.

This finding means that the lower the complexity of the ecological composition of the agroecosystem is, the more important are the ecological compensation areas to attract a wide variety of pollinators. But this is not a sufficient measure, and the next important step is to improve the agroecosystem structure with specific habitats that offer not only food, but also nesting sites, because their availability can be crucial for many species, becoming the main limiting factor of their reproductive success.

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Ecological Intensification: Managing Biocomplexity and Biodiversity in Agriculture Through Pollinators, Pollination and Deploying Biocontrol Agents against Crop and Pollinator Diseases, Pests and Parasites

Peter G. Kevan, Les Shipp, and Guy Smagghe

1 Introduction: Ecological Intensification Beyond Biodiversity

Ecological intensification as it applies to agriculture has been defined as "a knowledge-intensive process that requires optimal management of nature's ecological functions and biodiversity to improve agricultural system performance, efficiency and farmers' livelihoods" (FAO 2013) (http://www.fao.org/agriculture/crops/thematic-sitemap/theme/biodiversity/ecological-intensification/en/, accessed 15 February 2016). FAO (2013) has produced a useful, even if incomplete and hit-and-miss, annotated bibliography that makes reference to the broad generalizations of the value of biodiversity in agricultural systems [e.g. noting especially the important Royal Society report (2009) and pollination, pest regulation, soil nutrients/cycling and to cropping systems (http://www.fao.org/fileadmin/templates/agphome/documents/scpi/Deliverable_7_2_LiberationBibliography.pdf, accessed 15 February 2016)]. Although the benefits of ecological intensification in agricultural environments through attending to biodiversity, biocomplexity and ecosystem functions are presented, the reports (mentioned above) in documenting aspects of

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agricultural productivity, gives short shrift to measuring, and then relating, ecosystem function to that productivity.

Although modern agriculture has sought to simplify and intensify production, the inconvenient truth is that agroecosystems are complex webs of interactions that cannot be simplified and intensified *ad infinitum*. Sustainable agriculture argues that ecosystem services, including biological interactions, must be maintained for production to be maximized. Within the webs of biological interactions is pollination. There is now serious consideration about how biological control (Biever and Hostetter 1978; DeBach and Rosen 1991) and pollination should be more fully recognized in cropping systems for production (FAO 2013; IPBES 2014; Kevan 2015). In this review, we present additional dimensions to how biological control, pollination technology and managed pollinators can be used for crop protection against pests and diseases, for better crop production and how biological systems can be used to protect pollinators themselves.

The relatively recent concern for pollinator biodiversity and pollination as an ecosystem service is quite well addressed, but it will be through applications of fundamental ecological principles that a "knowledge-intensive process" can evolve. The relationships of diversity and abundance of pollinator and floral population dynamics, resilience and sustainability need to be understood for management of sustainable pollination services (Kevan 2015). Our review focusses on how applied ecology can be used for pollinator, pest and pollination management by combining ecosystem services for food and fiber security.

The combination of several ecosystem services for agricultural sustainability requires the recognition that biodiversity is coupled with biocomplexity, productivity, resilience, and ecosystem functionality. We focus on three aspects of the use, and possible protection of, beneficial arthropods (pollinators, predators and parasitoids) and additional benefits they may provide for pest management. One aspect resides in pollination ecology and the potential role of pollinators in pest management. A second is the potential for managed pollinators to disseminate biological control agents, provided to them, into their own nests to suppress parasites and disease. A third is the possibility that beneficial arthropods (predators and parasitoids) managed for biological control may also disseminate beneficial microbes that help suppress pests in crops. Research and development (ecological intensification) on pollinators and Arthropoda biocontrol agents is well established, but the compound benefits of the added microbiological biocomplexity just now becoming realized as further, and manageable, ecological intensification.

2 Pollinator-Mediated Entomovectoring

Hokkanen and Menzler-Hokkanen (2007) and Hokkanen et al. (2015) used that term as "entomovector technology" to describe the use of managed pollinators as disseminators of biological control agents against crop pests (see also Mommaerts and Smagghe 2011). However, "entomovectoring" has much broader connotations, including the other two positive aspects we discuss in this review, and it applies to

the negative issues of insect dissemination of a wide range of ailments that adversely affect plants (Harris and Maramorosch 1980) and animals [including insects and human beings, even for biological warfare technology (Lockwood 2008)] (Mullen and Durden 2009) directly and indirectly. We recognize that pollinators, and other flower visitors, themselves may transmit many microbes and infect crops with diseases while foraging at flowers (e.g. fire blight (van der Zwet and Kiel 1979; Farkas et al. 2011), grey mould (Elad et al. 2007), mummyberry (Woronin 1888; Batra and Batra 1985). It that has been indicated that pathogens and parasites of pollinators, notably bees, can be passed intra- and inter-specifically from individual to individual via flowers (Kevan et al. 1991; Durrer and Schmid-Hempel 1994; Otterstatter and Thomson 2008; Graystock et al. 2015) with consequent adverse effects. McArt et al. (2014) assess a selection of some of the available literature in respect of floral features that can influence such transmissions.

We use more precise designations for the types of entomovectoring with our emphasis on beneficial vectors that disseminate biological control agents against pests and diseases in agriculture. Few studies seem to have even suggested that flower visitors might disseminate plant pathogens that could beneficially suppress populations of weeds or other noxious plants (Kevan et al. 1989; Eisikowitch et al. 1990). Thus, this review focusses on the beneficial facets of entomovectoring.

3 Doubly Beneficial Arthropods: Pollinators in Crops, Domestic Self-Help in the Hive, and Biocontrol Predators and Parasitoids

Pollinators, through their very activities, carry microbial particles. The foremost of those particles are pollen grains themselves, which range in size from 6 to100 μm. Pollinators also carry fungal spores, bacteria, and viruses. Some of those are detrimental to the pollinators themselves, causing diseases, others are detrimental to the plants at which they attend the flowers. Why should modern agriculture embrace pollination, and how can modern agriculture expand the utility of pollination technology to include crop production and protection? Although those ideas are not new, there is growing general interest in using pollinators as vectors of biological control agents for crop protection (Aleklett et al. 2014; Kevan et al. 2003, 2007, 2008, 2014; McArt et al. 2014; Sutherland et al. 2016).

The putative demise of native pollinators in wild to highly managed agricultural systems around the world is cause for environmental and economic concern (Kevan 1999, 2001; Kevan and Phillips 2001; Kevan and Imperatriz-Fonseca 2002, 2006; US NAS 2007; Aizen et al. 2009; Garibaldi et al. 2014). In some cropping systems, there is clear evidence that adding managed pollinators or encouraging communities of wild pollinators boosts yields in quantity and quality, even in those for which many agronomists continue to erroneously claim that pollination management is not needed for self-compatible crops (Richards 2001; Kevan 2015). In other cropping systems, managed pollination is standard practice, such as in pome crops, many forage legumes, some oil seeds (including oil palm), and many soft and tender fruit

(Free 1993; Roubik 1995). It is now standard practice for some greenhouse crops, especially tomato (Veldhuis and van Doorn 2006). As agronomic practices expand and become more intense, the need for managing, protecting or encouraging pollinators is becoming more and more evident (IPBES 2014 ongoing).

Paucity of pollinators leads to crop yields and sometimes quality being below maximal. So do plant pathogens, pests and competitors. Much of modern agriculture relies on chemical interventions (e.g. herbicides, fungicides, insecticides, nematicides, miticides, anti-microbials) for crop protection. Those chemicals carry inherent well-known risks, such as non-target adverse effects, evolution of resistance, and residues that reach to the human food-chain and into ecosystems. Additional to that list of risks, are the adverse effects to pollinators and pollination (Kevan 1999, 2001; Fischer and Moriarty 2014). Moreover, pollinator management (especially honeybee beekeeping) uses its own suite of chemical agents to protect its micro-livestock.

4 Managed Pollinators As Vectors of Biological Control Agents against Crop Pests and Diseases

Based on the reasoning that pollinators disseminate microscopic particles, including pollen and various plant pathogens, several scientific teams initiated research on the use of managed pollinators for the dissemination of biological control agents against plant pathogens (Kevan et al. 2007, 2008; Mommaerts and Smagghe 2011). Table 1 summarizes much of the progress made to date, itemizing the biological control agents, the pathogen or pest against which they were tested, the kind of managed pollinator used, and the crop targeted for protection. Continuing research and development has brought this technology to the point of commercial application in Canada (Kevan et al. 2008, 2014) and Europe (Mommaerts and Smagghe 2011). Throughout the research and development process, a suite of issues and questions has been kept in mind (Fig. 1).

1. What biological control agents could be used against which pathogens and pests, and how those biological control agents could be formulated to minimize adverse effects to the pollinator vectors (see 2) yet be disseminated in sufficient amount to suppress the disease or pest population to acceptable levels (below the economic threshold) for growers? Moreover, can different agents be combined so that one acts as a diluent (at least in part) in formulation of the other, and vice versa?
2. What managed pollinator vectors can be used and under what circumstances, and, as mentioned, how to maintain vector safety? So far honeybees (*Apis mellifera*) have been used in North America, Europe, South America, and Asia. Bumblebees have also been deployed with success in North America (*Bombus impatiens*), Europe (*Bombus terrestris*) and Korea (*B. terrestris*). A small study from Italy used horn-faced mason bees (*Osmia cornifrons*) (Maccagnani et al. 2009). Plans are being developed for further using of alfalfa leafcutting bees (*Megachile rotundata*) in Canada and stingless bees (Meliponini) in Latin America. All those kinds of bees can be used on field crops, but in greenhouses, bumblebees are the most used pollinators (Velthuis and van Doorn 2006).

Table 1 Biological control agents vectored for crop protection by managed pollinating bees. This table is a summary presented in approximate chronological order (for references see also Kevan et al. 2007, 2008; Mommaerts and Smagghe 2011). Sutton (personal communication 2011) has catalogued about 80 species that have been suggested to have suppressive action against plant pathogens and pests. Only those listed below have been used with managed pollinators as the potential vectors

Biocontrol agent	Pathogen/Pest	Pollinator/ Vector	Crop	Location	References
Clonostachys rosea	Grey mold	Honeybee	Strawberry	Ontario, 1992	Peng et al. (1992)
		Bumblebee		Ontario, 2012	Sutton and Kevan (2012)
Pseudomonas fluorescens	Fireblight	Honeybee	Pome	USA, 1992	Thomson et al. (1992); Johnson et al. (1990), (1993a,b)
Pantoea agglomerans a.k.a. *Erwinia herbicola* + *Enetrobacter agglomerans*				New Zealand, 1996, 1998, 1999, 2002	Vanneste (1996); Vanneste et al. (1999), (2002); Cornish et al. (1998)
Heliothis nuclear polyhedrosis virus	Corn ear worm (*Helicoverpa*)	Honeybee	Crimson clover	USA, 1994	Gross et al. (1994)
Clonostachys rosea	Grey mold	Honeybee and/ or bumblebee	Raspberry	Ontario,1997	Yu and Sutton (1997)
Trichoderma harzianum			Strawberry	Italy, 1999	Maccagnani et al. (1999)
				USA, 2000	Kovach et al. (2000)
				Israel, 2006	Shafir et al. (2006)
				Quebec, 2009	Albano et al. (2009)
Clonostachys catenulatum			Raspberry & Strawberry	Europe	Hokkanen et al. (2009), (2011); Karise et al. (2016)
Metarrhizium anisopliae	Pollen beetle (*Meligethes aeneus*) (later with aphid)	Honeybee	Canola (rape seed)	UK 1994, 1998, 2007	Butt et al. (1994), (1998); Carreck et al. (2007)
Bt	Banded sunflower moth	Honeybee	Sunflower	USA, 1999	Joyoti and Brewer (1999)
Binab-T® (*Trichoderma harzianum* and *T. polysporum*)	Cucumber rot (*Didymella byoniae*)	Bumblebee	Greenhouse cucumber	Sweden, 2000	Svedelius (2000)

(continued)

Table 1 (continued)

Biocontrol agent	Pathogen/Pest	Pollinator/ Vector	Crop	Location	References
Trichoderma spp. incl. *harzianum*	Sunflower head rot	Honeybee	Sunflower	Argentina, 2002	Escande et al. (2002)
Bacillus subtilis	Mummyberry	Honeybee	Highbush blueberry	USA, 2004	Dedej et al. (2004)
			Rabbiteye blueberry	USA 2005	Ngugi et al. (2005)
	Fire blight	Honeybee & Mason bee	Pear	Italy, 2006	Maccagnani et al. (2006), (2009)
Coniothyrium minitans and *Trichoderma atroviride*	Alfalfa blossom blight	Alfalfa leafcutting bee	Alfalfa	Alberta, 2005	Li et al. (2005)
Trichoderma harzianum + *Gliocladium virens*	None indicated	Bumblebee	Greenhouse	Italy, 2005	Maccagnani et al. (2005)
Beauveria bassiana	Tarnished plant bug (TPB)	Honeybee	Canola & Sweet Pepper	Ontario, 2006	Al-Mazra'awi et al. (2006), (2007)
Metarrhizium anispoliae	Pollen beetle (*Meligethes aeneus*) + cabbage seed weevil (*Ceutorhynchus assimilis*)	Honeybee	Canola	UK, 2007	Carreck et al. (2007)
Beauveria bassiana + *Clonostachys rosea*	TPB, Green peach aphid, whitefly, Western flower thrips, Grey mold	Bumblebee	Greenhouse tomato and pepper	Ontario, 2008	Kapongo et al. (2008b)
Trichoderma atroviride + *Hypocrea parapilulifera*	None indicated	Bumblebee	None indicated	Belgium, 2010	
Beauveria bassiana	Coffee berry borer	Honeybee	Coffee	Ecuador, 2002–3	Ureña and Chuncho, (2002), (2003)
Gliocladium catenulatum	Grey mold	Bumblebee	Strawberry	Belgium, 2011	Mommaerts et al. (2011)

Clonostachys rosea	Mummyberry	Bumblebee	Lowbush blueberry	PEI & Nova Scotia, unpubl.	
Bt	Cabbage looper	Bumblebee	Greenhouse tomato and pepper	Ontario, unpubl.	
Autographa californica nucleopolyhedrovirus (AcMNPV)	Cabbage looper	Bumblebee	Greenhouse tomato and pepper	Ontario, unpubl.	
Bacillus subtilis + *Bt*	Grey mold cotton caterpillar, diamondback moth and leek moth	Bumblebee	Greenhouse tomato	Korea, unpubl.	
Clonostachys rosea + *Bt*	Sunflower head rot + banded sunflower moth	Bumblebee	Sunflower	Ontario, unpubl.	Sutton and Kevan (2013)
Streptomyces griseoviridis and *Gliocladium catenulatum*	Blueberry blossom blight	Bumblebee	Rabbiteye blueberry	USA, 2011	Smith et al. (2012)
Clonostachys rosea	*Botrytis* blight	Bumblebee	Lowbush blueberry	Nova Scotia	Reeh et al. (2014)
Clonostachys catenulatum	*Monolinia* brown rot	Honeybees	Cherry	S. Australia	Hoogendoorn (2014)

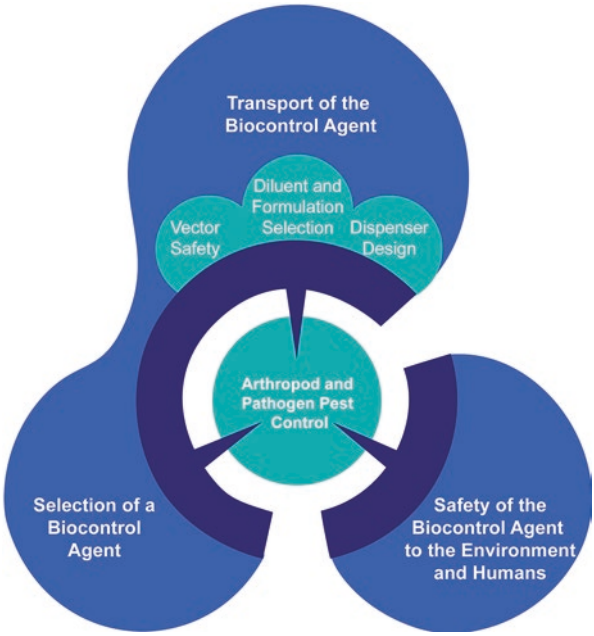

Fig. 1 The integrated and connected array of facets of the research and development process in pollinator biocontrol vector technology

3. How much of any particular biological control agent can be formulated and delivered in a particular way so that the pollinators deliver enough to the target crop to suppress the disease or pest population to acceptable levels (below the economic threshold) for growers? The combined nature of the delivery system (dispenser) and formulation, plus vector safety come together in dispenser design.
4. The issues of environmental safety and registration must be addressed to bring a technology that is safe in the human food supply chain, safe for growers to use, does not adversely affect the crop plants which it is to protect, and has minimal environmental side effects.

The components are presented as Fig. 1 taken from Kevan et al. (2008) and presented in slightly different form (with credit given) by Mommaerts and Smagghe (2011).

A wide variety of biological control agents have been suggested as possibilities for pollinator vectoring and at least a dozen variously tested (Tables 1 and 2). They range from viruses, to bacteria, and fungi as microbial biological control agents. Tests on the safety of those agents to pollinators vary from thorough to apparently untested (see Table 2 and accounts by species). Very few have been tested for their potential adverse effects on the botanical side of pollination *per se*. The effects on pollen viability and germination rates on stigmas have rarely been tested, and the potential for adverse effects on post-pollination fertilization processes, embryogenesis, seed and fruit set hardly assessed. The exception seems to be for *Bacillus subtilis* vectored by honeybees to rabbit eye blueberry (*Vaccinium corymbosum*) to suppress mummyberry fungus (*Monilinia vaccinii-corymbosi*) (Ngugi et al. 2005).

Table 2 Biological control agents applied directly for health of *Apis mellifera*. We have not been able to discover tests made for other managed pollinators

Biocontrol agent	Target pest	Formulation	References
Metarhizium anisopliae	*Varroa destructor*	Dusts and coated strips	Kanga et al. (2003)
		Coated strips	Kanga et al. (2006), (2010)
		Laboratory study	Kanga et al. (2002)
		Field trials	James et al. (2006)
			Rodriguez et al. (2009b)
Beauveria bassiana		With carnauba wax or candelilla wax, or both	Meikle et al. (2007), (2008a), (2008b), (2009)
		Laboratory studies	Rodriguez et al. (2009a)
Hirsutella thompsonii		Laboratory study	Kanga et al. (2002)
Bacillus isolates		Laboratory tests	Tsagou et al. (2004)
40 isolates of entomopathogenic fungi; isolates *of M. anisopliae*, *B. bassiana*, and *Verticillium lecanii* further assessed		Laboratory tests	Shaw et al. (2002)
Metarhizium anispoliae, Beauveria bassiana, Clonostachys rosea		In cells attended by bees in the colony	Hamiduzzaman et al. (2012)
Three microbials		Laboratory/field	Lodesani et al. (2003)
General concepts			Chandler (2008); Chandler et al. (2001)
Bacillus subtilis	Foul brood and chalk brood	Not tested	Sabate Daniela et al. (2009)
Metarhizium anisopliae, Beauveria bassiana, and *Hirsutella illustris*	*Aethina tumida*		Muerrle et al. (2006)
Pseudoscorpions	Varroa and others		Donovan and Paul (2005)

The diluents used for the various biocontrol agents have ranged from mineral (talc and clays) to vegetable (flours and starch) materials. In general, the mineral diluents have proven to be problematic, irritating the pollinator vectors so that they groom the formulation from their bodies before delivering it or causing mortality (Mand et al. 2015; Karise et al. 2015; Menzler-Hokkanen 2016). The vegetable diluents, notably flour has worked well in laboratory and experimental studies (Kevan et al. 2008). Animal derived diluents, such as milk powder, have not proven useful. The formulations used in various experimental trials, especially when commercial potential has been indicated, have been retained as proprietary, but are of food grade and/or organically certified (e.g. PWGSC 2008) materials. To be practical, commercial formulations need to have several properties: (1) able to adhere effectively to the bodies of the insect vectors but able to dislodge and dose the target flowers; (2) not be so irritating to the insect vectors that they groom the material from their bodies;

(3) not cause health problems for, or death of, the vectors; (3) remain dry and flowable in the dispensers (despite becoming moistened by feces as happens with *Bombus* spp.) for the duration of application (about 3 days for application with *Bombus* spp.); and (4) be readily applied into dispensers by beekeepers and growers.

5 Biological Control Agents Applied for Pollinator Health

Given that it is possible for managed pollinators to deliver biological control agents from their domiciles to protect crops from pests and diseases, and that managed pollinators suffer from their own suites of diseases and pests which they transmit into their domiciles, it is possible that biological control agents (as opposed to medicaments or antibiotics) can be disseminated by the pollinators for their own protection. As far as we can determine, this idea has been explored only for the western honeybee (*A. mellifera*) (see Chandler et al. 2001; Shaw et al. 2002; Davidson et al. 2003; Lodeseni et al. 2003; Grobov and Kosmachev 2005; James 2009; Rodriguez et al. 2009a; Hamiduzzaman et al. 2012 and Table 2), mainly for the control of *Varroa destructor*, a devastating ectoparasite that carries with it a suite of viral pathogens (Kevan et al. 2006; Chen et al. 2006; Chen and Siede 2007).

Hamiduzzaman et al. (2012) report the effects of *M. anisopliae*, *B. bassiana* and *C. rosea* against *V. destructor* in honey bee hives. The noted that although the first two mentioned fungi caused reduced weights of treated bees and caused infection, *C. rosea* seemed more benign to the bees. Mortality of *V. destructor* was noted as an effect of all three fungi, but the last named caused lower mortality.

One of the primary problems has been the dispersal of the biological control agents into the colony. Table 2 summarizes the information available on the biological control agent, the target pest, and the mode of delivery.

6 Potential for Pollinator and Pollination Related Problems Posed by Biological Control Agents

Ampelomyces quisqualis is a naturally occurring hyperparasite of powdery mildews. The European Commission EC HCPDG (2004) states, as an act of faith, that "Since AQ 10 is strictly specific to fungi of the family Erysiphaceae, there is no impact of the non-target organisms." It goes on to say that all the tests performed on aquatic organisms, birds and bees gave negative results, but provides no data. Similarly, the producing company's (IntraChem) report indicates no adverse effects on honeybees, but no data are provided (Franceschini undated). The formulation AQ10 has been tested as safe on *B. terrestris* in laboratory topical and feeding trials.

Bacillus thuringiensis is a Gram-positive soil-dwelling aerobic bacterial insect pathogen, also known as *B.t.* or simply Bt or BT. It has the capacity to produce endospores that contain crystalline (Cry) proteins such as δ-endotoxins. When those crystals encounter the alkaline pH of insects' guts, they interact with the epithelial cells and cause poisoning. Some twelve strains, or biotypes, that produce different Cry

proteins are available commercially and all have been tested for effects on honeybees (EPA 1998a, c, d, 2011a) A full review is beyond the scope of this article and would reveal that by and large, Bt is not harmful to honeybees. We make some general comments about the most familiar strains. *B.t. kurstaki* (Biobit, Condor, Cutlass, DiPel, Full-Bac, Thuricide, Bactospeine, Javelin, Leptox, MPV, M-Penil, Novabac, Steward, Victory) is particularly effective against the caterpillars of Lepidoptera (moths and butterflies). *Bacillus thuringiensis aizawa* (Certain, Agree, XenTari), even though it is highly toxic to honeybees (Kirkland 1991 in EPA 1998a) has been tested and is useful against wax moths that infest bee hives and beekeeping equipment. *Bacillus thuringiensis israelensis* (Teknar, Skeetal, Vectobac, Mosquito Dunks, Mosquito Attack, Gnatrol, Batimos) is commercially used against larval biting flies and other Diptera. Recently, *B.t.* san diego (Trident, M-One, M-Trak, Foil, Novodor) as *B.t. tenebrionis* has been tested and is effective against some beetles, notably boll weevils, Colorado potato beetles, and elm leaf beetles and seems innocuous to honeybees.

In short, none of the strains tested since the early 1960s, except possibly *B.t. aizawai*, has been shown to have any major detrimental effects on honeybees (Vandenberg 1990; Vandenberg and Shimanuki 1986; EPA 1998a, c, d, 2011a), including those *B.t.* toxins that have been genetically incorporated into crop plants, such as maize, potato, and cotton (Roh et al. 2007; OECD 2007). Seemingly minor sublethal effects of GMO cotton pollen (also expressing cowpea trypsin inhibitor gene) on larval and adult honeybees are questionable (Duan et al. 2008; Han et al. 2010a, b, 2012; Mussen 2015). Little testing has been done with other bee pollinators in mind. Mommaerts et al. (2009a) note that B.t.k. (as Dipel) was innocuous to *B. terrestris* when fed in sugar syrup, but that B.t.a. (as XenTari) killed workers of that species in high concentrations (0.1%) but was harmless at 0.01% concentration in sugar syrup.

Bee vectoring of B.t.k. (as Dipel) with *B. impatiens* for cabbage looper (*Trichoplusia ni*) control on greenhouse tomatoes in semi-field trials also had no negative impact on hive health (i.e., number of workers and brood) (Les Shipp, unpublished data). The mode of application of B.t. i. (applied mostly to aquatic larval stages of biting flies) is probably not a threat to most dipteran pollinators (Larson et al. 2001).

The incorporation of B.t. k. toxin into genetically modified crops heightened concern following publication by Loseyi et al. (1999) of the toxicity of B.t. k. toxin-expressing maize pollen to monarch butterflies. The series of studies that followed (Stanley-Horn et al. 2001; Lang et al. 2015) concentrated attention to the potential hazard that wide spread cultivation of B.t. maize might pose to butterflies and, even though negative effects were registered in both laboratory and field trials, concluded that the risks were small. A meta-analysis of 25 studies on the effects of B.t. Cry proteins on honeybee survival found no effect on either honeybee larvae or adults in the laboratory (Duan et al. 2008). However, risks associated with the release of clouds of B.t. k. toxin expressing pollen from other anemophilously pollinated plants that may benefit from protection from lepidopteran and/or coleopteran pests by genetic engineering using B.t. Cry expression have not been considered. Various economically important trees (especially *Populus*) and grains (e.g. wheat) have been considered and those may release pollen in huge clouds that can, and do, travel great distances from their sources on atmospheric convection and wind. Whether or not such clouds would land in sufficient concentration to adversely affect whole com-

munities of non-target, non-pestiferous, and possibly ecologically important as pollinators or in wildlife nutrition, seems not to have been considered.

***Bacillus subtilis* (Serenade and Rhapsody are both strain QST713)** has been tested in the laboratory and in field trials on managed pollinators and pollination (Hoxter et al. 1998). The EPA (2011b) documentations states that based on a single application at the 10 lb. product/acre, the Estimated Environment Concentrations (EECs) for the technical grade active ingredient are 250 ppm and 115 ppm. Thus, the LC_{50} of 5663 ppm for honeybees (*A. mellifera*) is approximately twenty-two (22) times the EEC based on foliar residue. However, honeybees directly sprayed with QST 713 would be exposed to approximately 8000 ppm which is well above the $LC_{50.}$ Trials on various crops for which honeybees were deployed and to which Serenade had been applied indicated no adverse effects on pollinators, pollination, or yields (EPA 2011b; Hoxter et al. 1998). In using honeybees, Ngugi et al. (2005) tested this material on the flowers of rabbiteye blueberry (*V. ashei*) with applications directly to laboratory-held flowers and found no adverse effects on pollen germination and growth on the stigmas. Although results of the field tests were less easy to interpret, their results indicate that application of Serenade did not have adverse effects on pollination and fruit characteristics. They cautioned that applying this product in conditions otherwise unfavorable for adequate pollination may pose risks to production. They make no mention of safety to honeybees. Similarly, the report by Dedej et al. (2004) makes no mention of any problems caused to honeybees in the field using this product dispensed for hives.

In laboratory tests by Mommaerts et al. (2009b), topical contact and oral delivery of Serenade via sugar water resulted in 88 and 100% worker mortality, respectively. With lower concentrations (11/2, 1/5 and 1/10 Maximum Field Recommended Concentration [MFRC]) the toxicity decreased, but the effect depended on the route of exposure. In addition to lethal effects, nests were also evaluated for sublethal effects after treatment with the seven microbial control agents (MCAs) at their respective MFRCs over 11 weeks. It also caused sublethal effects in reducing the production of drone brood.

***Beauveria bassiana* (BotaniGard is *B. bassiana* GHA)** is generally believed to be safe for pollinators (Zimmerman 2007a; PMRA 2009). Meikle et al. (2007, 2008a, 2008b, 2009) exposed colonies of honeybees (*A. mellifera*) in the field in France to two strains of *B. bassiana*, one was GHA the other a strain isolated from *Varroa* mites in Europe. They noted that their treatments had no direct adverse effect on the honeybee colonies, but their results were encouraging from the viewpoint of *Varroa* control. On the other hand, Al-mazra'awi et al. (2007) and Kapongo et al. (2008a) found that in high doses, *B. bassiana* killed *A. mellifera* and *B. impatiens*, respectively. In reporting the results of their field-tent and greenhouse trials to investigate the safety of using this biocontrol agent for pollinator vectoring, they pointed out the need for care in formulation so that the spore count of *B. bassiana* is low enough to reduce adverse effects to the bumblebees but high enough to be delivered to the crops for suppression of insect pests (tarnished plant bug, green peach aphid, whitefly, and western flower thrips). Espinosa Ortiz et al. (2011) recorded that different isolates of *B. bassiana* were variously virulent depending on the honeybees' life stages, larvae, pupae of adults. Mommaerts et al. (2009b) showed that exposure of *B. terrestris* to Botanigard via contact (topical application) at its MFRC caused 92%

mortality after 11 weeks, while the 1/10 MFRC killed 46% of exposed workers. It also caused sub-lethal effects in reducing the production of drone brood (Mommaerts et al. 2009b). Hokkanen et al. (2003, 2004) reported that the exposure route has a substantial impact on the infection rate of *B. bassiana* (local Finnish strain) on *B. terrestris*. In greenhouse trials, 7% of the dead bees were infected with *Beauveria* while foraging on plants that were spray with a *Beauveria* suspension (10^8 spores per ml). Direct exposure to this concentration resulted in >50% infection. Semi-field and field trials in Ontario in greenhouse tomatoes and sweet pepper at the recommended bee vectoring concentration (1.37×10^{10} spores/g of inoculum) found minimal impact on the bees. In commercial trials, we found that using dispensers at the rate of 5–10 hives per ha resulted in commercially-acceptable pollination, yield and fruit quality. Goerzen et al. (1990) and Brinkman et al. (1997) showed alfalfa leafcutting bees (*M. rotundata*) are highly susceptible to *B. bassiana* at all life stages.

Clonostachys rosea is an endophytic fungus. It has been used through pollinator biovectoring and direct sprays for the suppression of various fungal pests on crops, notably against grey mold (*Botrytis cinerea*) on small and tender fruit (Peng et al. 1992; Sutton et al. 1996) and more recently against mummy berry (Reeh et al. 2014) on lowbush blueberries and head rot (*Sclerotinia*) on sunflowers (Sutton and Kevan 2013). In various trials with *A. mellifera* and *B. impatiens* as vectors, it has not been found to adversely affect them (see Peng et al. 1992; Yu and Sutton 1997; Sutton et al. 1997) even in combination with the entomopathogenic fungus, *B. bassiana* (Kapongo et al. 2008b). Results from various trials that record spore loads on floral parts with crop yields (quantity and quality) indicate that this fungus does not interfere with the botanical processes in pollination and subsequent fertilization, fruit set, development and seed-set (e.g. Cota et al. 2008). Pre-Stop Mix® (containing *C. catenulatum*) is registered for use in Europe for apivectoring on various crops, especially for protection of strawberries against grey mould (Hokkanen and Mezner-Hokkanen 2009; Hokkanen et al. 2011; Karise et al. 2016) and is deemed safe for honeybees and bumblebees (Verdera 2015).

Coniothyrium minitans (***Paraconiothyrium minitans***) is an Ascomycete fungus. It has been deemed safe by the US EPA (1998b) and in Europe Commission EC HCPDG (2003) for honeybees on the basis that it is used as a soil treatment and specifically affects *Sclerotinia*. It appears to have not been tested. In trials in Alberta using *C. minitans* with *Trichoderma atroviride*, it appeared to be safe for alfalfa leafcutting bees that encountered the biocontrol product on alfalfa flowers where it had been applied to suppress alfalfa blossom blight (Li et al. 2005). The strain CON/M/91–08 has been isolated for use as Contans7.

Gliocladium catenulatum (marketed as PreStop-Mix) has been examined extensively by PMRA (1998, 2008). The evidence presented indicates that this organism is relatively safe for adult honeybees. It also appears to have no adverse effects on *B. terrestris* in laboratory trials in which it was administered in pollen, syrup and topically (Mommaerts et al. 2009b).

Hirsutella thompsonii is an Ascomycete fungus. It has been advocated as a possible biological control agent for use against *Varroa destructor* in honeybees (Kanga et al. 2002). Kanga et al. (2002) concluded that this fungal agent was harmless to the bees and did not affect queen fecundity.

Metarhizium anisopliae is an entomopathogenic fungus that occurs widely in the soil. It was first indicated as a possible biological control agent in 1993 (Zimmermann 1993). Although there are some taxonomic issues with closely related organisms, either subspecies, varieties (e.g., *M. flavoviride*, *M. acridum* or *M. a.* var. *acridum*) the tests that have been made indicate the fungus is harmless to honeybees (Zimmermann 2007b) to the extent that it is proposed as a biocontrol agent against *Varroa destructor* (Hokkanen et al. 2003, 2004; Kanga et al. 2006, 2010; James et al. 2006). Recent research by Espinosa Ortiz et al. (2011) indicates that some isolates of *M. anisopliae* are highly detrimental to larval and pupal honeybees even if not to adults, and that there are great differences in virulence between isolates. The most commonly considered strain is F52 which has been in review with PMRA (2010a, 2011) in which assessments on pollinators are not mentioned specifically. Recently, Smagghe et al. (2013) investigated the impact of strain F52 on *B. terrestris* using the miniature two-way dispenser bioassay as described by Mommaerts et al. (2012). Over a 6 week exposure period to concentrations of 10^7, 10^8 and 10^9, the only significant mortality to the worker bees occurred at 10^9 (100%). Sub-lethal effects on drone production was also only seen at the concentration of 10^9.

Pantoea agglomerans (also known as *Enterobacter agglomerans* and *Erwinia herbicola*) is a widespread bacterium has been used as an antagonist to fireblight (*Erwinia amylovora*). It is commercially formulated as Blossom Bless in New Zealand where it has been applied to pome fruit flowers through honeybee vectoring and sprays (Vanneste 1996; Cornish et al. 1998; Vanneste et al. 1999, 2002). It occurs abundantly in the environment of honeybees and is not noted as a pathogen (Loncaric et al. 2009). Technical information on Blossom Bless does not mention trials or problems with pollinators (www.kvh.org.nz/vdb/document/814 Product Testing Report 6 December, 2011 Blossom Bless and www.grochem.co.nz/Portals/537/labels/BlossomBless_300g.pdf).

Pseudomonas fluorescens is a naturally occurring bacterium. Strain A506 (Blightban) has been extensively reviewed by Canadian regulatory authorities (PMRA 2010b). It appears to be harmless to adult honeybees that have been used to vector *P. fluorescens* to the flowers of apple and pear trees to suppress fireblight (*E. amylovora*) (Thomson et al. 1990, 1992, Johnson et al. 1993a, b). It does not appear to have been tested on other bees, nor on various life stages of honeybees, except for adults.

Streptomyces griseoviridis is a Gram-positive bacterium. It seems not to have been tested on bees and that part of environmental assessment seems to have been regarded as inapplicable because of the use of the agent in soil (e.g. PMRA 2003). However, it has recently been applied to rabbit eye blueberry for control of Botrytis blossom blight (*B. cinerea*) through pollinator biovectoring technology with bumblebees (*B. impatiens*) (Smith et al. 2012). Thus, it is now advisable that testing be conducted on pollinators for toxicity, sublethal reproductive and behavioral effects, and for potential interference with botanical aspects of pollination, fertilization, and fruit and seed set and quality.

***Trichoderma* spp.** and related fungi in commercially available formulations have been used in several entomovectoring trials using pollinators. It appears that few, if any, detailed scientific trials have been conducted on the potential hazards of *Trichoderma* and related species to managed pollinators, except for that of

Mommaerts et al. (2009b) on *B. terrestris* in Europe. One of the first studies involving pollinator vectoring was that on Binab-T by Svedelius (2000). His agent was designated as a mixture of two species, *T. harzianum* and *T. polysporum*, but it is now also known as a mixture (1:1) of *Hypocrea parapilulifera* and *T. atroviride*. Svedelius (2000) noted that Binab-T was not effective against cucumber rot in greenhouses when delivered to flower by bumblebees (*B. terrestris*) and did not note a problem for the bees. Kovach et al. (2000) and Albano et al. (2009) working in New York and Quebec respectively with Rootshield did not note any adverse effects on the bees that they studied as vectors (*B. impatiens* and *A. mellifera*). Maccagnani et al. (2005) did not note adverse effects of *T. harzianum* on vectoring *B. terrestris* in Italy. Neither Shafir et al. (2006) in Israel nor Beasley et al. (2005) in Australia noted any adverse effects to honey bees as vectors of Trichodex for protection of the Geraldton waxflower (*Chamelaucium uncinatum*: Myrtaceae). Mommaerts et al. (2009b) indicate that it is safe for those bees. *Trichoderma harzianum* strain T22 is now noted as the active microbial ingredient in Trianum-P, Trichodex and Rootshield which Mommaerts et al. (2009b) also indicated is innocuous to *B. terrestris*.

Trichoderma virens (also known as *Gliocladium virens*) strain G-14 appears to have not been tested on any bee species.

Other investigators have used other isolates of *Trichoderma* spp. and not recorded problems for the vectors, honeybees and /or bumblebees (i.e. Maccagnani et al. (1999) in Italy when used for suppression of grey mold on strawberries). Escande et al. (2002), in Argentina, used *T. koningii*, *T. aureoviride* and *T. longibrachiatum* against head rot (*Sclerotinia*) on sunflowers with honeybees as the vectors and noted no issues.

Regulatory authorities have not seen fit to question the possible adverse effects of the Trichoderma-based biological control agents. For example, the rather cursory documentation from Health Canada (PMRA 2010c) for proposed registration of Rootshield reads as follows "A waiver rationale was previously submitted for terrestrial arthropod and non-arthropod invertebrate testing, based on the absence of published scientific literature in which *Trichoderma harzianum* caused infection or any other impact on insects or other invertebrates. In one published study, bees were used to disseminate *T. harzianum* strain KRL-AG2 without any apparent adverse effects. In another, no adverse effects were noted for hives treated with *T. harzianum* strain T-39 [this is probably the study by Shafir et al. (2006) on Trichodex]. Rather, literature indicated that insects, especially mites, consumed the hyphae of *Trichoderma* species. While terrestrial arthropods and other invertebrates may be exposed from the proposed outdoor uses of RootShield Biological Fungicide products, the risk to these organisms is considered low based on the lack of adverse effects in published scientific literature." The inadequacy of that review is evident from its lack of appreciation of the existing literature and non-citation thereof, even if the evidence at hand supports the apparent safety of *Trichoderma* spp. products to managed pollinators used as vectors or just exposed incidentally.

There appear to be no studies on the possible adverse effects to post-pollination events in plant reproduction.

Viral agents Few viral agents have been used in pollinator biovectoring studies and none have been found to adversely affect the pollinators. It can be assumed that

such viral agents are specific to the pest against which they are directed, such as corn earworm (Gross et al. 1994). Some viral agents have been tested for their effects on non-target organisms, and bees have been included. The *Autographa californica* MNPV, a nuclear polyhedrovirus for cabbage looper control, was recently evaluated for bee vectoring using *B. impatiens*. The virus was isolated from cabbage loopers and was found not to have any negative impact on hive health (i.e., number of workers and brood) (Les Shipp, unpublished data).

7 Summary of Tests of Biological Control Agents on Pollinators

Most of the studies that have tested the safety of pollinator vectored biological control agents have concentrated on adult honey bees (*A. mellifera*) in laboratory, cage and field trials. By and large, those tests have indicated that the biological control agents are mostly safe and only at high concentrations (above those expected to be encountered in crop protection use) have adverse effects. What have not been tested extensively are the potential comparative effects on honeybee larvae, pupae and adults. Given that the biological control agents vectored by honeybees to the outside for crop protection, or spread within the hive for colony protection, must, even in low concentrations as in the former use, contact the brood, more investigations are needed. Certainly, the recent results from the studies of Espinosa Ortiz et al. (2011) indicate that different strains of various biological control agents have different effects on different life stages of honeybees. Only a few studies have examined the sub-lethal effects of bee-vectored biological control agents on fecundity of the queen, and none seem to have addressed the sub-lethal behavioral effects on adult honeybees. It seems that even for honeybees, there remain uncertainties about safety, especially concerning differential effects on the various life stages and sub-lethal (both reproductive and behavioral) effects.

For bumblebees, Mommaerts et al. (2009b) have addressed effects on some biological control agents on *B. terrestris*. In general, the products that they tested seem safe at the concentrations used for biological control and bee-vectoring. The research by Al-mazra'awi et al. (2007) points out the need for establishing formulations that are minimally harmful to the bees yet bring about suppression of the targeted pest. They do note that different agents differ in their virulence, and that some can have sub-lethal reproductive effects. Similar comparative studies have not been made for *B. impatiens* in North America. Although, overall indications are that the biological control agents that have been tested are quite safe to bumblebees (two *Bombus* spp.), there remain uncertainties about safety, especially concerning differential effects on the various life stages and sub-lethal (both reproductive and behavioral) effects. It is important, however, to remember that the commercial bumblebee hives have a limited active lifespan of 8–12 weeks.

Alfalfa leafcutting bees (*M. rotundata*) have not been used for disseminating biological control agents (at least by direct application to their bodies). None the

less, it is worth noting that tests using entomopox virus from grasshoppers, baculovirus from cut worms, and nuclear polyhedrosis virus (NPV) from spruce budworm did not infect *M. rotundata* (Goerzen et al. 1990; Kaupp et al. 2011). On the other hand, the strain of *Beauveria bassiana* (SRS-Bb-86-5) tested by Goerzen et al. (1990) killed prepupae at all doses tested. They also tested *Aspergillus parasiticus*. Li et al. (2005) using *Coniothyrium minitans* and *Trichoderma atroviride* to suppress *Sclerotinia* blossom blight in alfalfa in the presence of *M. rotundata* did not comment of the effects of these biological control agents to the bees.

Maccagnani et al. (2006) used *Osmia cornuta* to disperse *B. subtilis* to pear blossoms in Italy, but did not record adverse effects on the bees.

8 Summary of Tests on Botanical Aspects of Pollination (Stigma and Pollen Functions, Fertilization, Seed and Fruit set)

Even though, it is the intent of pollinator biovectoring from a crop protection perspective to place biological control agents onto flowers where they then protect the developing fruit and, often, the rest of the plant (Kevan et al. 2007, 2008), few studies have looked into the possibility of the agents' adversely affecting post-pollination processes. That seems to be rather an oversight considering the simplicity of the research methodologies required. In many of the experiments conducted, one can assume that post-pollination processes were not adversely affected in that no reductions in fruit- and/or seed-set are noted. In using honeybees, Ngugi et al. (2005) tested *B. subtilis* delivered to the flowers of rabbiteye blueberry (*V. ashei*) with applications directly to laboratory-held flowers. They found no adverse effects on pollen germination and growth on the stigmas. Results of field tests were less easy to interpret, but they indicate that application of Serenade did not have adverse effects on pollination and fruit characteristics.

9 Pest Control Arthropods As Vectors of Biological Control Agents Against Crop Pests and Diseases

It has long been known that entomopathogenic viruses could be transported by insects other than their hosts (Steinhaus 1954; Bird 1955; Franz et al. 1955; Weiser 1957, Smirnoff 1959; Fuxa 1991; Fuxa et al. 1993). Researchers working with various beneficial insects for biological control, and even pest insects, have noted their capacities for transmitting beneficial, pest suppressing, microbes (Table 3). Although the first observations and experiments precede those on managed pollinators as vectors of biological control agents, no commercial technology seems to have been realized.

More recent studies have concentrated on the potential for (1) an additive effect when predatory insects and entomopathogenic fungi are used together to control

Table 3 Entomovectoring of secondary biological control agents by primary biological control agents, insect pests and scavengers. Lists are in chronological order

Primary biological control agent	Secondary (microbial) biological control agent	Target pest	Reference
Ephippiger bitterensis (Orthoptera: Tettigoniidae)	Nuclear-polyhedrosis virus	Unspecified	Vago et al. (1966)
Campoletis sonorensis (Hymenotera: Ichneumonidae)	Nuclear polyhedrosis virus	Larvae of *Heliothis virescens*	Irabagon and Brooks (1974)
Calosoma sycophanta (Coleoptera: Carabidae)	Nuclear-polyhedrosis virus	Gypsy moth larvae	Capinera and Barbosa (1975)
Apanteles melanoscelus (Hymenoptera: Braconidea)	Gypsy moth nuclear polyhedrosis virus		Raimo (1975)
Apanteles melanoscelus	Nuclear-polyhedrosis virus	Gypsy moth larvae	Raimo et al. (1977)
Apanteles glomeratus	Granulosis virus	Cabbage butterfly *Pieris rapae* (Lepidoptera: Pieridae)	Levin et al. (1979)
Zelus exsanguis (Hemiptera: Reduviidae)			Kaya (1979)
Podisus maculiventris (Hemiptera: Pentatomidae)	Nuclear-polyhedrosis virus	Cabbage looper *Trichoplusia ni* (Lepidoptera: Noctuidae)	Biever et al. (1982)
Nabis roseipennis (Hemiptera: Nabidae)	Nuclear polyhedrosis virus	Velvetbean moth, *Anticarsia gemmatalis* (Lepidoptera: Noctuidae) larvae,	Young and Yearian (1987)
Apanteles glomeratus (Hymenoptera: Braconidea)	Baculovirus	Larvae of *Pieris brassicae*	Hochberg (1991)
Apanteles telengai, *Aleiodes gasteratus* (Hymenoptera: Braconidae) and *Campoletis annulata* (Hymenoptera: Ichneumonidae)	Granulosis virus	*Agrotis segetum* larvae	Caballero et al. (1991)
	Nuclear polyhedrosis virus AgNPV	Velvetbean moth, *Anticarsia gemmatalis* (Lepidoptera: Noctuidae) larvae	Fuxa and Richter (1994)

(continued)

Table 3 (continued)

Primary biological control agent	Secondary (microbial) biological control agent	Target pest	Reference
Coccinella septempunctata (Coleoptera: Coccinellidae)	*Erynia neoaphidis* (Zygomycota: Entomophthorales)	Pea aphid,*Acyrthosiphon pisum* (Homoptera: Aphididae)	Pell et al. (1997)
Podisus maculiventris (Hemiptera: Pentatomidae)	Nuclear-polyhedrosis virus (AcNPV recombinants)	Unspecified	Lee and Fuxa (2000a, b)
Coccinella septempunctata (Coleoptera: Coccinellidae)	*Erynia neoaphidis* (Zygomycota: Entomophthorales)	Pea aphid, *Acyrthosiphon pisum* (Homoptera: Aphididae)	Roy et al. (2001)
Chrysoperla rufilabris (Neuroptera: Chrysopidae)and *Doru taeniatum* (Dermaptera: Forficulidae)	*Spodoptera frugiperda* nucleopolyhedrovirus		Castillejos et al. (2001)
Hippodamia convergens (Coleoptera: Coccinellidae)	*Paecilomyces fumosoroseus (= Isaria fumosoroseua)* (Ascomycota: Eurotiales)	Russian wheat aphid, *Diuraphis noxia* (Homoptera: Aphididae)	Pell and Vandenberg, (2002)
Spined soldier bug, *Podisus maculiventris* (Heteroptera: Pentatomidae)	*Vairimorpha necatrix* (Microspora: Microsporidia) and *Lacanobia oleracea* granulovirus (LoGV)	*Lacanobia oleracea* and *Spodoptera littoralis (Lepidoptera: Noctuidae)*	Down et al. (2004)
Lasius niger (Hymenoptera: Formicidae)	*Lecanicillium longisporum* (Ascomycota: Hypocreales)	Rosy apple aphid, *Dysaphis plantaginea* (Homoptera: Aphididae)	Bird et al. (2004)
Anthocoris nemorum (Heteroptera: Anthocoridae)	*Beauveria bassiana* (Ascomycota: Hypocreales)	Nettle aphid, *Microlophium carnosum* (Homoptera: Aphididae)	Meyling et al. (2006)
Dicyphus hesperus (Heteroptera: Miridae)	*Paecilomyces fumosoroseus* (= *Isaria fumosorosea*) Apopka-97 (Ascomycota: Eurotiales)	Greenhouse white fly *Trialeurodes vaporariorum* (Hemiptera: Aleyrodidae)	Alma et al. (2007)

(continued)

Table 3 (continued)

Primary biological control agent	Secondary (microbial) biological control agent	Target pest	Reference
Orius laevigatus (Heteroptera: Anthocoridae)	*Lecanicillium longisporum* and *L. muscarium* (Ascomycota: Hypocreales)	Peach aphid, *Myzus persicae* (Homoptera: Aphididae), Western flower thrips *Frankliniella occidentalis* (Thysanoptera: Thripidae) and Silverleaf whitefly *Bemisia tabaci* (Hempitera: Aleyrodidae)	Down et al. (2009)
Harmonia axyridis (Coleoptera: Coccinellidae)	*Pandora neoaphidis* (Zygomycota: Entomophthorales)		Wells et al. (2011)
Harmonia axyridis (Coleoptera: Coccinellidae) and *Crysoperla carnea* (Neuroptera: Chrysopidae)	*Beauveria bassiana* (Ascomycota: Hypocreales)	Peach aphild, *Myzus persicae* (Homoptera: Aphididae)	Zhu and Kim (2012)
Calosoma sycophanta (Coleoptera: Carabidae)	Microsporidia	Larvae of the gypsy moth *Lymantria dispar*	Goertz and Hoch (2013)
Eocanthecona furcellata (Hemiptera: Pentatomidae)	*Spodoptera litura* multiple nucleopolyhedrovirus		Gupta et al. (2014)
Heterorhabditis (Nematoda: Rhabditida)	*Photorhabdus* endosymbiotic bacteria	A wide variety of pests, from cockroaches to beetles, flies and ticks are attacked by these nematodes whose bacterial endosymbionts kill the hosts	Poinar (1972), Gaugler and Kaya (eds) (1990), Akhurst and Dunphy (1993)
Steinernema (Nematoda: Rhabditida)	*Xenorhabdus* endosymbiotic bacteria		
Pest insect			
Carpophilus freemani (Coleoptera: Nitidulidae) freeman dried fruit (sap) beetle	*Beauveria bassiana*		Bruck and Lewis (2002)
Bradysia sp. (Diptera: Sciaridae) fungus gnat	*Beauveria bassiana*		Kapongo et al. unpubl.
Scavenger			
Acheta domesticus	Nuclear polyhedrosis virus		Bergoin (1966)

(continued)

Table 3 (continued)

Primary biological control agent	Secondary (microbial) biological control agent	Target pest	Reference
Birds	Nuclear polyhedrosis virus	Cabbage looper	Hostetter and Beiver (1970)
Sarcophagidae spp.	Nuclear-polyhedrosis virus		Hostetter (1971)
Birds	Nuclear-polyhedrosis virus		Entwistle et al. (1977a, b), (1993).
Sarcophaga bullata (Diptera: Sarcophagidae)	Nuclear-polyhedrosis virus (AcNPV recombinants)	Unspecified	Lee and Fuxa (2000a, b)
Acteta domestica			

insect pest species (Jacobson et al. 2001; Alma et al. 2007), and (2) the dissemination of fungal entomopathogens by non-pest insect species on the improved efficacy of the pathogen (Pell et al. 1997; Butt et al. 1998; Roy et al. 2001; Bruck and Lewis 2002; Pell and Vandenberg 2002; Bird et al. 2004; Meyling et al. 2006; Carreck et al. 2007). The dispersal of entomopathogens by non-target insects often provides a targeted way of dispersing the fungal pathogen directly into the pest population.

The remarkable endosymbiotic relationships of entomopathogenic bacteria with entomopathogenic nematodes (Poinar 1979; Gaugler and Kaya 2018; Ankhurst and Dunphy 1993) has led research into the potential of transgenetically producing the microbial toxins in crop plants for insect pest control (ffrench-Constant and Bowen 2000).

10 Conclusions

The Anthropocene epoch is broadly defined as having started when human activities started to have a distinct and characteristic global impact on Earth's geology and ecosystems. As human beings have come to recognize their potential to change environments (i.e. the onset of agriculture) and latterly to mitigate negative effects resulting from agriculture, industry, warfare, and population pressures, conservation strategies have been invoked. The conservation strategies have ranged from being species-specific to recognition of the importance of habitats and spaces but now, increasingly, embrace concerns for functionality of ecosystems. Ecosystems function through the component species and their diversity (biodiversity), the abundance of those components (populations) and their activities within and between each other. Physical factors (e.g. climate, pollution, desertification) constrain ecosystem function, and are central to environmental concerns because they influence ecosystem function, i.e. "nature's services". It is generally agreed that biodiversity and ecosystem resistance to perturbation by adverse physical conditions are positively

related, possibly through functional redundancy and associated niche hierarchy. Thus, in the application of ecological principles to restoration of ecosystem function in degraded environments, biodiversity and abundance, coupled with inter- and intra-specific activities probably have a role in mitigating the effects of physical adversity. "Ecological intensification" (FAO 2013) needs to embrace more than simplemindedly adding biodiversity to degraded systems: the functions of the elements added should be understood in more depth than as independent taxocenes.

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Bee Pollination of Crops: A Natural and Cost-Free Ecological Service

Otto Boecking and Eve Veromann

1 Today's Knowledge About Pollination: Still at a Starting Point?

The principal appreciation of pollination as a key factor for stable and sustainable agricultural crop production, wild plant diversity maintenance, habitat stability and restoration, and thus one of the most important contributions to human life and world economics, is without any doubt higher today and fortunately reached the public awareness, especially if compared to times of Christian Konrad Sprengel (°22 September 1750 – †7 April 1816) the founder of flower-ecology (Sprengel 1793). During his lifetime, he invested a lot of effort into educational work explaining the principles of pollination and raising people's awareness of the importance of pollination. But no one really could appreciate his outstanding work and knowledge at that time. Today, while understanding more and more about the critical role of pollination and pollinators, especially bees, aspects of pollinator declines and landscape changes are shifting increasingly into focus. The public and scientists have realized that the naturally cost-free pollination services like they were available a century ago and not a topic of concern, must today be compensated by providing cost-intensive pollination services in many cases.

At present, there is a long list of publications about pollination research and extension services available worldwide, however it is obvious that many gaps still

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exist in our knowledge on this topic. Thus, it is worthwhile to raise and try to answer the critical questions still open in this field. For example, information about the pollination requirements, insect pollinators and necessary pollinator densities for most crops is still extremely limited in order to provide scientifically proven recommendations for practical use (Allsopp et al. 2008; Delaplane and Mayer 2000; Garratt et al. 2016; Henselek et al. 2018; Schulp et al. 2014). Furthermore, the existing basic scientific findings must finally reach application in agricultural practice, practical solutions and recommendations for the farmers. Most existing recommendations concerning pollination for many crops used in extension service might have some scientific basis, but are mainly deduced only from practical experiences and can be influenced by contrasting interests. In order to highlight this fact one example should help: someone who provides pollination services with his honeybee colonies will appreciate a higher pollinator density (number of bee colonies per hectare) in the field, compared to the farmer who has to pay for this service. However, it will get more complex if someone interferes and asks for a balance and solid pollination services, thus, will hinder "over-pollination". This illustration might get more complex when an alternative and more attractive crop for the honeybees will bloom nearby during the same time, when the honeybees should fill their duty as pollinators in the target crop the beekeeper/owner has paid for.

Here we will not rewrite and thus duplicate the state of the art details in the field of pollination by bees as many authoritative papers and books are available. For further reading we recommend, for example, the following publications: Free (1993): Insect Pollination of crops; Delaplane and Mayer (2000): Crop pollination by Bees; James and Pitts-Singer (2008): Bee Pollination in Agricultural Ecosystems and Abrol (2012): Pollination Biology – Biodiversity Conservation and Agricultural Production.

On the contrary, we would like to highlight here in this chapter the widespread concerns about pollinator declines and thus the potential loss of pollination services.

2 The Growing Knowledge About the General Importance of Pollinators Is Followed by the Concerns About Pollinator Declines

Today there is no doubt about the general importance of honeybees as providers of pollination (e.g. Gallai et al. 2009; Klatt et al. 2014; Klein et al. 2007; Kremen et al. 2007; Lautenbach et al. 2012; Potts et al. 2016). However, the value of wild pollinators (especially solitary bees and bumblebees) might have been significantly underestimated until now, since the focus was mainly on honeybees. Garibaldi et al. (2013) have shown for many crop systems worldwide that flower visitation by wild insects increases fruit sets significantly and that wild bees (solitary bees and bumblebees) pollinate some crops more efficiently compared to the most common

investigated honeybees (Fig. 1). Based on their results, the authors suggested that new practices for integrated management of both honeybees and diverse wild insect assemblages will enhance global crop yields. Recent publications showed that in some cases wild bees (bumblebees and solitary bees) can be more effective than honeybees and significantly improve the fruit set while they apparently change the honeybee flight behaviour and thus boost cross-pollination (Brittain et al. 2013;

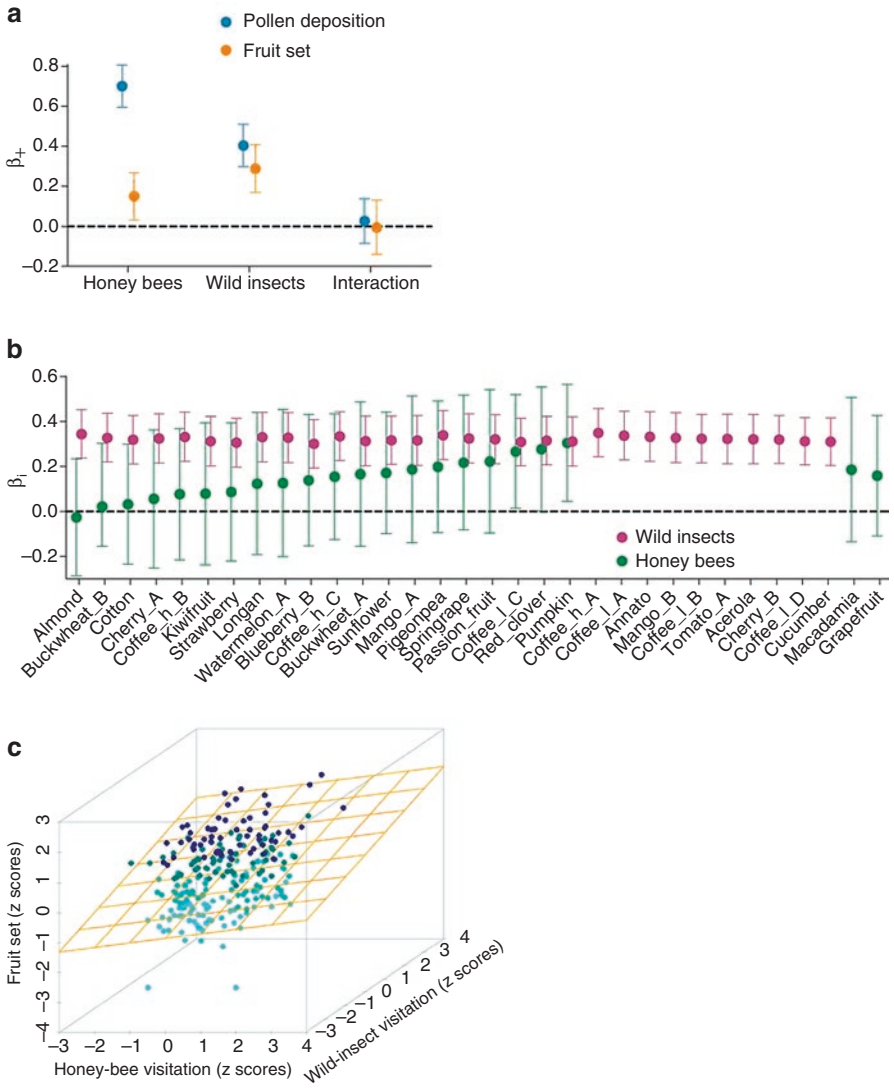

Fig. 1 The visitation of crop flowers by wild bees increases the fruit set in all examined crops (regression coefficient $\beta_i > 0$), whereas honey bee visitation has weaker influence. From Garibaldi et al. (2013)

Garratt et al. 2016; Martins et al. 2015). In orchards with non-*Apis* bees (blue orchard bees, *Osmia lignaria*), the foraging behavior of honeybees changed and the pollination effectiveness of a single honeybee visit was greater than in orchards where non-*Apis* bees were absent, because honeybees switch between planted tree rows due to the presence of the orchard bees. This change led to a greater proportion of fruit set in these orchards (Brittain et al. 2013). Therefore, species interactions can alter the behavior of insects and as a consequence increase the functional quality of the dominant pollinator species, here the honeybees. Garratt et al. (2016) have shown that the proportion of pollination service for apple trees in the UK provided by wild bees (bumblebees and solitary bees) varied from 70–77% while honeybees constantly contributed between 23–28% of pollination services. They also found that the presence of solitary bees in the studied orchards was the most constant and they never totally disappeared while the presence of honeybees and bumblebees depended on the variety of apples and location of the orchard. Therefore the importance of solitary bees as the most reliable pollinator service provider in apple orchards should be highlighted. Moreover, it is known that behavioural differences between honeybees, bumblebees and solitary bees can alter the likelihood of pollen transfer from their bodies to the plant stigma and thus curranty better pollination service. Bumblebees and solitary bees tend to have greater rates of stigmal contact compared to honeybees (Woodcock et al. 2013).

There has been a significant decline in the species richness and abundance of pollinators in recent years in the whole world. The decline is attributed to land-use change and intensification, habitat loss (Fig. 2), habitat fragmentation, increased field size, climate change, pesticide application, introduced alien species, the spread of pests and pathogens, disease switchover and other environmental changes that threaten the biodiversity of insect pollinators and the plants they collect food from. Changes in agricultural practices, the shift to more intensive agriculture, especially since the 1950s, has led to a sharp decline in the area of wildflower-rich habitats, such as hay meadows and pastures where insects can usually find shelter, overwintering and nesting sites, nesting material and food resources, which are all the requisites they need. Decrease in diverse floral resources has led to the decrease in the

Fig. 2 The decline of the brown-banded carder bee *Bombus humilis* Illiger is closely linked to the agricultural intensification and loss of field margins. Today, this bumblebee species is endangered in whole Europe. (Photo: Peeter Veromann)

diversity of wild pollinators. Globally, the reproduction of the majority of flowering plant species (90%) is dependent on animal pollination (Ollerton et al. 2011). Therefore, much concern is about the decline in pollinators, which is followed by the decline in insect-pollinated plants and *vice versa* (Biesmeijer et al. 2006; Garibaldi et al. 2011; González-Varo et al. 2013, Ouvrard and Jacquemart 2018). Today it is also accepted knowledge, that the interactions between insects and plants are highly complex and therefore it is a challenge to predict how these interactions can be affected by changes in pollinator species composition. Moreover, a recent publication suggests that ongoing pollinator declines may have more serious negative implications for plant communities than it is currently assumed. Brosia and Briggs (2014) showed that the loss of a single pollinator species within a pollinator network/community reduces floral fidelity in the remaining pollinators, with significant implications for ecosystem functioning in terms of reduced plant reproduction, even when potentially effective pollinators remained in the system. These findings are based on manipulative field experiments in which a single pollinator species was temporarily removed from study plots in subalpine meadows.

Wild bees have been shown to be efficient crop pollinators around the world and the economic value of this ecosystem service provided is equal with that provided by managed honey bees (Kleijn et al. 2015; Winfree et al. 2007; Potts et al. 2016). Increasing trend to grow mass-flowering crops (e.g. oilseed rape, sunflower etc.) has a positive effect on pollinator densities (Holzschuh et al. 2013; Riedinger et al. 2015) but the effect on different pollinator guilds is unclear. There is evidence that blooming oilseed rape fields promote the abundance of solitary bees (Riedinger et al. 2015) but have an inconsistent impact on bumblebees. However, the growing of mass-flowering cultures is favoring only a small minority of common bee species that prevail in cultural fields and provide most of the crop pollination services (80% of pollination services are provided only by 2% of the wild bee species; see list of dominant bee crop pollinators in the Table 1) (Kleijn et al. 2015). Thus, the methods implemented for conservation of abundant and common wild bee species do not support the biodiversity conservation measure and non-abundant or rare species. What is more, the oligolectic species are still under continuous threat (Fig. 3). In addition, Holzschuh et al. (2016) have raised an important question of whether the increased pollinator densities in mass-flowering crops are caused by their population size increase or if they are simply attracted to huge food resources. So, they found that mass-flowering crops dilute pollinators' abundance because they found a consistent negative correlation between the growth area of mass-flowering crops and pollinator densities in mass-flowering fields across the Europe. Thus, it means that despite of the rapid increase of mass-flowering crops across the Europe, the size of wild pollinator population will not win from this land-use change in general.

Changes in land-use intensity and agricultural practices have also resulted in greater habitat fragmentation, i.e. the spatial detachment of habitat patches which causes reduced and isolated populations that are at an increased risk of inbreeding. Spatial separation affects wild bees on different scales: (i) at large scale (up to hundreds of kilometers), it reduces connectivity of nest sites, isolates bee populations and thus reduces gene-transfer between different populations; and (ii) at small scale,

Table 1 The dominant bee crop pollinators in Europe according to Kleijn et al. (2015). Listed are all species whose abundance formed at least 5% of all specimens of wild bees on crop flowers at least one study

Species	Species	Species
Andrena carantonica	*Bombus hortorum*	*Hylaeus punctulatissimus*
Andrena chrysosceles	*Bombus lapidarius*	*Hylaeus taeniolatus*
Andrena cineraria	*Bombus pascuorum*	*Lasioglossum malachurum*
Andrena decipiens	*Bombus pratorum*	*Lasioglossum pauxillum*
Andrena distinguenda	*Bombus subterraneus*	*Lasioglossum politum*
Andrena dorsata	*Bombus terrestris/lucorum*	*Lasioglossum subhirtum*
Andrena flavipes	*Ceratina cucurbitina*	*Lasioglossum xanthopus*
Andrena haemorrhoa	*Ceratina mandibularis*	*Melitta leporine*
Andrena helvola	*Eucera clypeata*	*Nomada lathburiana*
Andrena labialis	*Halictus resurgens*	*Osmia bicolor*
Andrena lagopus	*Halictus rubicundus*	*Rhophitoides canus*
Andrena nigroaenea	*Halictus scabiosae*	
Andrena nitida	*Halictus simplex*	
Andrena ovatula	*Halictus tetrazonianellus*	
Andrena subopaca		
Anthidium septemspinosum		

Fig. 3 An oligolectic solitary bee *Adrena hattorfiana* (Fabricius) feeding its young on pollen of *Knautia arvensis*. This solitary bee species is threatened in several European countries because of loss of habitats and food plants. (Photo: Peeter Veromann)

at the local habitat patches, it reduces connectivity between foraging and nesting sites that influences food seeking success. Looking at natural habitats it is obvious that the isolated populations are threatened, since the species decline of wild bees will reduce the wild plant diversity very fast followed by instability of the ecosystem itself and its potential for restoration (Potts et al. 2010). However, the impact of fragmentation can differ depending on the habitat preferences of bees. For instance, Williams et al. (2010) have shown that bees nesting below ground are less sensitive to disturbance factors and less influenced by small scale fragmentation than bees that nest above ground. At the same time, the density of bees nesting above ground can be higher in smaller habitat patches (Hinners et al. 2012).

Fragmented landscapes can be redesigned keeping the needs of different pollinators in mind. There are different reasonable measures to connect isolated habitats, for instance, in addition to being food resources, flowering strips inside the fields or in field edges can work as connecting corridors between habitats. For example, pollinator-specific wild flower seed mixes have clearly proven to contribute to wild bees' diversity and abundance (Carvell et al. 2006; Grab et al. 2018; Redpath-Downing et al. 2013; Rundlöf et al. 2018). Woody linear landscape elements like hedgerows, ditches with coppice, lanes with trees etc. can also act as the connecting corridors to reduce isolation between the nesting habitats of wild bees. The importance of hedgerows as a long term set-aside for native bees has been highlighted by several authors e.g. Morandin and Kremen (2013) and Williams et al. (2015), however, this kind of manipulation with agricultural landscape element requires significant input to establish.

The first public and political steps to acknowledge the importance of pollinators and their interactions with plants and to raise awareness were undertaken within the Convention on Biological Diversity (CBD) on the 5th Conference of Parties (in 2000) with the "Sao Paulo Declaration on Pollinators" (International Pollinator Initiative 1999). An action plan (decision VI/5) was developed, and the International Pollinator Initiative was formed under the leadership of the Food and Agriculture Organization (FAO). However, somehow it was unsurprising that someone once asked the principle question: "*Buzziness as usual? Questioning the global pollination crisis*" as Jaboury Ghazoul did in 2005 with a provocative topic concerning the uncertainty about the dynamics of pollinator populations (Ghazoul 2005). Unfortunately, until now there is no adequate answer available concerning this critical question and it will be difficult to answer this in principle, since long-term investigations in this field are lacking totally.

In 2011, key unanswered questions for future research in the field on the greatest knowledge gaps that need to be addressed were postulated by Mayer et al. (2011) in order to inspire new ideas in research on pollination ecology and pollination-related topics. These topics ranged from (1) plant sexual reproduction, (2) pollen and stigma biology, (3) abiotic pollination, (4) evolution of animal-mediated pollination, (5) interactions of plants, pollinators and floral antagonists, (6) pollinator behavior, (7) taxonomy, (8) the breadth and depth of our current understanding of plant-pollinator assemblages, (9) geographical trends in pollinator diversity, (10) drivers of pollinator loss, (11) pollination as an ecosystem service, (12) managing pollination services, (13) conservation and (14) implementation of conservation of plant-pollinator interactions.

The Millennium Ecosystem Assessment (http://www.millenniumassessment.org/), a global initiative launched by the United Nations, demonstrated the vital importance of ecosystem services for human well-being and found that two thirds of them are in decline or threatened. Bees provide direct ecosystem services. The on-going initiative on "The Economics of Ecosystems and Biodiversity" (TEEB, http://www.teebweb.org/) analyses the value of ecosystems and biodiversity to the economy, to society and to individuals. It underlines the urgency of action, as well

as the benefits and opportunities that will arise as a result of taking the value of ecosystems and biodiversity into account better in policy decisions.

Thus, today we can conclude, that the focus changed in the last century from principle pollination questions to a more broad view on ecosystems and biodiversity and therefore, to the critical field of economy and society.

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Solitary Bees As Pollinators

Bettina Maccagnani and Fabio Sgolastra

1 Working Mothers: This Makes the Difference

What makes the difference between solitary and social bees? Every female makes her own nest, and has her own brood to rear without the assistance from other females. This means that each female collects pollen, and the need of nectar is limited to the amount required to get enough energy for individual activities plus little amounts used to mix up the pollen for the brood (Ladurner et al. 1999). This biological trait has the direct consequence that solitary bees can profit also from flowers that are not particularly rich in nectar or in its sugar concentration, while they are much more interested in the pollen rewards offered by the flowers (Nepi et al. 2005).

The pollen collecting organs of the solitary bees can be very different from the well-known structures honey bees have on the hind legs, which build up a compact pollen mass around a spine, mixing a little nectar to the pollen in order to fix it (Fig. 1a-b). In Antophoridae bees, for example, the hind legs are covered by dense and long hairs which allows them to collect huge amounts of pollen (Fig. 2a and Fig. 3a-d). Megachilidae bees collect pollen through series of special hairs in the ventral part of the abdomen Fig. 3a-b; in (Fig. 1b, Fig. 3a-b); in other species hairs are distributed both in the legs and in the ventral parts of the abdomen and of the thorax. The most important difference with the pollen mass produced by honey bees

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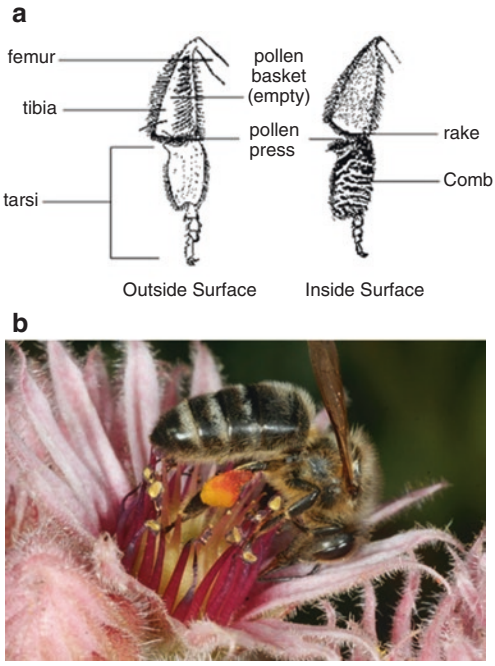

Fig. 1 The hind leg of a honey bee (**a**) and a honey bee with a bi-color pollen load (**b**)

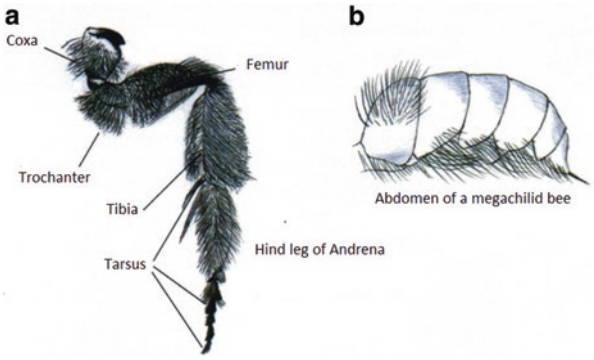

Fig. 2 Pollen collecting organs in *Andrena* (**a**) and *Megachile* (**b**) (Alessandra Montanari)

and bumble bees is that the pollen remains dusty, and easily can detach from the body of the bee when passing from one flower to another. This morphological aspects plus the behavioural trait of landing on the reproductive organs in the central part of the flower (Fig. 5a-b) are present in many solitary bee species, which bring the collected pollen directly in contact with the stigmas. This increase the pollinating efficiency compared with the performance of the nectar foraging honeybees, which very often land on the petals, unless they are contemporarily collecting pollen and nectar, or in case of flower shapes that oblige them to land in the central part of the flower to reach the nectar (Fig. 6a-b) (Fig. 4).

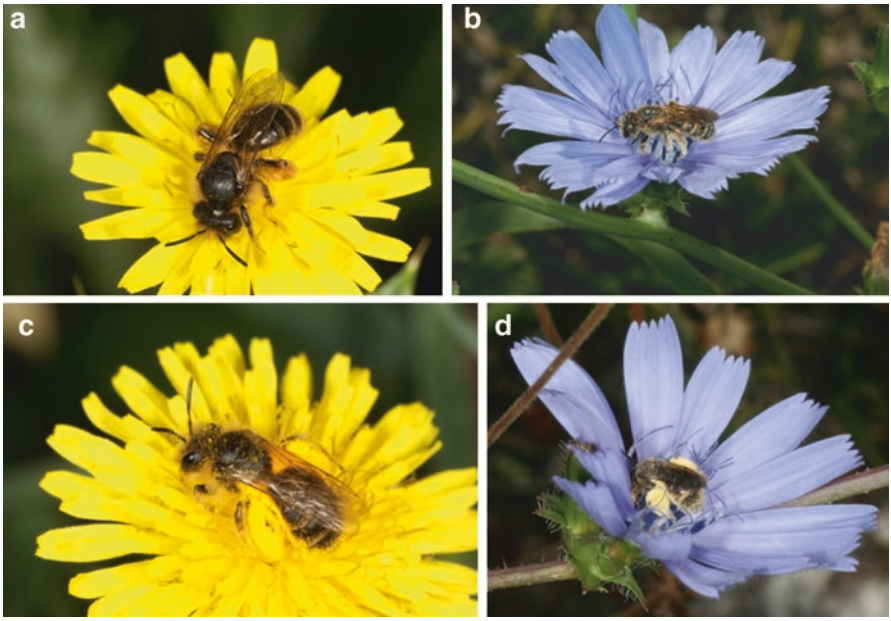

Fig. 3 *Andrena* sp. bees foraging on *Picris hieracioides* (**a**) and *Cichorium intybus* (**b**) before and after (**c-d**) pollen collection

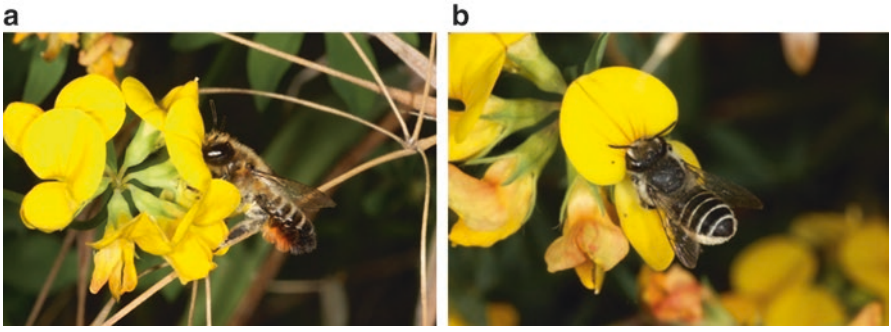

Fig. 4 Megachilid bees foraging on *Lotus corniculatus* before starting to collect pollen (**a**) and with the full pollen load (**b**)

Being a solitary bee species does not mean to be territorial: on the contrary, many species show gregarious nesting habits. In fact, if the site offers optimal nesting conditions, hundreds of nests of solitary bees can be established in a very restricted area. The nesting habits of solitary bee species can be very different, but three main groups can be described: species that dig nests in the soil (Fig. 7), species that nest in pre-existing cavities (Fig. 8a-b), mainly in the stem of plants or in cavities excavated by other insects (or in old house walls, in the modern age), species that excavate their nests in the wood (Fig. 9).

Fig. 5 Pollen collecting (**a**) and nectar collecting (**b**) *Ceratina* sp. on *Borrago officinalis*

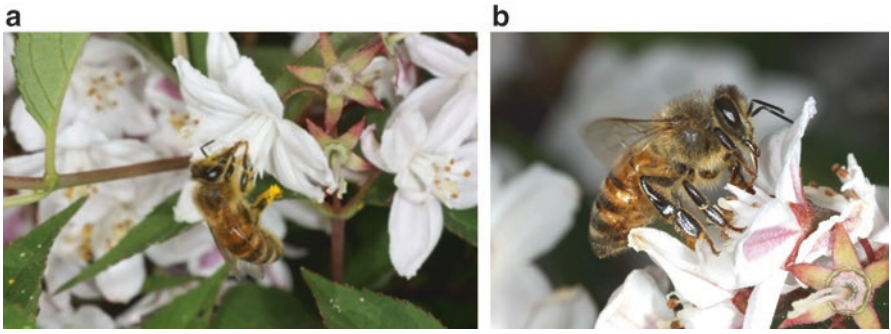

Fig. 6 Pollen collecting honey bee (**a**); nectar collecting honey bee (**b**)

2 Which Bee for Which Crop

Many studies have been run in recent years to find out which pollinator features are to be considered to determine which pollinator species could be candidate for improving the pollination service of a certain crop (Cane 1997; Thomson 2001; Kevan and Phillips 2001; Cane and Tepedino 2001; Bosch and Kemp 2002; James and Pitts-Singer 2008). These features consider the ecological link between plant and insect, the synchronism between foraging activity and blooming period, between flower attractiveness and exploitation modalities put into effect by the insect (McGregor 1976; Buchmann 1983; Free 1993; Benedek and Nyeki 1996; Goodell and Thomson 1998; Javorek et al. 2002) (Table 1).

The degree of effective contact between the pollinating insect and the reproductive organs of the flower depends on these modalities, and also on the ratio between the size of the insect and the size of the flower (Figs. 10; 11a-b). The relative values of pollinators to crops depend on how much pollen they remove from anthers as well as how much they deposit on stigmas. According to Goodell and Thomson (1998) in a comparison on apple pollination efficacy, *Apis* workers and *Bombus*

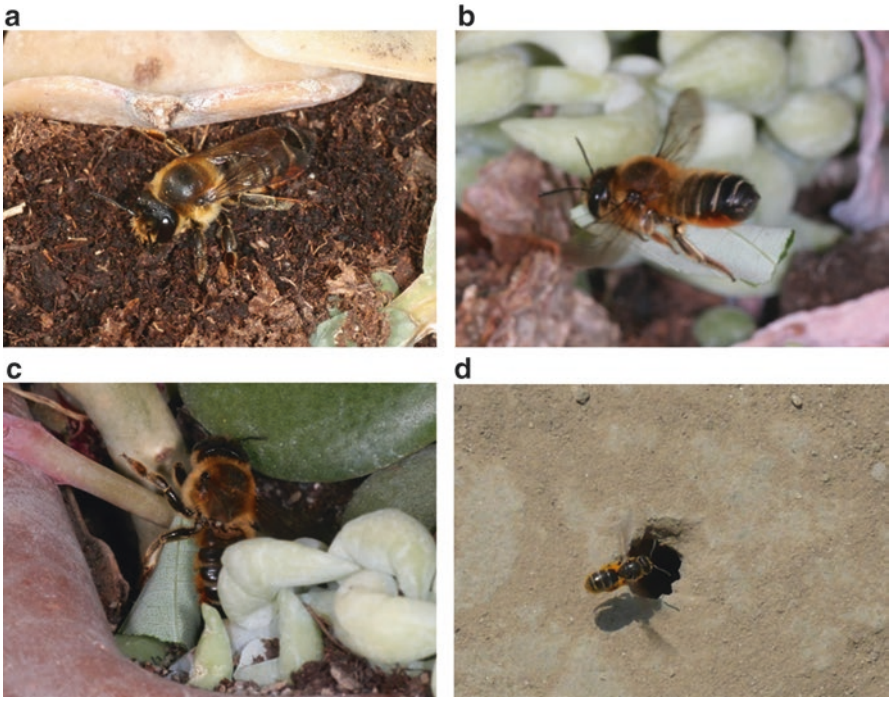

Fig. 7 Megachilid leaf-cutting bee (**a-c**) and *Andrena* sp. (**d**) nesting in the soil

queens differ in both pollen deposition and removal because they frequently adopt different foraging behaviors. Nectar-collecting *Apis* removed fewer pollen grains from the anthers of cv Delicious than did nectar- and pollen-collecting *Bombus*. As these differences are part of the complex reproductive strategy of entomophilous flowered plants, they are applicable to all the other crop/pollinator species comparisons (Kendall 1973; Kendall and Solomon 1973; Harder and Thomson 1989; Harder and Wilson 1998; Thomson and Thomson 1999).

Many studies have been published on the potential pollinating efficiency of native solitary bees on several crops (Willians 2002; Shuler et al. 2005; Klein et al. 2007) which undoubtedly and essentially contribute to maintain an unwavering background ecosystem service of crop pollination. The populations of wild bees can be very abundant if sufficiently large conservation areas are preserved (Kremen et al. 2004; Potts et al. 2010; Abrol 2011).

In some cases, effective and manageable native solitary bees were identified. For example, in US, the squash bee *Peponapis pruinosa* Say is a very effective pollinator of *Cucurbita pepo* (Tepedino 1981; Artz and Nault 2011), while the southeastern blueberry bee, *Habropoda laboriosa* (Fabricius) can very well compensate for inadequate honey bee pollination the rabbiteye blueberry, *Vaccinium ashei*; on this crop, a new adaptable pollinator *Osmia ribifloris* Cockerell was successfully reared and used in captivity (Sampson and Cane 2000). Despite the high number of

Fig. 8 Cavity nesting species: (**a**) *Heriades* (top left) closed the nest with small stones kept together with resins; *Anthidium* sp. female (central) is entering her nest; on the right, a mud wall closed the nest of *Osmia cornuta*. (**b**) *Anthidium* sp. female resting at the nest entrance

crop-pollinator interactions studied with the aim of highlighting how effective pollinators can maximize crop yield, only few native bee species are commercially available. This discrepancy is due to some limiting factors, related to the possibility to develop a mass reared populations of pollinators: the number of reproductive cycles completed by the insect in one year, the duration of the diapause, the adaptability to artificial nesting sites, the gregariousness degree during nesting (Kevan and Phillips 2001; Cane and Tepedino 2001; Cane 2008). On the basis of these criteria, several authors give indications on how to re-establish a numerically adequate population of the pollinating insect in the cultivated area (Bosch and Kemp 2001; Kevan and Phillips 2001).

Up today among the few reared solitary bee species present in North America, the alfalfa leaf-cutter bee *Megachile rotundata* Fabricius (Pitts-Singer and Cane 2011), the blue orchard bee *Osmia lignaria* Say and the alkali bee *Nomia melanderi* Cockerell (Cane 2008) are commercially available on the large scale, or can be reared by the growers themselves. *Osmia cornifrons* (Radzowski) has been managed in Japan for apple pollination since the 1940s (McKinney and Park 2012), and is used also in Korea and China (Da-Yong and Long-Shi 2007; Lee et al. 2008); it was introduced into the United States for pollination in 1977. In Australia, a promising study on tomatoes pollination in greenhouses has been performed by rearing the blue banded bee, *Amegilla chlorocyanea* Cockerell on artificial substrates in captivity (Hoogendorn et al. 2007) (www.beaware.org.au; http://www.aussiebee.com.au).

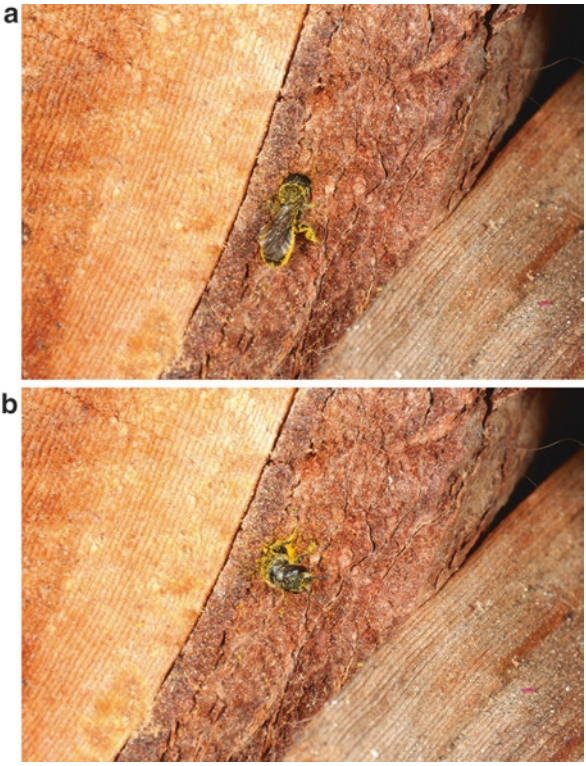

Fig. 9 Small carpenter bee excavating her nest (**a**) and entering backwards with her pollen load on the abdomen (**b**)

Table 1 Foraging flight duration and provision weight of *O. cornuta* populations foraging on pears

Year/Reference	Provision weight (g)
2000/Maccagnani et al. (2007)	0.509 ± 0.056 (females)
2001/Maccagnani et al. (2007)	0.591 ± 0.177 (females)
1994/Bosch (1994)	0.260 (males)
	0.451 (females)

In Europe, *O. rufa* L. (syn. *O. bicornis*) and *O. cornuta* Latreille are reared and used for orchard pollination and seed productions (Pinzauti et al. 1997; Ladurner et al. 2002; Maccagnani et al. 2003a, b; Krunic et al. 2005; Gruber et al. 2011).

3 Solitary Bees: A Focus on *Osmia* sp.

The genus *Osmia* Panzer (Hymenoptera Megachilidae) comprises 300 species (Michener 2000). Among those already commercially available as crop pollinators, *O. cornuta*, *O. rufa* and *O. cornifrons* are Palearctic species; *O. cornuta* (Fig. 12)

Fig. 10 The giant and the small, *Xylocopa violacea* and *Ceratina* sp. on *Cynara cardunculus*

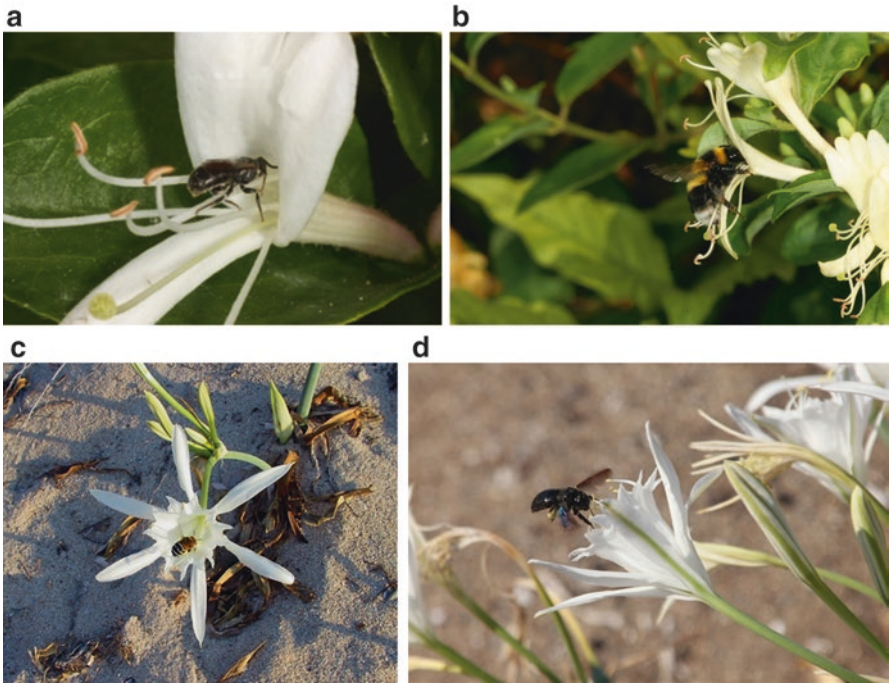

Fig. 11 Too small bee size (**a, c**); proper bee size (**b, d**)

occurs in Central and Southern Europe, Turkey and Northern Africa; *O. rufa* (Fig. 13) shows a more Northern distribution, while *O. cornifrons* (Fig. 14) is native from Japan. *O. lignaria* (Fig. 15), with different subspecies, is a Nearctic species, widely distributed in the U.S., where also *O. cornuta* has been introduced in the late eighties for almond and apple pollination, and is now established (Torchio and Asensio 1985; Vicens and Bosch 2000).

Fig. 12 *Osmia cornuta* on *Pyrus malus* (apple) flower

Fig. 13 *Osmia rufa* (syn. bicornis) on *Papaver rhoeas*

Osmia species show gregarious nesting behavior and build their nests in pre-existing cavities (Fig. 16), where the female produces a linear series of cells divided by mud walls (Tasei 1973; Peters 1977) (Fig. 17).This is why they are called generically "mason bees"; in each pedotrophic cell, one egg is laid on a nectar-pollen based mixture (Nepi et al. 2005) which the five larval instars will grow on.

Concerning the relationship with the plant food source exploited, *Osmia* species are oligolectic or polylectic, and for many of them the timing of the life cycle is related to the flowering of the Rosaceae botanical family (Márquez et al. 1994), whose pollen is highly preferred in comparison to others (Tasei 1973; Márquez et al. 1994; Maccagnani et al. 2003a, b). Their efficiency as pollinators, which has been compared to that one of honey bees by many authors (Bosch 1994; Monzon et al. 2004; Maccagnani et al. 2003a, b) is due to several eco-ethological traits. The most noticeable trait is that their foraging activity is mainly oriented to collect pollen, and in this aspect *Osmia* species are substantially different from honeybees. In fact, the flower visit performed by honeybees in search of nectar implies a limited contact with the reproductive parts of the flowers, as foragers land very often on petals than elongate the proboscis towards the nectaries, at the base of the stamens (Barth 1985; Free 1993, Fig. 9). Instead, mason bees are "top-foragers" and collect

Fig. 14 *Osmia cornifrons*

Fig. 15 *Osmia lignaria*

Fig. 16 Nesting females at the entrance of their nests in the early morning, waiting to warm up before flying

the pollen with their ventral abdominal sets of hairs, using either the forelegs or the abdomen to scratch the anthers, according to the flower morphology (Tasei 1973). Direct observations of *O. cornuta* foraging on apple flowers showed that all visits are performed landing on the flower reproductive organs to collect pollen from the anthers, contemporarily feeding a small amount of nectar, which is possible thanks to a much longer tongue with respect to honey bees (personal observations). The dimensions, the kind of approach to the flower and the efficiency of the ventral

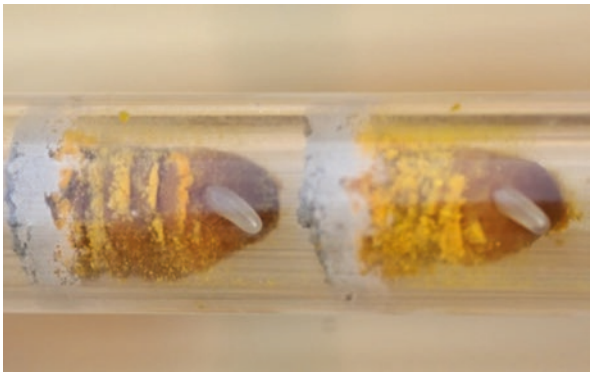

Fig. 17 Portion of an *Osmia cornuta* nest within a transparent plastic tube. Two mud walls are clearly visible. Two pollen provisions, made up by adding pollen and secretions, show the succession of dry pollen and wet components. One egg is visible on each provision

scopa allow *Osmia* species to collect the pollen with quick and effective movements, ensuring, at the same time, very fast flower handling and high pollinating efficiency, thus reaching also 100% flower pollination (Bosch and Blas 1994; Maeta 1978; Vicens and Bosch 2000). In fact, the number of pollen granules transferred to the stigma by mason bees is much higher with respect to the honeybees, which transport the collected pollen with the hindlegs, a modality that prevent any direct contact between the collected granules and the reproductive flower organs (Harder and Wilson 1998). Pinzauti (2001) demonstrated in *Osmia* that the pollen germination potential is conserved until the deposition of the mass in the larval cell. In fact, only in this moment the female adds to the discharged pollen little drop of stomodeal content, composed by nectar with the addition of glandular secretions that, someway, reduces the pollen viability (Nepi et al. 2005).

Osmia species have been assessed for crop pollination in different countries, both in open field on fruit trees like almond, apricot and apple (Bosch 1994; Bosch and Blas 1994; Krunic et al. 1995; Pinzauti et al. 1997) and in confined environments (Ladurner et al. 2002; Felicioli and Pinzauti 2008). Excellent results were obtained using *O. cornuta* for pear pollination (Maccagnani et al. 2003a, b, 2007; Monzon et al. 2004). Vicens and Bosch (2000) found that this species is a more efficient pollinator on almond and apple flowers than *A. mellifera*, because of its higher rate of stigma contact. Mason bees move from tree to tree more readily than honey bees, are less prone to orient along rows, thus enhancing cross pollination. Moreover, *O. cornuta* can adapt to a number of artificial nesting materials: another important aspect for the development of a pollinator management system (Krunic et al. 1995; Bosch 1995; Bosch and Kemp 2002).

4 Osmia cornuta

Osmia cornuta is an obligate monovoltine species distributed in the South-Central Europe and in Northern Africa, and is a highly gregrarious nesting species.

Females emerge from cocoons in March (some variations depend on the latitude), and the nesting period is concluded within 1 month. In natural environments, nests are constructed in broken giant cane stems (*Arundo donax* L.), but females easily accept many different substrates if dimensions are appropriated (8–10 mm diameter). Visual stimula are very important for the females to orient their choice during the search for a suitable place to nest. *Osmia cornuta* showes no difference in visual perception and colour discrimination ability when compared to honey bees (Menzel et al. 1988). Thus, every dark point visible from the distance is explored. In its lifetime, the female lays around 30 eggs, producing, on average, one cell per day (Tasei 1973; Krunic et al. 2005). The pollen is collected and transported on the abdomen, thanks to a ventral hairy structure named scopa (Fig. 8). Upon return to her nest from a foraging flight, the female compacts the pollen discharged during the previous flight by using the head and the frontlegs, and while doing this, she deposits drops of stomodeal content on the pollen mass. Then, she turns back within the nest and discharges the new pollen load. When the diameter is too small, the female gets out, turn back at the nest entrance, and reach the provision walking backwards.

Nepi et al. (2005) hypothesized that adding stomodeal regurgitation (plus glandular secretions?) to the pollen, to prepare the provision the larva will feed on, may serve to initiate germination or pseudo-germination. Pollen grains are probably the most difficult food from which nutrients can be obtained, because the pollen walls are among the hardiest structures in the biological world. Thus, pollen feeding animals have evolved different and complicated mechanisms to obtain the nutrients and the energy stored in the pollen cytoplasm (Roulston and Cane 2000; Nepi et al. 2005). The morphological and biochemical changes occurring in *O. cornuta* pollen provisions might be necessary for "activating" the pollen grains for the digestive process. Other active substances could play a role in the pollen digestion process in the solitary bee larvae, such as mother-derived secretions produced by the female and added to the provision (Ladurner et al. 1999; Heroin-Delauney 1966).

It is worthwhile to point out that solitary bee larvae feed directly on the mother provision, and this makes a big difference with respect to the social species like *Bombus terrestris*, in which adult workers contribute to larval nutrition pre-digesting the pollen (Ribeiro et al. 1999; Pereboom 2000), and *A. mellifera*, in which nurse bees feed the larvae with a glandular secretion after having themselves eaten and digested the pollen (Roulston and Cane 2000). The possible role of commensalistic microbial fauna of the larval cell in inducing biochemical changes in pollen-nectar provision remains to be investigated.

The energetic investment in the two sexes is quite different, as female cocoons weight, on average, around 0.4–0.5 g while male cocoons only 0.2 g (Bosch 1994; Maccagnani et al. 2007). As a result, a sex-dipendent dimorphism is considerably high in *O. cornuta*, and implies a much stronger foranging effort to produce female cell provisions than to produce males. Coherently, the female progeny originated from fertilized (diploid) eggs is produced at the beginning of the nesting period, when the mother female is at her maximum efficiency, while males are produced at the end, from unfertilized eggs. The pre-imaginal development lasts several weeks,

and go through five moults, the first occurring within the egg. The first larva that can be observed feeding on cell provision is the second stage larva,; the following larval stages consume the provision in a few weeks. Than the fifth instar larva spin its cocoons, overpass the summer, and undergo the methamorphosis into adult before Autumn. Overwintering occurs as adult in the cocoon.

In the sister species *O. lignaria*, Sgolastra et al. (2011, 2012) found that adult eclosion follows the end of the summer diapause occurred during the prepupal stage; few weeks later, the adults lower their respiration rates (to ~0.1 ml/g h) and undergo the winter diapause, that lasts until February–March, depending on the latitude. Winter diapause development in *Osmia* spp. occur in two phases. In the first phase, cold temperatures are required to increase the respiration response, which reaches a plateau in mid-winter. In the second phase, the respiration response follows an exponential increase, and when it has reached 0.45 ml/g/h the adult emerge promptly when exposed to 20°C, indicating diapause completion (Sgolastra et al. 2010).

In *O. bicornis*, a study by Wasielewski et al. (2013) found a complex dynamic of different energetic resources and enzymes that could explain the changes in the respiratory trends.

As each female can occupy simultaneously more cavities, and female eggs are layed at the beginning of the nesting period, female cocoons are generally found at the bottom of the cavity, while male cocoons occupy the front part of it. This behaviour has the biological significance of inducing a pronounced proterandry, resulting in the activation of males some days before females (likely related to the warmer temperatures in the front portion of the nesting tunnels, and to a higher oxigen diffusion, in comparison to the adults overwintering in the bottom portion), and to protect the female progeny (half of the male progeny, sex-ratio ff:mm 1:2) by rearing females in the most protected part of the nest.

In the perspective of the use of solitary bees to enhance pollination, it has to be considered the time needed for adult diapause termination in early spring. This duration depends on overwintering conditions and on early spring weather conditions, mainly the mean daily temperature.

If the release of *O. cornuta* populations occurs at the beginning of crop flowering, females might not be "ready" for an efficient pollination service before the end of the flowering period. Early flowering crops with short flowering periods would profit from a good landscape management with the introduction of ecological infrastructures with nectariferous/polliniferous botanical species can support the bee population prior and after the blooming period of the target crop, thus enhancing both crop pollination and bee reproduction (Maccagnani et al. 2007).

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Bumble Bees and Entomovectoring in Open Field Conditions

Marika Mänd, Reet Karise, and Guy Smagghe

1 Introduction

Management of bumble bees for delivering biocontrol agents has been studied for more than 20 years (Peng et al. 1992; Yu and Sutton 1997). Most of the research, however, has been done mainly in laboratory or greenhouse conditions (Kevan et al. 2003; Mommaerts et al. 2011). The reason for using bees as vectors for biocontrol agents (BCA) stays in their morphological and behavioural characteristics. Bumble bees have relatively large body surface covered with branched hair, which aids trapping and transporting pollen grains (Free and Williams 1972; Batra et al. 1973). Similarly to pollen, the spores of microorganisms can stick to the fur, which characteristic has been put in use in entomovector technology to deliver BCAs to the target crop (Fig. 1). The commercial availability of bumble bee colonies has enabled the increase in usage of the buff-tailed bumble bee *Bombus terrestris* L. in Europe and the common eastern bumble bee *B. impatiens* Cresson (Hymenoptera: Apidae) in North America not only in greenhouse (Mommaerts et al. 2011) but also in field conditions (Kovach et al. 2000; Dedej et al. 2004; Karise et al. 2016a). In this

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Fig. 1 A bumble bee covered all over the body with a large amount of Prestop Mix powder, at the time of homing all the powder was gone (Photo: Reet Karise)

chapter the potential of bumble bees as vectors of BCAs in open field conditions will be considered. In addition, we talk also about several aspects, which have to be taken into account if harnessing bumble bees as vectors in open fields.

2 Bumble Bee Efficacy in Open Fields

2.1 Grey Mould Suppression

The data, collected during BICOPOLL project, confirmed that the bumble bees proved to be effective in mediating biofungicide Prestop-Mix (*Gliocladium catenulatum* Strain J1446 as active organism, Verdera OY, Finland) in open field conditions. Prestop Mix is a biological preparation, which is safe both to humans and beneficial organisms visiting the fields (Verdera 2015). The infection rate decreased approximately 1.5–3 times when pathogen pressure was light or moderate, but no change was seen when there was high pathogen pressure due to heavy rain and cool temperature conditions (Fig. 2) (Karise et al. 2016a). Higher rainfall and colder temperatures during the fruit maturing period create particularly good conditions for the pathogen *B. cinerea* (Wilcox 1994; Cota et al. 2009) by which the infection rate on berries might rise up to 70–80%. In these conditions, also chemical control most likely could fail without proper decision supporting systems (Evenhuis and Wilms 2009). The efficacy of honey and bumble bee-vectored biocontrol has been found to be comparable to synthetic fungicide spraying (Kovach et al. 2000).

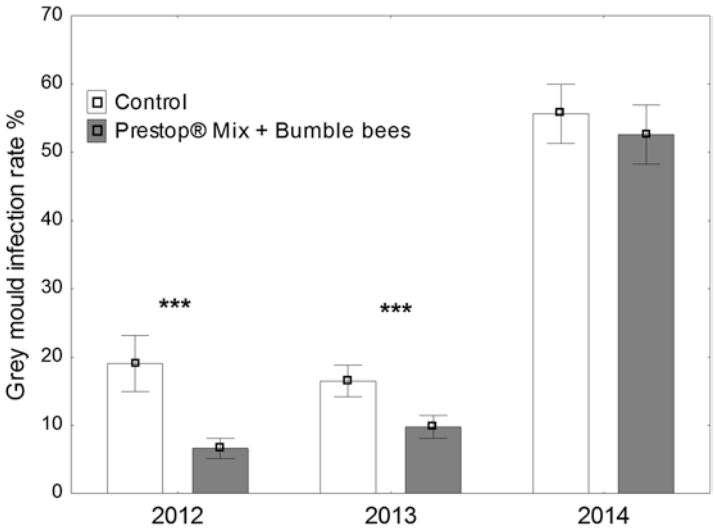

Fig. 2 Grey mould infection rate on control plots (not treated) and plots treated with bumble bee-mediated Prestop-Mix. The means with standard error bars are presented. Asterisks (***) indicate a significant decrease ($P < 0.001$) in the infection rate during the years with low pathogen pressure (from Karise et al. 2016a)

2.2 Inoculum Dissemination: Safety and Efficacy

The basis for dissemination task is the ability of the preparations to adhere to and get released from the hair of a bee easily. The amounts of powdery preparations, stuck to the hairs of the bees exiting the nest box, are variable. Some bees are covered only ventrally, some others however all over their bodies, although the larger amounts of the powders may disturb the vectoring bees, they lose most of it within first 60 s (Mommaerts et al. 2010). Both bumble bees and honey bees might suffer some-what when covered with larger amounts of the powders containing kaolin in closed experimental conditions (Karise et al. 2016b, 2018). Though, the effect was never noticed on the field nor in greenhouses when performing the BCA vectoring task. In addition, the commercial bumble bee hives have short life-span and are not meant to function longer than the time needed for pollination of the target crop. Even with the shortened individual life-span, the number of days a bee lived, was suitable to fulfil the pollination and vectoring tasks (Fig. 3). Commercial bumble bee hives are well suitable also with bioinsecticides, despite some of the entomopathogenic fungi can infect also the vectoring bees (reviewed by Mänd et al. 2010).

The efficient biocontrol can be achieved only when the BCAs are spread evenly over the entire field. In this purpose, it is important to study the bumble bee density on the flowers of the target crop. According to BICOPOLL project results, the bumble bee dispersal over the field was equable over a distance of 100 m (Karise et al. 2016a).

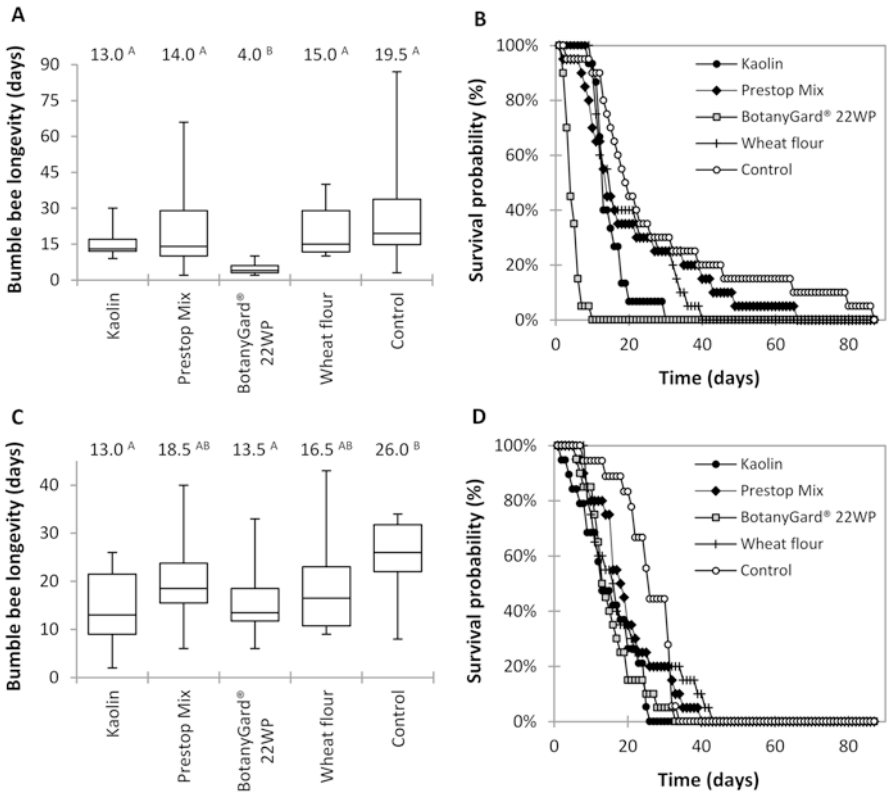

Fig. 3 Lethal effects of exposure to kaolin, Prestop-Mix, BotaniGard and wheat flour on the survival on bumblebees (*B. terrestris*). A and B present the longevity of bumblebees (days) and the survival probability (%) at 18 °C, while C and D the respective data at 28 °C. The longevity data are expressed as box plots with the minimum, lower quartile, median, upper quartile and maximum values. The numbers upon the boxes denote medians and different letters indicate statistically significant differences between groups at $p < 0.05$. From Karise et al. 2016b

The second important aspect is the dispersal rate of the BCAs. The results showed also the even distribution of the *G. catenulatum* on flowers. Indeed, this study was conducted on relatively small strawberry fields, but the good distribution of *Clonostachys rosea* (Link) Schroers by *B. impatiens* has been observed up to 150 m (Reeh et al. 2014). Wolf and Moritz (2008) have shown the mean foraging distance of *B. terrestris* being 267 m, whereas 40% of bumble bees foraged within the radius of 100 m. Thus the distance between bumble bee hives on larger fields could be around 200–300 m to guarantee the good visitation rate for the strawberry.

2.3 The Secondary Transmission of BCA

The greenhouse experiments of BICOPOLL project indicate, that bumble bees lose about 81% of Prestop-Mix already within the first 60 s of their flight (Mommaerts et al. 2010). The sequential visits of any other flower visitors aid the transportation of BCAs from flower to flower (Maccagnani et al. 2009). This phenomenon has been called the secondary inoculation and may play important role in efficacy of the technology (Nuclo et al. 1998). The rate of newly opened flowers and weather conditions, which favour insect visitation of flowers, affect the efficacy of secondary transmission of BCAs (Nuclo et al. 1998; Maccagnani et al. 2009). In the northern regions, the strawberry flowering occurs at the time when there are almost no other numerous wild bumble bees foraging, since the newly emerged overwintered queens are establishing their colonies yet. Still, at that time the numbers of foraging honey bees, several solitary bee species and also different species of dipterans might be quite high. The BICOPOLL field study showed, that the most abundant groups in Estonia were dipterans including syrphid flies which formed 49% of the number of all flower visitors, followed by honey bees 29% and solitary bees 13%. Ahrenfeldt et al. (2015) showed that the wild bee species diversity and community composition on strawberry has a north-south gradient from Mid-Norway through Denmark to Germany. The diversity of these insects is higher in the southernmost regions. So, it is possible that the effect of the secondary dissemination on the entomovectoring is higher in regions with higher insect abundance and diversity.

2.4 Additive Value from Pollination

The additive value from applying the entomovector technique comes through enhancing the pollination of target crop. The direct benefit from bee-pollination depends on the crop species and cultivar. There are plant species, which give almost no yield without animal pollination: for instance, blueberries, raspberries, apples, cherries and plums benefit largely from insect pollination. Strawberries, however, belong to those species by which the effect from pollination depends on the cultivar (Klatt et al. 2014a, b; Tuohimetsä et al. 2014). There are more than 200 stigmas in the strawberry flowers (Free 1993; Perkins-Veazie 1995) and each of these needs to be fertilized in order to achieve high quality fruits. In some strawberry cultivars the pollen grains can be released from the anthers with the help of wind. Other cultivars need a high functional insect diversity to get the flowers fully pollinated (Klein et al. 2007). Fruits from properly pollinated flowers are not only greater but also have longer shelf-life and highest commercial value (Klatt et al. 2014a, b). In BICOPOLL experiment, still, no increase was found in fruit weight due to insect pollination (Karise et al. 2016a). This suggests that the cultivar ‘Sonata’ used in those experiments can cope with wind pollination.

3 Bumble Bee Foraging Behaviour

When using bumble bees on open fields it is very important to understand basics of bumble bee foraging behaviour in order to find out right solutions.

3.1 Flower Selection

Bumble bee flower preference is based on several aspects. Food plant choice of bees depend on the amount and quality of nectar and pollen provided by the flowers present in the foraging area. The nectar production of flowers can vary between the cultivars (Bertazzini and Forlani 2016) and also depends on the weather conditions (Nicolson and Nepi 2005). For example, in moist conditions the nectar sugar content is low and larger amounts of nectar are needed to satisfy the colony needs (Shackleton et al. 2016). The effect of cultivars varies across the years, since different weather conditions favour different properties of the cultivars, best seen in hybrid lucerne (Karise et al. 2006). The food resource quantity and quality may also be manipulated by farmers, as demonstrated by Viik et al. (2012): proper fertilization increases both the numbers of flowers and the amounts of nectar per flower, which serve as main attractants for bees. In case of toxic compounds, e.g. pesticides in bee feed, the behaviour of foragers can be changed (Koskor et al. 2009).

The other important characteristic is the tongue length of the bee, which influences the flower selection and in turn the breadth of the diet (Teräs 1985). Short-tongued bumble bees usually have wider diet compared to long-tongued bumble bees, who prefer flowers with narrower and deeper corollas (Alford 1975; Goulson et al. 2008). Bees with short tongues do not pollinate properly flowers of red clover and field bean for instance, however open flowers of strawberry have no demands on the tongue length of the pollinator. The commercial bumble bee *B. terrestris* has relatively short tongue, however, the bee is able to feed on different flower types. In order to get access to the nectaries hidden in the bottom of deep corollas, *B. terrestris* often bites holes into corolla tubes and thus robs nectar without pollinating the flower. This bumble bee species very easily broadens their forage plant selection to non-native garden plants and mass-flowering crops (Goulson et al. 2002). The wide diet of *B. terrestris* enables them easily to react on changes in agricultural landscape and human activity. This also provides us the possibility to force bumble bees to forage on plant species not naturally in their diet. Some crops flower in the time, when there is no *B. terrestris* foragers available naturally.

3.2 Flower and Forage Patch Constancy

Bumble bee *B. terrestris* is a food generalist visiting several (2–4) plant species within the same foraging trip (Carvell et al. 2006; Parmentier et al. 2014; Somme et al. 2014). Bumble bees have no innate plant species preference. Unlike honey bees they constantly search for new profitable flower types, even in case of plentiful food resources, as it happens on large fields. It is suggested, that flower constancy may have emerged to save energy and/or time of the foragers (Free 1970; Dukas 1995; Gegear and Thomson 2004). Compared to honey bees the probability of spreading the BCAs on the non-target crop is quite high with *B. terrestris*.

Bumble bees generally prefer larger patches, and at the same time avoid less rewarding patches regardless of size (Makino et al. 2007). They also have shown strong constancy on sites, at which they already have found nectar and pollen resources (Osborne and Williams 2001; Cartar 2004), and visit same patches repeatedly until the site is still rewarding (Makino and Sakai (2007). In purpose of intensification of the forage effort, bumble bees recruit their nest mates to beneficial food sources by touching each other and releasing pheromone signals (Dornhaus and Chittka 1999, 2001; Ayasse and Jarau 2014). Beside the recruitment behaviour, the foragers systematically search for new flower resources. The distribution of foragers among patches depends on the relationship of recruitment rate and patch size and also on how long the individual forager spends in the particular patch (Renner and Nieh 2008).

3.3 Colony Size and Foraging Range

Colony size and flight range of the foragers affects the number of flower visiting bumble bees. In commercial crop production, it is very important to gain as much pollinating individuals as possible. The large colonies with lots of brood have higher need for pollen and nectar. According to this *B. terrestris* is one of the best succeeding bumble bee species with up to 400 workers in a colony, whereas in the colonies of *B. muscorum* and *B. sylvarum* e.g. only 20–100 workers are present (Benton 2006).

Bumble bees are central place foragers, which means that they collect food from the surroundings of their nest. *B. terrestris* has the maximum detected foraging range compared to other bumble bee species. Depending on different study methods, the observed foraging distances of the workers vary greatly even up to 4 km from the nest (Goulson and Stout 2001). The mass-marking experiments (Osborne et al. 2008) and radio-tracking (Hagen et al. 2011) have shown that *B. terrestris* workers can forage up to 2.5 km, although generally it remains between 500 and 1750 m from the nests (Walther-Hellwig and Frankl 2000; Westphal et al. 2006).

The species with small colonies have usually foraging areas up to 500 m in radii (Benton 2006). In context of entomovectoring, however, there is concern, that longer foraging distance of *B. terrestris* can cause dispersing of bumble bees on larger areas, which in turn will affect the efficiency of the technology.

3.4 Compatibility With Temperate Climate Conditions

Probably commercial bumble bees are most effective at the time there are few other pollinators available and the flowering occurs early in the season, when the daily temperatures stay cool. Bumble bees are pollinators, who are able to forage in cool temperatures. The heat generation ability in bumble bees is different from most of insects. During warming-up, bumble bees use their flight muscles without moving the wings (Heinrich 1979), thick body hair insulates the temperature into the thorax allowing to keep the heat (Newsholme et al. 1972; Peat et al. 2005). Some arctic bumble bees are able to forage even when the air temperature is below zero. They can forage even with light rain or fog if needed. In temperate regions, the bumble bee foraging starts at 5 degrees if there are no food supplies in hives left. A bumble bee colony stores very little nectar in the hive (Alford 1975) and this forces bumble bees to forage whenever it is possible. They even expand the daily foraging also into early morning and late evening periods.

4 Steering Bumble Bees to the Target Crop

Bringing bees as extra pollinators to open fields always holds the risk that they prefer some other plants species over the target crop. Steering them to any crop needs some certain knowledge on the behaviour and requirements of the particular bee species. Most often honey bees are used as extra pollinators due to their bigger and longer-living colonies. Commercial bumble bees, on the contrary, are easier to use, their colonies are smaller and perish soon after the pollination task is fulfilled. Bumble bees are usually not as aggressive in protection their hive compared to honey bees.

4.1 Handling of Bumble Bees

The handling of commercial bumble bees is easy. Bumble bee hive is small and compact. The producers supply the hives suitable for outdoor using: hives are placed in waterproof and insulaedt boxes. The entrances of the hives can be closed and opened, so that the bees can only enter or move in- and out-wards. For example,

Biobest Flying Doctors Hive (*B. terrestris*) has already built-in compartment for the BCA preparation.

The commercial bumble bee hives are supplied with liquid sugar solution. When taking the hives on field there are pros and cons on eliminating the sugar syrup. Eliminating it would encourage bumble bees to forage more actively. However, the extra food might be necessary in case of unsuitable weather conditions.

4.2 Synchronizing Pollination Service with Crop Flowering

Using commercial bumble bee colonies allows to synchronize the availability of flowers of target crops and insects, by taking the hives onto the fields exactly after flowering has started. This is important, because forager bees develop flower constancy and may stick on other plant species, which were in flower before the target crop. For strawberry, it is suggested that bumble bees should be taken to the fields when 5–10% of flowers are open, thus there is enough available forage for them. When there are too few flowers available, bees start searching alternative food source and learn to forage elsewhere. For the strawberry grower, it is also important to have bees on fields in a very right time, since the first flowers are those giving fruits with the highest quality and the protective effect of bumble bee-mediated Prestop-Mix was highest for the yield from the first flowers (Karise et al. 2016a).

4.3 Crop and Cultivar Selection

The attractiveness of flowers of different crops vary from cultivar to cultivar. In addition, the attractiveness of a crop to bees depends on which alternative food plants are available in the foraging area. To guarantee higher visitation rate, it is suggested to grow different cultivars within the same field. Varying sugar concentration of the nectar among cultivars has been seen to affect the bumble bee visitation rate (Yu and Sutton 1997; Kovach et al. 2000; Escande et al. 2002).

It is not needed to endeavour a 100% flower constancy. Our results indicate that effective disease control was achieved with about 22% strawberry pollen (Figs. 4 and 5) gathered by bumble bees (Karise et al. 2016a). Taking into account the bumble bee habit to try other plant species for food, it could be suggested to provide even some flowering plants nearby the strawberry field. During the 3 years of BICOPOLL project, the bumble bee preference for simultaneously gathered pollen, which was collected beside strawberry, varied between the years and places depending on which plants were available. The caragana, white clover, white nettle or Rosaceae species were gathered during the same foraging trips. The regional differences in environmental conditions within Europe are huge and therefore the

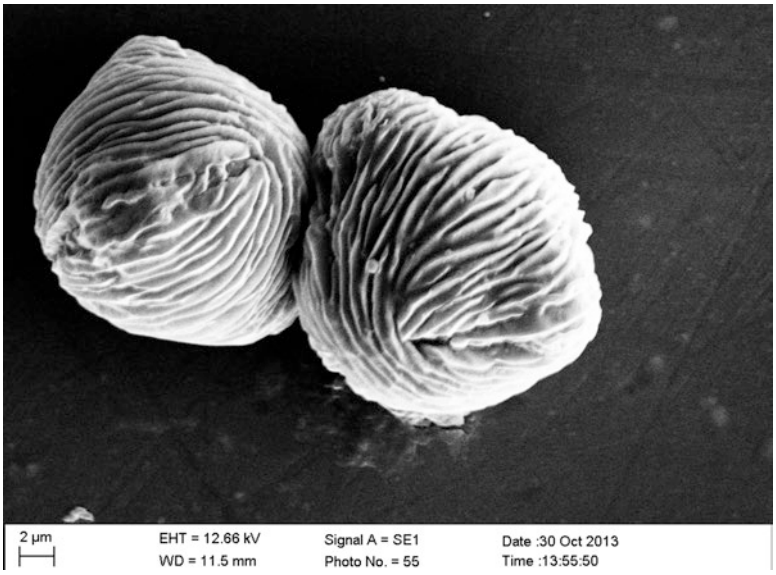

Fig. 4 Strawberry pollen grain. (Photo: Märt Rahi)

Fig. 5 A bumble bee carrying pollen into the hive. (Photo: Reet Karise)

region-specific data is needed for successive agricultural practice. The winter oilseed rape as a very attractive forage plant is usually suspected to draw bumble bees away from other crops. In case of strawberry this effect was not observed. The amount of strawberry pollen in bumble bee corbiculae was 20–25% each year independent of surrounding plant communities.

5 Conclusion

The success of using bumble bees in outdoor conditions depends on several interacting factors. However, knowing the specific behavioural aspects of the bee species used and the characteristics of target crop, make it possible to achieve reliable control of the disease.

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Dispensers for Entomovectoring: For Every Bee a Different Type?

Bettina Maccagnani, Matti Pisman, and Guy Smagghe

1 Introduction

A crucial step in the vectoring of supplemental pollen and/or a biocontrol organism (BCO) by pollinators is to load the vector in an efficient manner to ensure a sufficient loading. To achieve this, designing a suitable dispenser is essential. The main goal of the dispensers is to load the vectors (the bees) with the powdery product (the pollen and/or formulated BCO product) as they walk through it on their way out of the hive so they can disperse it to the target crops. An efficient dispenser should not only optimize the loading of the vector, but also have a low dispenser reloading interval, be able to be mounted on the hive easily, and have no influence on the vector's foraging behavior (Mommaerts and Smagghe 2011).

The dispensers used so far in entomovectoring studies can be divided into two groups, being one-way dispensers and two-way dispensers (Smagghe et al. 2012). In one-way dispensers, the chamber through which the vectors leave the dispenser is the same or not completely separated from the chamber through which the vectors enter the dispenser. Therefore, the vector will walk through the powder both when exiting and entering the hive. In two-way dispensers, the exit and entrance chambers are completely separated and only vectors exiting the nest will come into contact with the powder (Fig. 1). In this chapter, an overview will be given of the different

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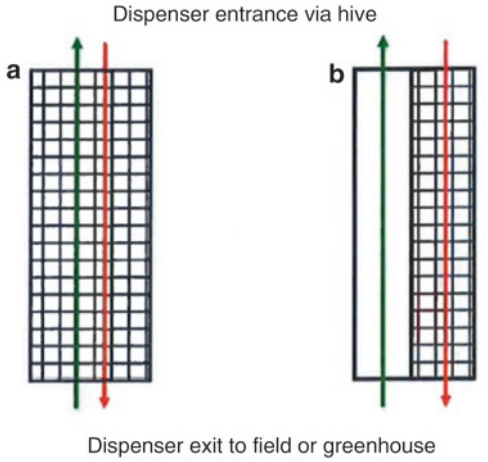

Fig. 1 Schematic view of (**a**) one way- type dispensers where the chamber through which the bees enter or leave the dispenser is the same (or is not completely separated), and (**b**) two-way dispensers where the chamber (with control agent) through which bees leave the dispenser is separated from the chamber (without control agent) via which they enter the dispenser. (red arrow = outgoing bees, green arrow = incoming bees, checked area = powder formulation. Adapted from Smagghe et al. (2012)

dispensers that have been developed over the past years for each vector of honey bees (*Apis mellifera*), bumble bees as *Bombus terrestris* and solitary bees as those of the genus *Osmia*.

2 Dispensers for Honey Bees

The first two dispensers developed for honey bees, the Harwood dispenser (Antles 1953) and the Tub dispenser, were one-way dispensers originally used for loading honey bees with pollen to achieve cross pollination (Dag et al. 2000; Mayer and Johansen 1988).

The Harwood dispenser consists of a wooden box with an internal roof curved towards the floor. Bees have to crawl through the slot formed by the bottom of the box and roof while passing over the powder, which is located in a trough on the floor before climbing over a Plexiglass strip to exit the dispenser.

The Tub dispenser is made of two wooden cubes holding a flexible acetate sheet to form a tub that can be filled with powder. Experiments to see whether or not these dispensers could be used for biological control resulted in poor loading of the honey bees used in the studies (Johnson et al. 1993; Thomson et al. 1992). Bilu et al. (2004) confirmed these findings and found that this was mainly due to the fact that the bees opened passages through the powder and bee activity became focused here, leading to a reduced contact with the powder and thus a reduction of the load. This phenomenon was also observed earlier in pollen dispensers (Legge 1976). Because

of the relatively poor performance of one-way dispensers, more appropriate dispensers were needed.

The dispenser optimization process continued with the development of four two-way dispensers suitable for experiments with honey bees, being the Peng dispenser (Peng et al. 1992), the Gross-dispenser (Gross et al. 1994), the Triwaks dispenser (Bilu et al. 2004) and the Houle dispenser (Albano et al. 2009).

The Peng dispenser consists of a wooden platform with a Plexiglass tray containing the powder, which can be placed on the bottom of the hive. A Plexiglass panel is attached vertically to the platform, and the light that passes through it from outside the hive attracts bees to crawl through the powder and onto the panel towards an exit slot, that is a few centimeters above the wooden platform. Returning honey bees enter the hive through a slot which is located beneath the wooden platform and avoid walking through the powder upon entering the hive.

The Gross dispenser is designed to be inserted in the front center of a modified bottom board of a honey bee hive and has a removable tray that can be inserted from the side to load the powder in the dispenser. Exiting honey bees will walk through the tray on their way out and enter the hive through a separate pathway upon their return.

The Triwaks dispenser consists of a wooden box with an extended base that can fit in the opening of a standard Langstroth hive. The dispenser is diagonally partitioned into two triangular compartments, one through which the honey bees enter the hive and one through which they exit the hive. The exit compartment is divided into three sub-compartments containing the powder formulation; it has its largest side into the hive, and end with the shortest side forming the exiting part of the dispenser that attracts exiting bees thanks to the light coming from the outside. Returning foragers find a large landing platform, which constitutes the largest side of the entrance compartment and ends with its shortest side into the nest, ensuring that honey bees enter the hive through the powder-free part of the dispenser.

The last dispenser is the Houle dispenser, which can be attached to a bee hive and is divided horizontally in an upper compartment containing a powder tray and a lower powder free compartment. Exiting honey bees leave through the upper compartment, while returning bees enter the hive through the lower compartment, avoiding the powder.

There is one other dispenser reported in a study by Kovach et al. (2000). They used a “Tray applicator” to inoculate the honey bees in their study, however, details about this applicator were absent in the paper.

So far, there is also one commercially available dispenser, called BeeTreat, which has been developed by Hokkanen et al. (2012, 2014). The BeeTreat is a two way dispenser which proved to be efficient in loading exiting honey bees in several field trials performed in the frame of the CORE Organic 2 (EU ERA-NET) project BICOPOLL, to provide a pan-European case study on protecting organic strawberry from the grey mold using the fungal antagonist *Gliocladium catenulatum* (Prestop® Mix). An overview of the different dispensers is given in Fig. 2.

Comparative studies of these different dispensers are not available as far as we know. The only direct comparison was made by Bilu et al. (2004). They compared the Triwaks dispenser to the Peng dispenser and the two one-way dispensers (Harwood dispenser and Tub dispenser). They found that the Triwaks dispenser

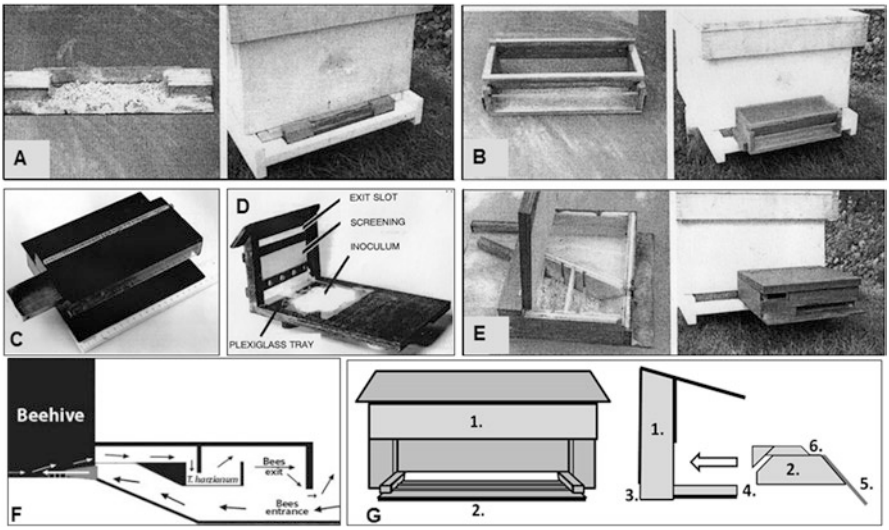

Fig. 2 Overview of the different hive-mounted dispensers developed for honey bees. One-way type dispensers: (**a**) Tub and (**b**) Harwood. Two-way dispensers: (**c**) Gross (**d**) Peng (**e**) Triwaks and (**f**) Houle. Adapted from Peng et al. (1992); Gross et al. (1994); Bilu et al. (2004); Albano et al. (2009) and Hokkanen et al. (2012)

achieved a significantly higher load then the other three dispensers. On top of that, it was found that the Triwaks dispenser would need to be reloaded only once a day, or even less when the initial amount of powder is increased, compared to 12 h intervals for the Peng dispenser. Although direct comparisons are not available for the other dispensers, we can evaluate their performance based on the results of past studies. For example, the Peng dispenser may have been found to be less effective than the Triwaks dispenser, yet it is still a suitable dispenser to use for the loading of honey bees as shown by Yu and Sutton (1997) and Peng et al. (1992). In these studies, the Peng dispenser resulted in a sufficient loading of the honey bees with *Gliocladium roseum* and a suppression of *Botrytis cinerea* on strawberry and raspberry. The Gross dispenser was also used in a number of studies and succeeded in loading the honey bees with sufficient amounts of inoculum to suppress pathogens and decrease pest survival (Dedej et al. 2004; Gross et al. 1994; Jyoti and Brewer 1999). The Houle dispenser was also found to perform well enough to ensure a suppression of pest species and plant pathogens (Albano et al. 2009). However, the Houle dispenser did have some minor issues in open field trials because moisture can accumulate in the dispenser and cause the powder formulation to cluster and this may then result in a lower loading of the honey bees.

So far, it looks that all the developed two-way dispensers perform satisfactorily for the loading of honey bees. It should be noted that we did not list any exact numbers on the CFU per honey bee measured in each study, as this would not be representative due to the differences in the initial loading of the dispensers. These differences also make it difficult to perform clear comparisons between the different

dispenser types. To optimize the vectoring by honey bees, a comparative study testing all available dispensers under uniform conditions like the one conducted by Bilu et al. (2004) would be interesting and useful.

3 Dispensers for Bumble Bees

Alongside honey bees, bumble bees are also used as vectors for the dissemination of microbiological control agents and supplemental pollen. However, the amount of research on dispensers compatible with bumble bees is rather limited. The first study with bumble bees was performed with *Bombus impatiens* by Yu and Sutton (1997). They used a dispenser consisting of a lower compartment filled with powder and an upper compartment without powder formulation. An illustration of the dispenser can be found in Fig. 3. Both compartments were separated by a sheet of Plexiglass. Bumble bees exiting the hive crawled through a hole connecting the main chamber of the hive with the lower compartment and passed through a zig-zag passageway, formed by diagonal walls, containing the powder formulation. Through a corner hole in the Plexiglass they could reach the upper compartment and leave

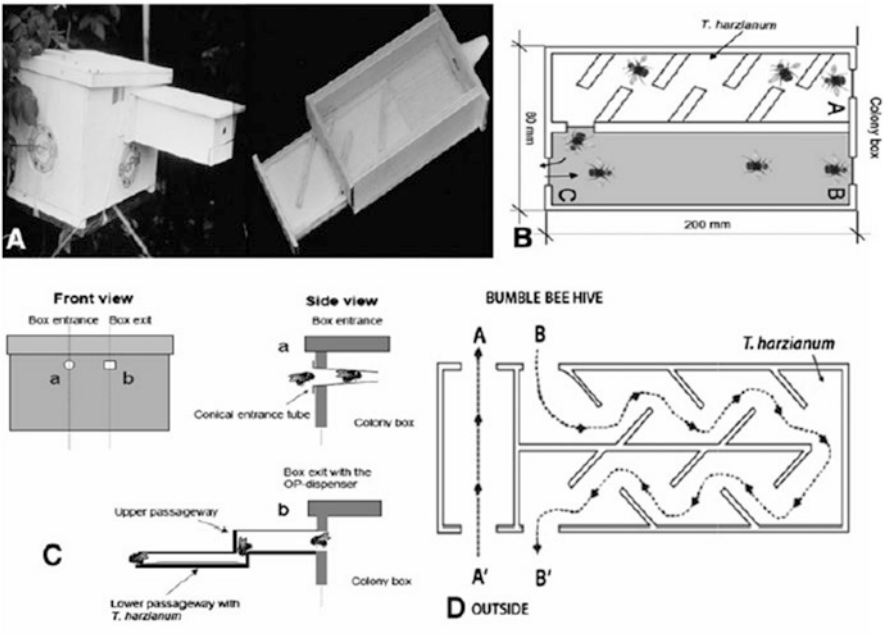

Fig. 3 Overview of dispensers developed for the bumble bee *Bombus impatiens* (**a**) One-way type dispenser by Yu and Sutton (1997); two-way dispensers developed for *Bombus terrestris*: (**b**) SSP and (**c**) OP dispensers by Maccagnani et al. (2005) and (**d**) Houle dispenser. Adapted from Yu and Sutton (1997); Maccagnani et al. (2005) and Albano et al. (2009)

the dispenser through a frontal hole. Incoming bumble bees entered through the same hole and proceeded to walk over the Plexiglass towards a hole leading to the main chamber of the hive. A tapered tube discouraged the bumble bees in the hive to use this hole as an exit. As such, exiting bumble bees were forced to come into contact with the inoculum and returning bees could avoid the powder completely. The study found that the loading of the bumble bees with *Gliocladium roseum* was comparable to the loading of the honey bees that were loaded using the Peng dispenser during the same experiment.

A second study by Albano et al. (2009) tested the Houle dispenser, adapted to commercial hives for bumble bees. The dispenser was a two-way dispenser where exiting bumble bees had to make a zig-zag walk through the inoculum before exiting the hive. Returning bumble bees entered through a different opening and could reach the hive directly. The Houle dispenser for bumble bees achieved a loading of 10^4 CFU per bumble bee. One major limitation proved to be the fact that the liquid excretions of the bumble bees covered the inoculum after a while, preventing further loading on other bumble bees.

Two other dispensers, the one-way side-by-side passageway (SSP) dispenser and the two-way overlapping passageway (OP) dispenser were developed and tested by Maccagnani et al. (2005) with *Bombus terrestris*. The SSP dispenser (Fig. 3) was a modification of the one devised by Yu and Sutton (1997). It consists of two side-by-side passageways, one containing a zig-zag passageway with diagonal walls and loaded with inoculum for exiting bees, the second one being a straight passageway for bumble bees that return to the nest. In order to ensure that bees take the right passageway when exiting the hive, the zig-zag passage was illuminated to attract outgoing bees, whereas the straight passageway was dark. However, this design showed to have several functional limits, as only 12.5% of the bumble bees exiting the dispenser were loaded with powder and those that did carry the inoculum only carried low concentrations. This seemed to be caused by the fact that the dispenser failed to separate outgoing and incoming bees. In addition, it looks that the bumble bees were poorly loaded, lost their way in the zig-zag passageway and/or a grooming behavior was induced before exiting/flying out. Many bees exited the hive through the dark straight passageway, thus avoiding the inoculum, or walked alongside the walls of the zig-zag tunnel. Moreover, like in the Houle dispenser, bumble bees seemed to secrete fluids onto the powder making it no longer suitable for transferring the biocontrol agent.

The OP dispenser (Fig. 3) consisted of two overlapping passageways. In the overlapped portion, a hole put the upper and lower passageways into communication. Exiting bees walked through the upper passageway, entered the hole down towards the lower one where they crawled through the inoculum towards the exit. Returning bumble bees entered the hive through a separate entrance hole on the nest wall. The entrance hole also hindered bumble bees trying to exit the hive this way, ensuring they pass through the inoculum when leaving the hive. This setup seemed to be successful in loading the vectors, as all exiting bumble bees carried powder with them and all bees used the correct way for entering or exiting. The load carried by the bees exiting the OP dispenser was higher if compared to the SSP dispenser,

but it should be noted that the initial loading of the dispenser was 10 times higher in the OP dispenser. Despite the seemingly good results of the OP dispenser, both dispensers resulted in an inoculum concentration in the flowers lower than the one achieved through the spraying of the control agents.

One missing piece of information in all the above mentioned studies is the effect of the dispensers on the behavior of the bumble bees (e.g. induced grooming after contact with the inoculum) and their foraging activity, which might have a great influence on their ability to disseminate the inoculum to the target crops. Mommaerts et al. (2010) investigated the SSP dispenser in a comparative study with their newly developed dispenser ("the Mommaerts dispenser") and found that the SSP dispenser indeed had a negative effect on foraging activity of the bumble bees when it was attached to the bumble bee nest. The mean number of foraging bumble bees dropped from 16.3 ± 0.7 to 4.7 ± 0.6 between 7 am and 9 am in the morning and from 12.5 ± 1.6 to 5.7 ± 0.8 between 4 pm and 6 pm in the afternoon when the SSP dispenser was attached, which was a significant reduction in the amount of foragers. For the OP dispenser, no data is available on this subject.

Another dispenser for bumble bees of *B. terrestris* was developed by Mommaerts et al. (2010) (Fig. 4). This dispenser consists of two rectangular compartments: an exit compartment (length = 20 cm, width = 5 cm, and height = 4 cm) with a grid on the bottom that contains the powder formulation and a smaller entrance compartment.

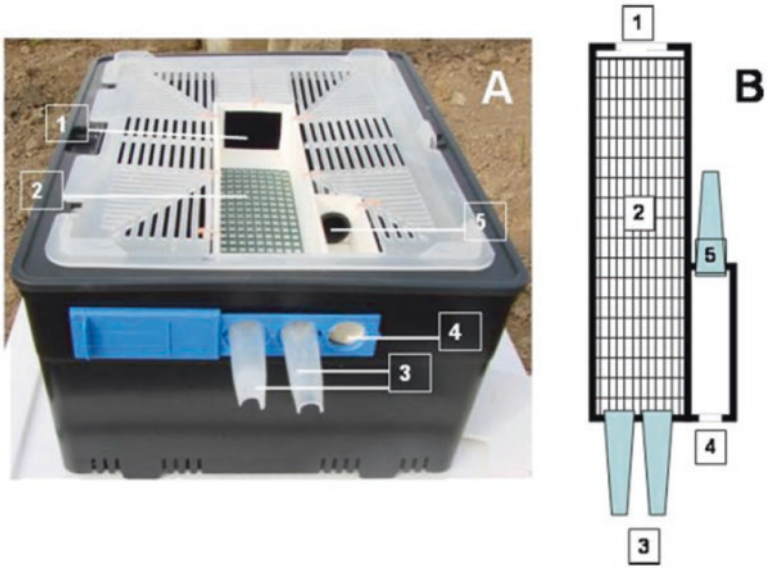

Fig. 4 The Mommaerts two-way dispenser. (**a**) Photograph from the front without the cover lid, and (**b**) schematic drawing, top view. (1) Connection of the exit compartment to the bumble bee hive; (2) exit compartment with a grid at the bottom that contains the powder BCO formulation; (3) exit holes with bumble bee-in-closer; (4) entrance hole, (5) bumble bee-in-closer, connecting the entrance compartment to the bumble bee hive. Dispenser length 20 cm. Adapted from Mommaerts et al. (2010)

The grid ensures that the powder formulation is distributed homogeneously in the dispenser for the entire duration of the charge.

The length of the exit compartment was optimized based on a preceding test to investigate the effect of the dispenser length on vector loading. For this test, workers of *B. terrestris* walked a selected distance, i.e. 5, 10, 15, 20, 25, 30 or 40 cm, through a dispenser loaded with "Binab-T-vector" that is a BCO powder formulation of a 50/50 mixture of *Trichoderma atroviride* and *Hypocrea parapilufera* and specifically developed to be delivered by pollinators. By assessing the colony forming units (CFU) found on the bumble bee workers that walked different distances, the optimal dispenser length was determined. It was found that distances of 5–15 cm were too short to ensure sufficient loading, while distances over 25 cm induced grooming behavior of the bumble bees, resulting in a significant loss of loading and a longer passage time through the dispenser. A length of 20 cm and 25 cm resulted in a significantly higher load compared to the other distances and was deemed ideal (Fig. 5). As there was no significant difference between 20 cm and 25 cm, the dispenser was given a length of 20 cm. The significant effect of the length of the dispenser on the loading of bumble bee workers of *B. terrestris* is an important finding that should be considered during the development of potential future dispensers.

Experiments with the Mommaerts dispenser also showed that a much better whole-body loading was achieved compared to the tested SSP dispenser. Interestingly, the legs of workers that walked through the Mommaerts dispenser carried a much higher load compared to bumble bees that walked through the SSP dispenser. This is important as the vector's legs make contact with the flower organs during the flower visiting. There was also no effect on foraging intensity when the dispenser was attached to the hive or when it was filled with "Binab-T-vector". A refill of the dispensers at 3 day intervals was recommended by the authors, but note

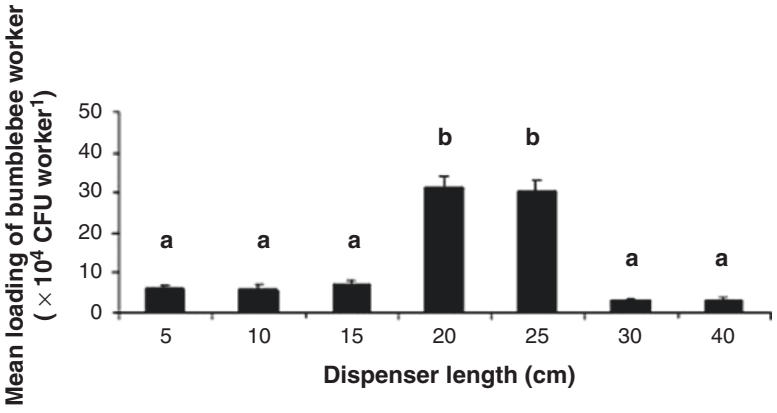

Fig. 5 Loading of bumble bee workers for each dispenser length. A length of 20–25 cm seems to be the optimal length. This length gives a sufficient loading of the bumble bee, while a shorter dispenser length does not expose the bumble bee's body to enough product, and longer distances enhance the grooming behavior which results in lower loadings. Adapted from Mommaerts et al. (2010)

that initial filling, foraging activity of the vectors and the size of the hive all play a role in determining the optimal refill frequency. More recent, a commercial dispenser "Flying Doctors" was created by Biobest based on the design of the Mommaerts dispenser (see more details in paragraph 6.2).

4 Dispensers for Solitary Bees

Among the numerous solitary bee species, only those nesting in pre-existing cavities and showing gregarious behavior (see "Solitary Bees As Pollinators") can be considered as candidates to vector BCOs. The dispenser must be integrated into the shelter hosting the nesting material and have a two-way design: outgoing bees must be obliged to walk on the BCO and returning bees must avoid getting in contact with it.

The first study aiming at developing a dispenser for solitary bees was conducted after the spread of *Erwinia amylovora*, the causal agent of the fire blight, on pears in Emilia-Romagna, the main pear cultivation area in Northern Italy. The research was motivated by the fact that honey bees could not be used as a vector for biological control, as in some pear varieties flowers are not attractive to honey bees. In addition, as a consequence of fire blight infection, the movement of honey bee colonies from infected to uninfected areas was forbidden to prevent its diffusion, making the use of honey bees as pollinators (or vectors for entomovectoring) not possible. An alternative was identified by Maccagnani et al. (2003), as they found that *Osmia cornuta* was a very efficient pear pollinator. The first prototype of the dispenser (Figs. 6 and 7) was developed by Maccagnani et al. (2006) in a comparative study on the efficacy of *O. cornuta* and *A. mellifera* to deliver a powder formulation of the strain "BS-F4 ^{Rif}" of *Bacillus subtilis* to pear flowers in the control of *E. amylovora.* It consisted of a simple wooden box in which a plastic ramp was inserted. The dispenser could be positioned in the lower part of the shelter and a

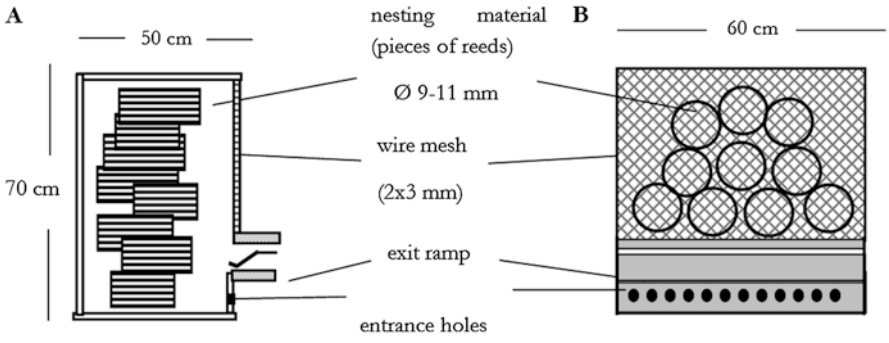

Fig. 6 Side (**a**) and front (**b**) view of the dispenser for *Osmia cornuta* mounted on nesting shelter

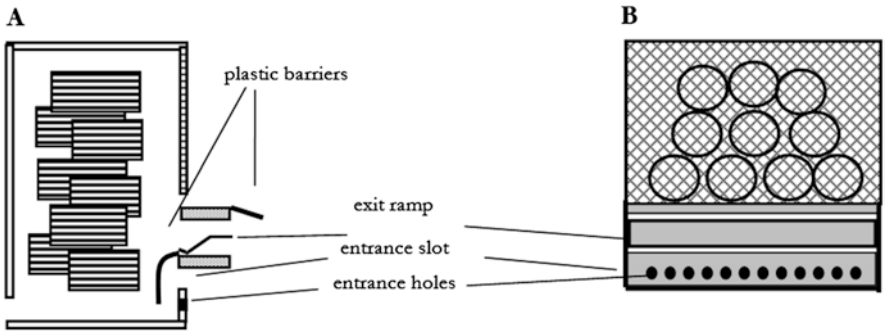

Fig. 7 Side (**a**) and front (**b**) view of the modified dispenser for *Osmia cornuta*

metal mesh closed the remaining portion of the front side of the shelter. The BCO powder formulation was placed in a channel at the base of the ramp. Outgoing bees were expected to fly from their nesting tunnels towards the mesh and walk downwards to get to the first available opening, provided by the dispenser ramp. They were thus obliged to overpass the channel with the powder formulation before climbing up the ramp to exit the dispenser. Returning bees could enter the shelter through a series of holes drilled below the dispenser and avoid contact with the powder formulation. *Osmia cornuta* females, which had already started nesting, got used to the system shortly after its installation in the shelter. Females' behavior was observed for 1 week, four times per day for 30 min. The percentages of ingoing and outgoing females using the correct pathway were significantly higher compared to those that took the wrong one. Returning bees showed no interest in entering the shelter through the dispenser ramp and learnt very easily to fly to the holes set below the plastic ramp, using the proper returning pathway. However, some outgoing bees started to use the entrance holes as an exit way. To improve the correct use of the exit/entrance ways and to force exiting bees to pass through the dispenser, an extra entrance slot was created between the entrance holes and the dispenser. In addition, a plastic barrier was attached to the base of the ramp, inclined toward the bottom of the shelter, making the entrance invisible to females exiting their nests. A second barrier was attached on top of the dispenser to prevent returning bees from landing on the exit ramp (Figs. 6 and 7). Despite the different efforts to force the bees in using of the correct pathways, habituation was observed for every modification, and after a certain time bees learned to exit the shelter without crawling through the powder formulation. At the end of the week of observations, the percentage of ingoing bees using the correct entrance was 98% (n = 917), while the percentage of the outgoing bees using the exit ramp was significantly lower (81%; n = 796).

In addition, Maccagnani et al. (2006) evaluated the efficiency of this model of dispenser to load *O. cornuta* females with "BS-F4^{rif}" preparations in comparison to *A. mellifera.* To load honey bees, a commercial dispenser model developed for guided pollination was used, which was substantially analogous to that one for *O. cornuta*. For both species, the dispenser was loaded with 1.5 g of the powdered

"BS-F4^{rif}" preparation (10^7–10^8 CFU/mg). At increasing time periods after loading (0, 30 and 60 min), 10 insects exiting the dispenser were individually captured, killed, and processed according to the protocol described in Maccagnani et al. (2006). Results are reported in Fig. 8. The number of "BS-F4^{rif}" found on the body of *O. cornuta* exiting the dispenser ranged from 10^4 to 10^7 CFU/insect; *Apis mellifera* carried an average load of 10^4 CFU/insect. A decrease in the bacterial cell number was observed as the time after dispenser loading increased. *O. cornuta* bees sampled at T = 0 min had the lowest presence of "BS-F4^{rif}", likely because the exiting females initially tried to avoid the powder, passing through the dispenser in points where it was less concentrated; however, such behavior ceased in a short time.

Based on the model described above, Maccagnani et al. (2008) modified the shelter + dispenser design to increase the number of females using the proper exit pathway by placing the dispenser in the higher part of the shelter (Fig. 9). An inclined metal mesh closed the front opening of the shelter and ended in continuity with the channel containing the BCO. As females are attracted by the bright upper portion of the shelter, the new position of the dispenser and the slope of the mesh favored the use of the dispenser as the exit way. A wooden platform was placed upon the top of the dispenser to divide the shelter in an upper open space, functioning as the entrance, and a bottom space containing the nesting materials. The dispenser was mounted after adult bees emerged from cocoons, mated and initiated the nesting activity (Figs. 10, 11 and 12). When the metal grid was installed, forcing the females to pass through the dispenser on their way out, females were initially disoriented and needed some time to find out how to reach the nests through the free space above the dispenser. In a short time, bees got oriented, and, at the successive foraging flight, they were able to return to their nest without crossing the dispenser. At the same time, all exiting bees passed through the dispenser without any hindrance.

As a conclusion, the installation of the dispenser in the upper part of the shelter increased the efficiency in diverting outgoing bees to the proper pathway with respect to the models in which the dispenser was placed at the bottom.

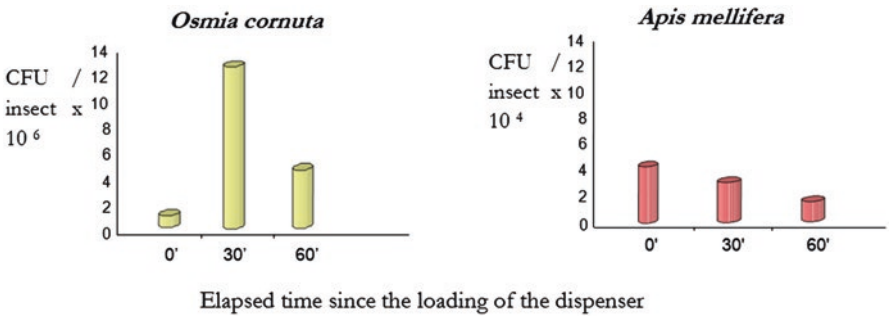

Fig. 8 Efficiency of *Osmia cornuta* and *Apis mellifera* as vector of BCO: BS-F4^{rif} contamination of exiting individuals (Mann Whitney U Test, all $P < 0.001$)

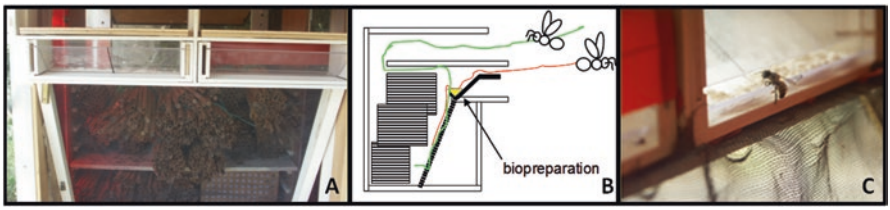

Fig. 9 Front view (**a**) and schematic drawing (**b**) of the shelter and dispenser by Maccagnani et al. (2008); (**c**) *Osmia bicornis* exiting the shelter passing through the dispenser charged with the BCO

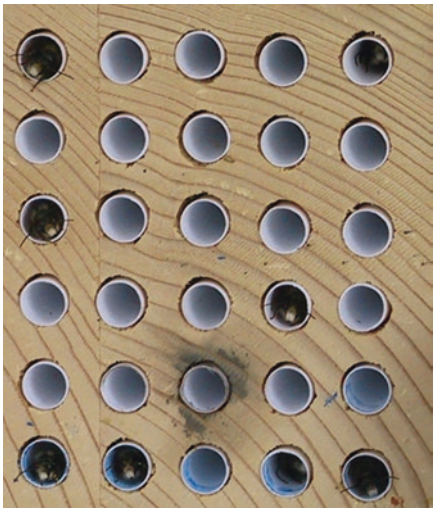

Fig. 10 *Osmia cornuta* males, emerged before females, waiting for them in the nests

Fig. 11 *Osmia cornuta* mating on pear flowers

Fig. 12 *Osmia cornuta* nesting female returning with pollen

This dispenser model was used to determine the efficiency of *O. cornuta* in loading up a strain of *Bacillus subtilis* (BD170) compared to *A. mellifera* during a research funded by the SafeCrop Center (Trento, Italy) (Maccagnani et al. 2008). The dispenser used for *A. mellifera* was based on the prototype presented by Bilu et al. (2004). The average number of colony forming units found on the body of *O. cornuta* (n = 31) was in the order of several billions (10^7) while on honey bee bodies 10–50-fold lower amounts of active bacterial cells were found (Figs. 13 and 14).

In 2012–2014, thanks to the CORE Organic 2 Project BICOPOLL, a low cost and easy to manage dispenser+shelter system for O. cornuta was developed. The main aim of the BICOPOLL project was to improve the dispenser for *O. cornuta* in the direction of setting up an effective, low cost and easy to manage shelter + dispenser system. The model named MB13 (Mason Bees 2013) was constituted by a polystyrene-made honey bee hive (height = 37 cm, width = 24 cm; depth = 48 cm), adapted as a shelter by removing the cover and positioning the box vertically (only for this component of the model, see Fig. 18). The true dispenser device was similar to the one described above in Fig. 9b and was inserted in the upper part of the shelter box, leaving a free space of around 8 cm between the dispenser and the top of the shelter box. The dimensions of the dispenser were adapted to the polystyrene shelter and the plastic ramp was elongated to height = 6 cm, width = 24 cm and depth = 7 cm in order to hold higher amounts of BCO. The upper wooden layer, placed on the top of the dispenser to divide the shelter into two parts and to create the entering space, was significantly elongated to prevent females from using this way as an exit.

The efficiency of two prototypes of the MB13 model was tested by providing nesting materials for 50 females and 100 males, which were released in their cocoons within a polystyrene small box placed inside each shelter. As in the previous experiments, when most of the females had initiated the nesting activities, a metal grid, connecting the bottom of the shelter to the base of wooden box housing the dispenser ramp, was installed. This way, females were obliged to pass through the dispenser on their way out. Leaving their nest, they crawled on the powder at the base of the ramp climbed on the ramp and exited the dispenser to go foraging. The free space between the dispenser and the top of the shelter gave female bees the possibility of returning to their nest without walking through the dispenser.

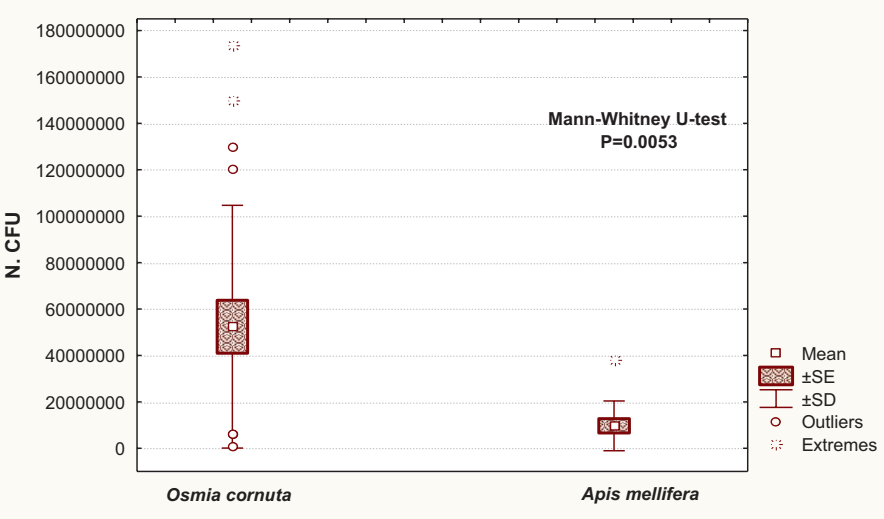

Fig. 13 Mean number of CFU found on the entire body of *Osmia cornuta and Apis mellifera* females exiting the dispenser

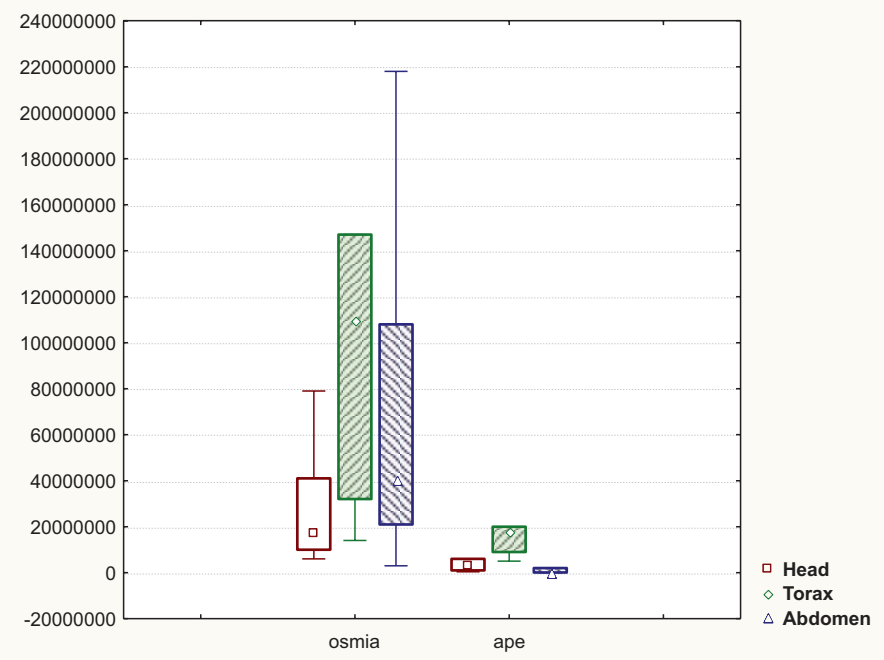

Fig. 14 Mean number of CFU found on different body parts (head, thorax and abdomen) of *Osmia cornuta* and *Apis mellifera* exiting the dispenser

Experiments were run to evaluate the loading efficiency of this dispenser model, which was loaded with 5 mL and 2.5 mL of "Amylo-X" (Intrachem-Italia). "Amylo-X" is a powdery biopreparation based on *Bacillus amyloliquefaciens* strain D747, containing 5×10^{10} CFU/g, that is an efficient antagonist of *E. amylovora*. The powder was distributed at the base of the dispenser, forming a layer of nearly 1 mm high. The transect that the bees had to walk through was around 1.5 cm for the first trial. Eight *O. cornuta* females exiting through the dispenser and crawling on the powdery preparation were captured, anesthetized with ether, and their body was divided into three parts: head, thorax and abdomen. The body parts were treated according to a protocol for bringing the bacterial cells attached to the hairs into solution. Body parts were separately washed into an Eppendorf containing 1 mL solution of $MgSO_4$, and centrifuged for 3 min. A diluting series was performed until the 10^{-6} dilution: the dilutions 10^{-3} and 10^{-6} were used to inseminate Petri dishes containing a culture medium (Nutrient Agar) suitable for the development of *B. amyloliquefaciens.* Plates were incubated at 36 °C for 24 h, after which the number of developed colonies was counted (Fig. 15). The results of both trials (dispenser charged with 5 or 2.5 mL of "Amylo-X") showed that exiting bees loaded up very high amounts of BCO with 10^6 CFU for each body part (head, thorax, abdomen). When the dispenser was loaded with 5 mL, the statistical analysis (ANOVA) showed no difference in the amount of BCO loaded up by the *O. cornuta* body parts (Fig. 16), while when the "Amylo-X" load was of 2.5 mL then the abdomen loaded up a significantly higher amount of CFU with respect to the head and the thorax (Fig. 17). These results indicate that each bee can potentially transport several

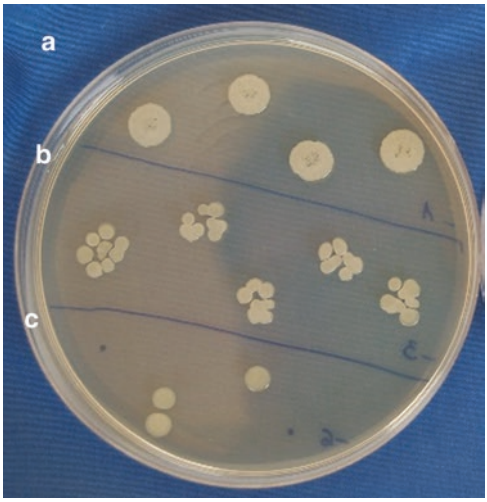

Fig. 15 *Bacillus amyloliquefaciens* colonies developed on Nutrient Agar after 24 h of incubation at 36 °C. (**a**): not diluted washing solution was used to inseminate the plates; several colonies developed completely overlapped. (**b**): 10^{-3} dilution, several colonies together partially overlapped. (**c**): 10^{-6} dilution, the colonies developed separately and were thus counted and multiplied for the factor of dilution to obtain the number of colony present in the initial washing solution

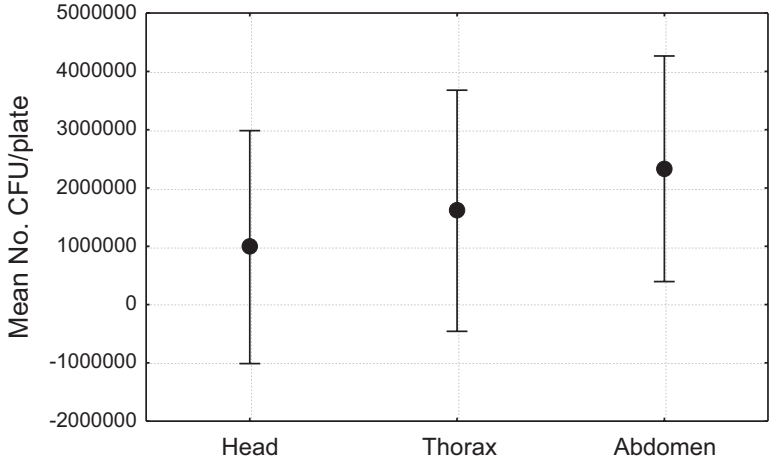

Fig. 16 Mean amounts of BCO on different body parts of *Osmia cornuta* workers when the MB13 dispenser model was charged with 5 mL per 20 cm^2. There was no statistical difference (ANOVA; F2,42 = 0.047720; P = 0.6238) over the different body parts

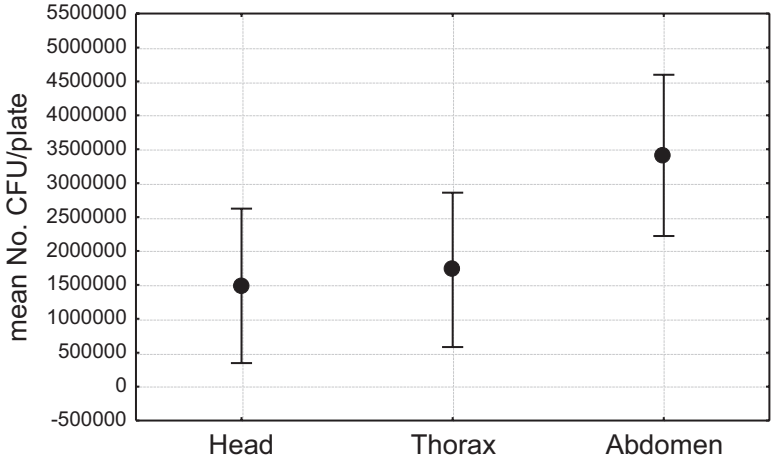

Fig. 17 Mean amounts of BCO on different body parts of *Osmia cornuta* workers when the MB13 dispenser model was charged with 2.5 mL per 20 cm^2. With "Amylo-X" load at 2.5 mL in the dispenser, the abdomen was loaded with a significantly higher amount of CFU compared to the head and the thorax (ANOVA; F2,32 = 3.324; p = 0.0488)

millions of inoculum cells to the visited flowers, even when the dispenser load is partially consumed.

The further development of the dispenser lead to the MB14 model, the most efficient one. In this version all the components remained the same as in the previous model, but the plastic ramp was eliminated, so that the powdery preparation of

the antagonist could be placed on a horizontal support (Figs. 18 and 19). The horizontal support allowed to load up the dispenser with much higher amounts of BCO in comparison to the ramp.

The MB14 model was tested in a study on the efficiency of *O. cornuta* in the primary dissemination of BCO to pear flowers under semi-field conditions (Fig. 20). A net screened plastic tunnel (10 × 5 × 3 m) with early flowering plants in pots (*Brassica naps*, *Viburnus album*, *Prunus spinosa*, *Ranunculus* sp.) allowed *O. cornuta* to start nesting activity. On the day of the trial, forage plants were removed and one pear plant, whose flowers had been numbered, was introduced in the tunnel. The dispenser was charged with 5 mL of the BCO, and one female at a time was set free to pass through the dispenser. The behavior of eight females was observed, and the sequence of the visited flowers on the plant was recorded. The flowers were then collected and treated according to the protocol described above to assess the number of CFU of the BCO deposited by the bees.

The amount of bacterial cells found in the first six flowers was, on average, in the order of magnitude of 10^6 (Fig. 21). Data showed a very high variability due to the behavior of the bees. Behavioral differences concerned the way of exiting the dispenser (some of the bees walked on the roof of the dispenser, avoiding the powder formulation at the bottom) and the approach of the flowers.

Recently, Biddinger et al. (2010) adapted the model developed by Maccagnani et al. (2006) to a rosaceous specialist pollinator, the Japanese orchard bee *Osmia cornifrons*, for delivering the BCO "Serenade" (*Bacillus subtilis* strain QST 713) to control fire blight on apple (Fig. 22). The authors found that *O. cornifrons* used the

Fig. 18 The MB14 dispenser model for mason bees developed in 2014 for mason bees with the dispenser device placed in the upper part of the shelter. The structure of the shelter box is the same than in MB13. In the MB14 model the exiting way obliges mason bees to walk on the bottom of the dispenser to reach the exit slot. The BCO is placed on a horizontal plastic support laying on the bottom of the dispenser and the powder can be distributed over a surface of 20 (length) × 5 (width) cm. Returning bees learn easily to enter flying above the dispenser

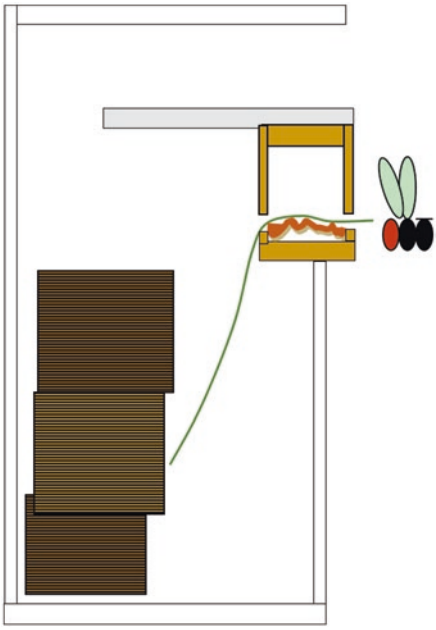

Fig. 19 Schematic view of the MB14 dispenser model for mason bees

Fig. 20 *Osmia cornuta* female exiting the MB14 dispenser. The BCO was distributed on a horizontal plastic support at the bottom of the dispenser

correct exit pathway, but only 50% of the returning bees entered through the entrance holes (Fig. 23). This finding is opposite to what Maccagnani et al. (2006) observed several times, i.e. bees used the correct entrance but failed to follow the correct exit pathway. Biddinger et al. (2010) concluded that, in a way, the wrong behavior of entering bees contributed as well to their contamination with the biocontrol agent. This conclusion is not completely acceptable, as females carefully clean up their body to detach the pollen, so it is likely that the large majority of the BCO loaded up while entering through the dispenser is deposited in the larval cell. Anyway, Biddinger et al. (2010) found that the amount of biocontrol agents carried by *O. cornifrons* females exiting the dispenser was high, and approximately 18 times to

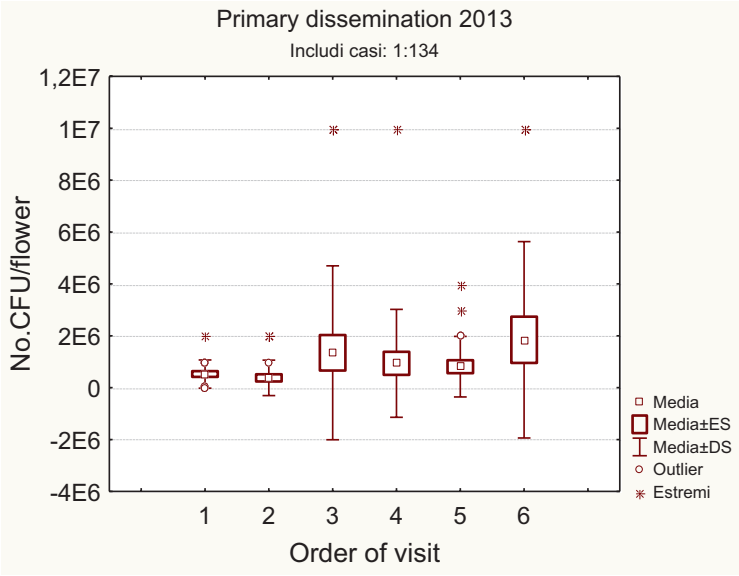

Fig. 21 Mean number of CFU found on pear flowers consecutively visited by *Osmia cornuta* (N = 8)

that one carried by honey bees for protection of blueberries for mummy berry disease (Scherm et al. 2004) (Fig. 23).

5 Miniature Dispenser for Laboratory Tests

The abovementioned dispensers were all used in field and greenhouse tests to determine the ability of the vectors to disseminate BCOs to the target crops and determine the suppression of the pest species and plant pathogens. However, testing is also required to determine the possible effects of the BCOs on the vectors that carry them. To achieve this, a simple laboratory setup was developed by Mommaerts et al. (2012). This bioassay is based on a miniature dispenser and allows for easy testing of possible side effects on micro-colonies of bumble bees. Three different bioassays were developed: a one-way-passage miniature-dispenser bioassay, a two-way-passage miniature-dispenser assay and a flight cage bioassay with a two-way-passage miniature-dispenser. The one-way-passage miniature-dispenser bioassay consists of a miniature dispenser (length = 20 cm, width = 5 cm, and height = 5 cm) connecting two micro-colony nest boxes. The upper lid can be opened to fill up the dispenser with powder. The 5 × 5 cm-sidewalls each have a round hole in them to connect the dispenser to the micro-colony nest boxes. One of the nest boxes contains a micro-colony while the other one contains no bumble bees but provides pollen and sugar water as food. Bumble bees crawl back and forth through the dispenser

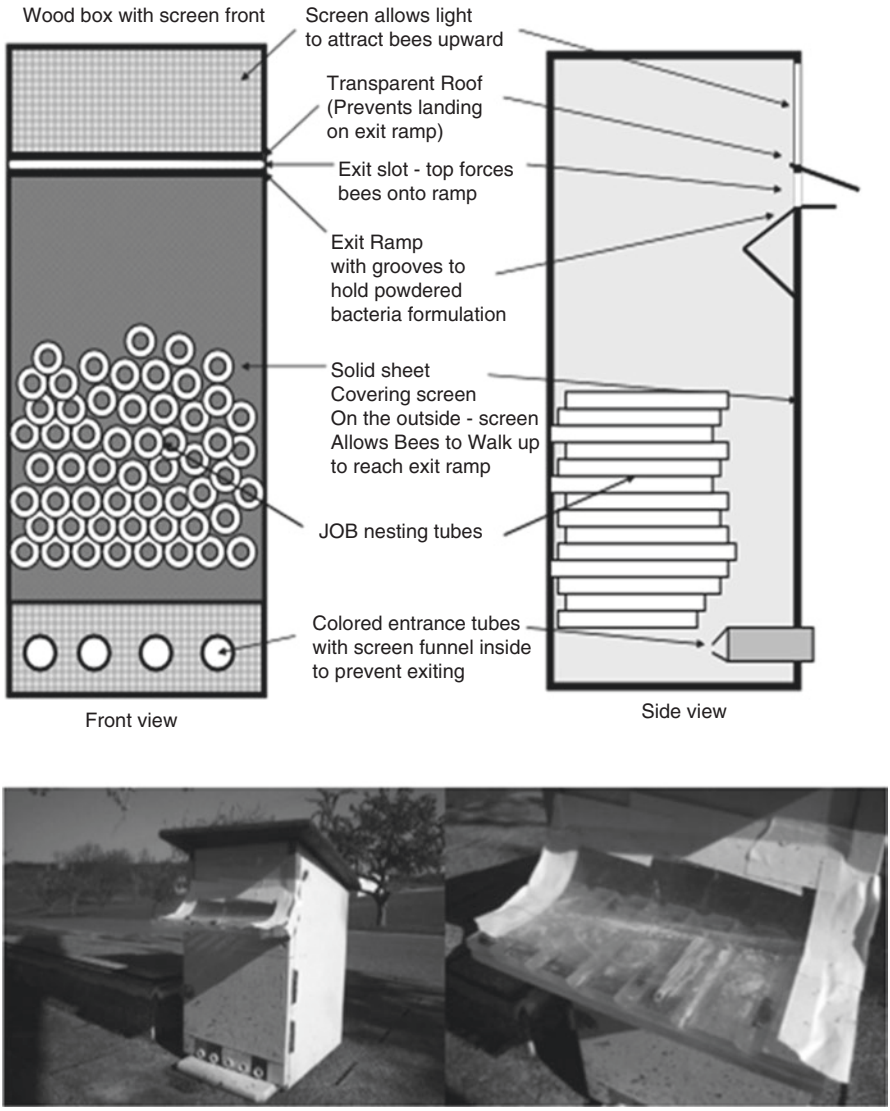

Fig. 22 The model developed by Maccagnani et al. (2006) has been used for the Japanese orchard bee *Osmia cornifrons* to deliver "Serenade" (*Bacillus subtilis* strain QST 713) to control fire blight on apple by Biddinger et al. (2010)

to forage and come into contact with the BCO powder formulation. The two-way-passageway miniature-dispenser bioassay consists of a similar setup as the one-way bioassay but now a plastic tube of 20 cm also connects the two nest boxes. This way, bumble bee workers exit the colony-containing nest box to forage through the powder-containing dispenser and return to the nest through the tube. A

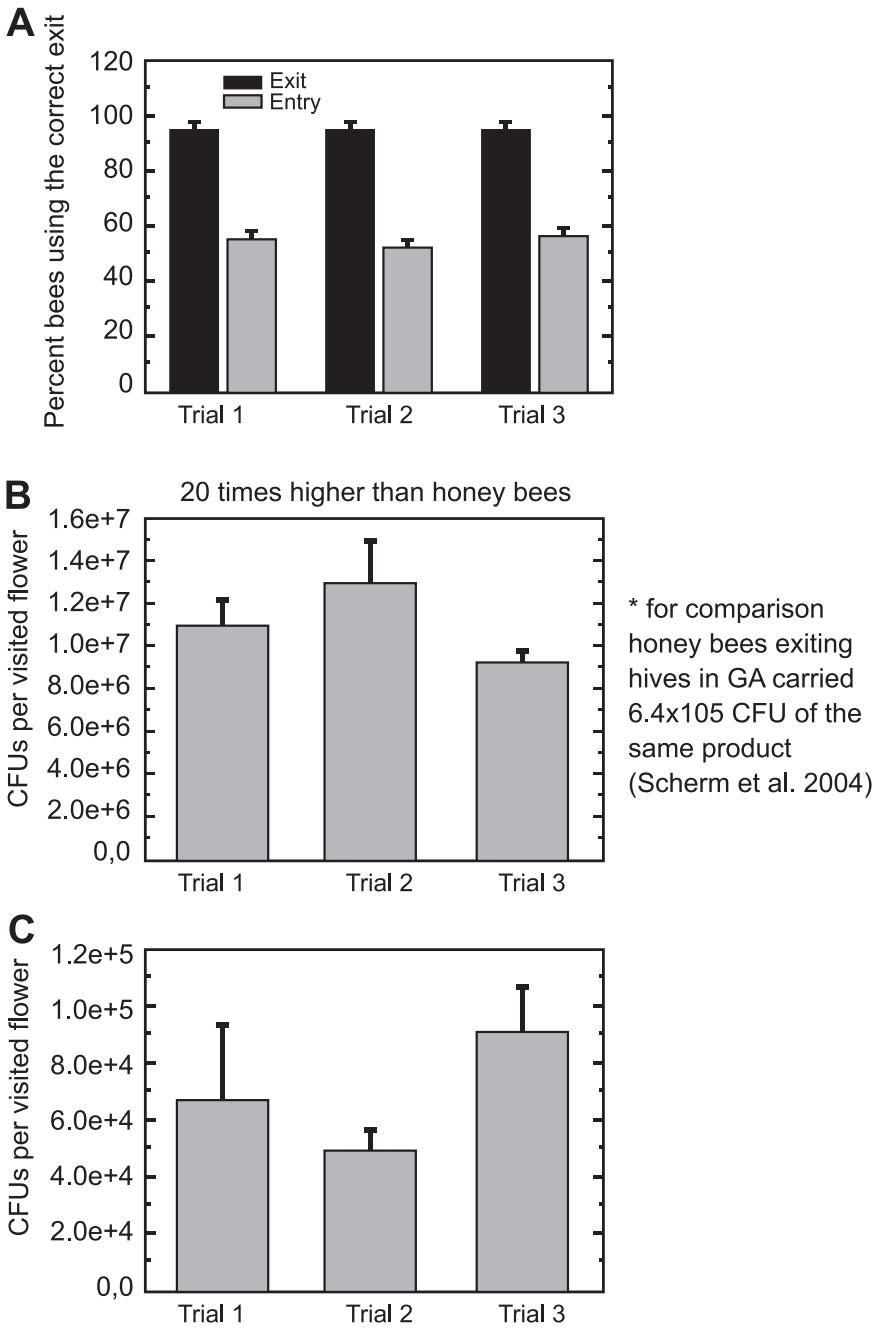

Fig. 23 (**a**) Utilization of the proper pathway in the dispenser by entering/exiting *Osmia cornifrons* females. Results demonstrated that most bees used the right exit but only about the half returned into the dispenser using the correct entrance; (**b**) Mean number of CFU (*Bacillus subtilis*) per exiting bee by Japanese orchard bees exiting the nest dispenser. (**c**) Mean number of CFU (*Bacillus subtilis*) per one flower deposited by Japanese orchard bees in crabapple blossoms (from Biddinger et al. 2010)

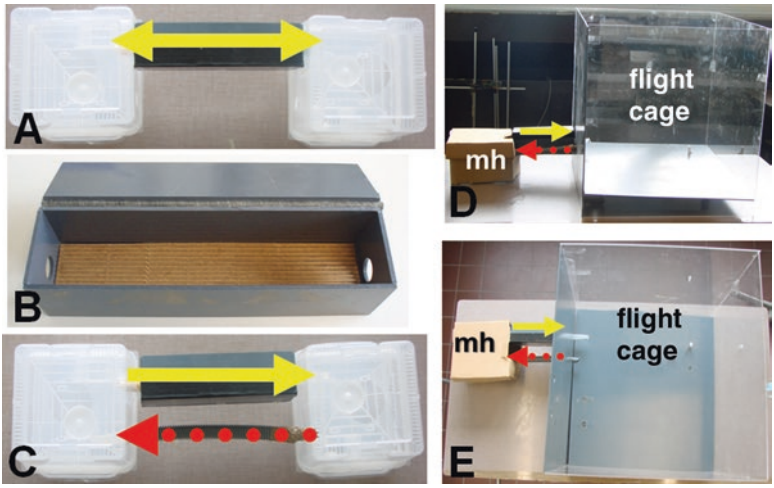

Fig. 24 Overview of the experimental set ups. **(a)** the one-way miniature dispenser bioassay showing a plastic micro-colony nest boxes connected with another micro-colony nest box by a miniature dispenser (20 × 5 × 5 cm); (**b**) a detail of a miniature dispenser containing ribbed carbon paper on the bottom; (**c**) the two-way miniature dispenser bioassay whereby an additional connecting is made between the two micro-colony nest boxes by use of a 20-cm long plastic tube; and (**d-e**) the flight cage experiment showing a mini-hive (mh) connected with a flight cage (50 × 50 × 50 cm) with D the side view and E the top view. The full yellow arrow indicates the route with the miniature dispenser containing the formulated powder product, that the bumble bee workers follow to go from the micro-colony nest box or mini-hive to the other next box or flight cage; while the dotted red arrow indicates the route without miniature dispenser and free of powder formulated product that the bumble bee workers take to return from the food compartment to the nest box/mini-hive. Taken from Mommaerts et al. (2012)

unidirectional passage through the tube is ensured by using bumble bee enclosers. The flight-cage bioassay was developed to include the impact of flight on possible side-effects of the BCOs. Here, a mini-hive containing 50 workers and their brood is connected to a flight cage (50 × 50 × 50 cm) through the abovementioned dispenser and a plastic tube free of powder. Pollen is provided in the nest and sugar water in the flight cage. The setups are pictured in Fig. 24. For more specific details on the setup we refer to the original paper by Mommaerts et al. (2012).

Testing of different BCOs showed that the best predictions of lethal and sub-lethal effects were achieved using the two-way miniature-dispenser bioassay or the flight-cage set-up. The two-way bioassay has the advantage that it is easy to setup in the lab, while the flight cage setup manages to take into account the loss of powder during flight for more accurate results. Both tests seem to be capable of performing a first screening of potential lethal or sub-lethal effects of BCOs and carrier powders, but further validation is recommended as some indicators for colony health, such as the free flight foraging efficiency, have not been considered so far in these risk assessments.

6 Commercially Available Dispensers

The goal of the entomovectoring technology is to improve crop protection and yield to increase their economic value. To achieve this, commercially available dispensers supporting the entomovector technology have to be widely available. So far, three commercial dispensers have been developed: the BeeTreat dispenser, the Flying Doctors dispenser and the BVT dispenser as developed by Bee Vectoring Technologies International Inc.

6.1 BeeTreat Dispenser

The BeeTreat dispenser is a two-way dispenser for honey bees developed by Hokkanen et al. (2012). It is made out of a wooden frame and a landing platform made of wood and a Plexiglass plate. The different components of the dispenser are shown in Figs. 25 and 26. The BeeTreat dispenser is designed to perfectly fit on Langstroth-type beehives but it is also compatible with other hive types. The frame can be easily attached to the beehives using a simple rubber strap. Once the frame is attached the landing platform can be put into place. After a few days where the bees can familiarize with the dispenser, the BCO formulation can be placed at the exit of the dispenser. Exiting honey bees will walk through the powder and under the Plexiglass plate to leave the hive. Upon returning, they will land on top of the Plexiglass plate and enter the hive through an entrance which is separated from the exit, to avoid contact with the BCO formulation (Fig. 27). The application of the BeeTreat dispenser in the field is shown in Fig. 28.

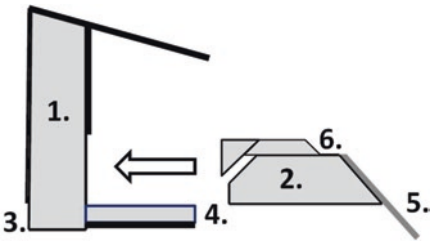

Fig. 25 Side view of the BeeTreat dispenser (www.aasatek.fi). 1 = body of the dispenser (back against the beehive); 2 = detachable steering part, to be inserted into the body; 3 = opening joining the dispenser with the hive opening; 4 = exit area for the bees; 5 = landing platform for the returning bees; 6 = entrance corridor for bees to return to the hive (crawl over the solid block 2 to access opening 3). The area between 3 and 4 forms the exit corridor, where the material to be dispensed is placed on the bottom. The solid block 2 forms the ceiling of the corridor. All parts are made of untreated wood or plywood, except 5, which is clear Plexiglas to allow daylight to be seen from the hive opening at 3 (upper surface is slightly roughened for bees to get a grip). Taken from Hokkanen et al. (2012)

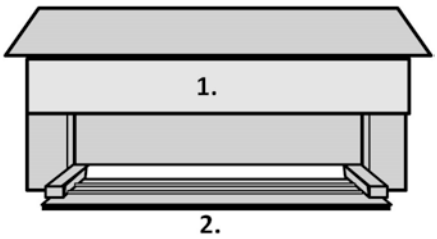

Fig. 26 Front view of the BeeTreat dispenser, without the steering part. 1 = body of the dispenser; 2 = bottom of the exit corridor, on which the material to be dispensed is spread between two 4 mm high ribs, 6 cm apart, at the full breadth of the dispenser. Note the exit/entrance slot to the beehive between parts 1 and 2 (corresponding to part 3 in Fig. 25). Taken from Hokkanen et al. (2012)

Fig. 27 Exit (under the Plexiglass plate) (**a**) and entrance (above the Plexiglass plate) (**b**) of the BeeTreat dispenser. Taken from Hokkanen and Menzler-Hokkanen (2009)

6.2 Flying Doctors®

The Flying Doctors® (Fig. 29) is a commercial dispenser for bumble bees of *B. terrestris*. This patented system consists of a cardboard box containing a specially developed hive with an integrated two-way dispenser system, based on the Mommaerts dispenser described earlier in this chapter. Bumble bees exit the dispenser through a loading tray and enter the hive through a separate entrance. The tray can be loaded with either a BCO to achieve crop protection or with

Fig. 28 BeeTreat dispensers in the field. Taken from Hokkanen and Menzler-Hokkanen (2009)

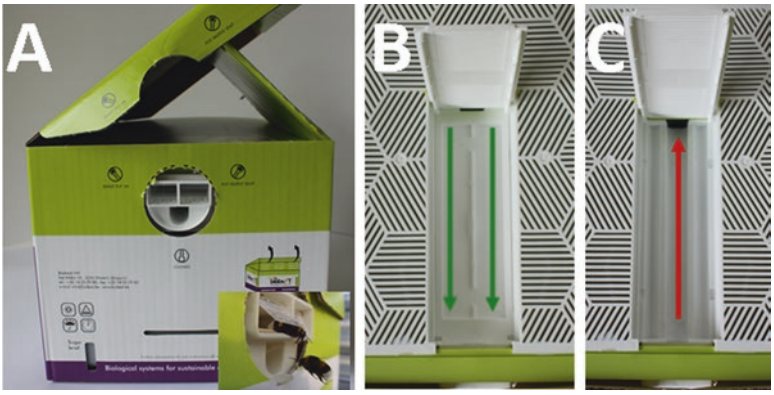

Fig. 29 The Flying Doctor® system from Biobest. (**a**) Bumble bee nest with dispenser on top, a bumble bee entering and exiting the dispenser in the right corner; (**b**) top view of the loading tray where the antagonist is placed (**c**) top view of the route for entering the hive. Taken from www.biobest.com

commercially available pollen to improve pollination of the crops. A transparent sealing flap guarantees a one-way traffic in the dispenser by sealing of the exit for bees returning to the hive. The hive entrance has an enclosure at the back to prevent bumble bees from leaving the hive by this way.

6.3 BVT Inoculum Dispenser

The BVT inoculum dispenser consists of a tray, incorporated into the lid of commercial beehives. Exiting bumble bees walk through the tray and get coated with the powder formulation. The tray is designed to ensure that bumble bees are forced to walk through it, resulting in an efficient loading of the bumble bees. The tray can be easily replaced upon depletion, which approximately takes 3 days. The dispenser is patented by Bee Vectoring Technologies International Inc.

7 Conclusions and Future Research

Over the past 25 years, the development of dispensers for use with the entomovector technology has shown promising results. Efficient dispensers are commercially available for both honey bees and bumble bees, allowing the entomovector technology to go beyond the stage of laboratory testing. Tracking the results that commercial growers achieve by using this technology should provide interesting results for future analyses of the dispensers and show potential areas for further improvement. Investigating separate traits of dispensers, as done by Mommaerts et al. (2010) for the length of the dispenser, might prove to be an interesting way to optimize dispensers. These tests give us clear results on which traits are important in an optimal dispenser and can easily be incorporated in future dispenser models.

Developing a standard protocol for the testing of new dispensers should also be a priority for a viable comparison among different dispenser types. We propose a standard protocol that investigates the following traits:

1. Does the dispenser influence the amount of workers leaving the nest when attached or build in to the hive?
2. Does filling the attached dispenser with powder influence the amount of workers leaving the nest?
3. What percentage of the vectors leaving the nest is carrying the powder formulation?
4. What is the average CFU found per vector leaving the nest?

For these tests, a standard powder formulation with a known CFU/g should be used in equal quantities in all studies in order to make reliable comparisons. The use of different powder formulations in different studies makes it difficult to compare the achieved loading, as the amount of powder and the initial CFU/g will greatly influence the amount of CFU carried by the insect vector.

Another important subject for future research should be the further development of an efficient dispenser for mason bees. Mason bees have proven to be efficient pollinators of fruit trees such as apple and pear, which are otherwise difficult to pollinate using honey bees (Monzon et al. 2004; Vicens and Bosch 2000). As these trees often have to deal with the destructive disease fire blight (caused by *Erwinia*

amylovora), entomovectoring by mason bees could provide a potential solution by combining efficient pollination with the dispersal of a BCO in the orchards (Maccagnani et al. 2006, 2008, unpublished data). To achieve this, an optimization of the mason bee disperser will be necessary, making it a priority for future research.

As a final conclusion, we can state that the dispensers that are currently available have proven to be efficient for use with the entomovectoring technology and commercial applications, but some optimizations might still be possible. Now, being the dispenser designs almost optimized, the next step to improve the loading of the vectors is to improve the powder formulations that should contain the BCOs. Optimizing the formulation for its use with the chosen vector and dispenser can greatly improve the loading of the vector and further increase the efficiency of the inoculum transfer. After all, the success of entomovectoring does not rely on a single component of the design, but on the interaction between all of them.

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Case Studies on Entomovectoring in the Greenhouse and Open Field

Guy Smagghe

1 Introduction

Pollination plays a key role in the establishment of successful fruit setting in agriculture and horticulture, which is why managed pollinators are often relied upon to improve yield in greenhouses and open fields. Using the entomovectoring technology, pollinators can potentially provide a second service, being the dispersal of biological control agents (BCOs) to the crops to suppress pest species and plant pathogens. Starting with the first study by Peng et al. (1992) on the possibility to protect strawberries against grey mould using honey bees, multiple studies have investigated the potential of entomovectoring to protect crops. In this chapter, different case studies are presented both in open field and greenhouse conditions aiming to protect different target crops. These case studies give an overview on the knowledge that is available on using pollinators to vector BCOs to target crops and suppress diseases. It should be noted though that, because of the fact that the effectiveness of entomovectoring is determined by the interaction of many components, results cannot be extrapolated automatically to designs using different target crops or control agents (Fig. 1).

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Fig. 1 Bumble bee of *Bombus terrestris* covered with a biological control agent (BCA) powder formulation, foraging on a strawberry flower. (Source: Veerle Mommaerts)

2 Entomovectoring for the Protection of Strawberries

Strawberry is a worldwide grown fruit crop in both open field and greenhouses. However, yields are often limited by diseases, the most destructive one being grey mould. Grey mould is caused by the airborne plant pathogen *Botrytis cinerea* and is most destructive on mature or senescent tissues of a variety of dicotyledonous hosts, including strawberry (Williamson et al. 2007). Symptoms become visible when fruits are ripening but infection with the pathogen occurs at flowering, as the stamens are considered to be the principal infection court. Therefore, treatment of the newly opened flowers seems to be the most effective strategy to prevent infection by *B. cinerea* (Mertely et al. 2002). As pollinators can potentially deliver control agents directly to the flower as soon as they are open and available for pollination, entomovectoring has been investigated as a way to protect strawberry plants against *B. cinerea*. The first study by Peng et al. (1992) investigated whether honey bees could disperse *Gliocladium roseum*, a fungus which suppresses spore production of *Botrytis cinerea*, to strawberry crops in open field and greenhouses by loading their Peng dispenser with a mix of talc-corn meal and spores. They found that honey bees emerged from the dispenser, carried the powder and transferred it successfully to the strawberry flowers. The amounts of transferred inoculum seemed sufficient to suppress *B. cinerea*, except when honey bee activity was reduced due to bad weather conditions.

The potential of another BCO, *Trichoderma harzianum*, was investigated in three different studies. The first one was conducted by Kovach et al. (2000) over a period of 4 years and used both honey bees and bumble bees as vectors. During the experiment, strawberry fields on several locations near New York (USA) were monitored and the effectiveness of *Trichoderma harzianum* 1295–22 spraying and vectoring was investigated. The authors reported that flowers in patches where the BCO was vectored by bees had lower concentrations of *T. harzianum* compared to flowers in patches that were treated with BCO's through spraying application. However, it was apparent that the level of control achieved through entomovectoring with bees

was higher than the spraying. It was also comparable or sometimes even higher to the control level as provided by commercial fungicides that were applied by spray at bloom. Moreover, it was remarked that the bee visits increased the seeds on collected strawberries with 22% and caused an increase in weight of up to 40% compared to strawberries in non-visited plots. Based on these findings, the authors concluded that the bee-vectored *T. harzianum* can be considered as a viable strategy for growers who wish to minimize the use of fungicides in the fight against *B. cinerea*. For more detailed information on the different experiments conducted during these 4 years, consult the original paper of Kovach et al. (2000).

A second study was conducted by Shafir et al. (2006) where a different strain, *Trichoderma harzianum* T39, was vectored by honey bees under open field conditions in Israel over two consecutive growth seasons. The authors compared the effect of the spraying of commercial fungicide with the vectoring of *T. harzianum* T39 (commercially developed as "Trichodex" for the control of grey mould). Honey bees were loaded with the powder formulation using the Triwaks dispenser as developed by Bilu et al. (2004).

Over the two seasons, the same protocol was used. It consisted of a randomized complete block design with four different treatments, being (1) fungicide only, (2) bee-vectored only, (3) both fungicide and bee-vectored, and (4) control. Sufficient levels of *T. harzianum* (10^4 CFU per flower) were found on flowers up to 200 meters from the hives in the bee-vectored treatments. It was concluded that the transmission of *T. harzianum* by honey bees is effective, but the ability to suppress grey mould was not constant throughout the season. The efficiency of both the fungicide and the vectored *T. harzianum* was best at the start of the season and started to fail towards the end, when the number of symptomatic fruits became too high. A third study was conducted by Albano et al. (2009) using honey bees (*Apis mellifera*) and bumble bees (*Bombus impatiens*) to vector the biofungicide "Rootshield" to strawberries in fields (honey bees) and greenhouses (bumble bees). They tested the ability of the vectors to get dusted with powder when walking through the Houle dispenser and deliver the powder to the strawberry crops. They found that both honey bees and bumble bees were capable of dispersing the powder efficiently. However, no data was reported on the level of disease suppression.

A third BCO that has been tested to protect strawberries against grey mould through entomovectoring is *Gliocladium catenulatum*, a fungus which is originally isolated from the soil. It is now produced by the Finnish company Verdera and commercially available as "Prestop". The first study investigating the potential of "Prestop", conducted by Hokkanen et al. (2012), started in 2005 and lasted over a period of 4 years. Research took place on different locations in Finland and used a newly developed dispenser, the BeeTreat dispenser, to load honey bees with "Prestop". Experiments took place in open field conditions and compared the disease incidence and marketable yield between four different treatments, being (1) bee-vectored "Prestop", (2) chemical fungicides, (3) chemical fungicides combined with bee-vectored "Prestop", and (4) control group. Looking at disease control, the bee-vectored "Prestop" decreased the disease incidence on average by 50% compared to 65% for chemical fungicides and 80% for the combined treatment. Based

on disease incidence, a combination of fungicides and bee-vectoring appeared to be the best option. However, when total marketable yield was investigated, the authors found that bee-vectored biocontrol was as effective, or in some years even more efficient, compared to the fungicides and the combined treatment. Comparing the marketable yields between the treatments collected in 2008 showed that it was only marginally larger in the fungicides treatment compared to the control. The bee-vectored biocontrol provided the highest yield with a 90% overall increase compared to the control group. Combining biocontrol with fungicides did not increase the yield any further despite the fact that disease suppression was better in this group (Fig. 2a). This suggests that sprays might have an impact on the yield potential of strawberry plants. The increased yield could also partially be attributed to the improved pollination of the flowers, as shown by the results of the trials on organic farms (Fig. 2b). Enhanced pollination by honey bees increased the yield by 58%, while combining pollination with bee-vectored biocontrol increased yield by 105% compared to the control group. All treatments were also reported to improve the shelf-life of the strawberries after harvesting, approximately doubling their durability, with the combination of fungicides and bee-vectored biocontrol increasing durability the most.

A second study was conducted under greenhouse conditions by Mommaerts et al. (2011) using the bumble bee *Bombus terrestris*. The authors used their own dispenser, the Mommaerts dispenser to load bumble bees with "Prestop-Mix" (*Gliocladium catenulatum* Strain J1446) and investigated the ability to suppress grey mould (*B. cinerea*) in manually infected strawberry plants. The experiments were conducted in a greenhouse with four fine-meshed tents, each subjected to a different treatment: (T1) Control (no pollination or biocontrol), (T2) "Maizena-Plus" (pollination and dissemination of Maizena-Plus), (T3) "Prestop-Mix" (pollination and dissemination of Prestop-Mix), and (T4) "Prestop-Mix" + "Maizena-Plus" (pollination and dissemination of a 1:1 "Prestop-Mix":"Maizena-Plus" formulation). All plants were manually infected with 10 μl of a water solution with a concentration of 10^5 *B. cinerea* spores per ml (Fig. 3). A comparison with a control group that was inoculated with water only showed that manual inoculation can lead to the development of *B. cinerea* under the greenhouse conditions, which were considered optimal for the development of the fungus. The efficacy of the treatment was determined by comparing the numbers of flowers that were visited by the bumble bees during the first 4 weeks with the numbers of red fruits formed during the following 4 weeks (pre-harvest yield). Strawberries were also incubated for 2 days in the laboratory after picking and examined afterwards to determine post-harvest effects and yield of the treatments.

As essential results of this greenhouse test, the authors reported a preharvest yield which was higher for T3 and T4, with 72 ± 17% and 71 ± 9% of the visited flowers developing into strawberries, respectively. For T1 and T2, the yield was lower with 54 ± 21% and 51 ± 9%, respectively, indicating a positive effect of the vectored "Prestop-Mix" on preharvest yield. The post-harvest yield was also better for T3 and T4, as 67 ± 13% and 79 ± 17% of the harvested berries did not show any signs of rot after incubation, respectively, compared to 43 ± 13% for T1 and

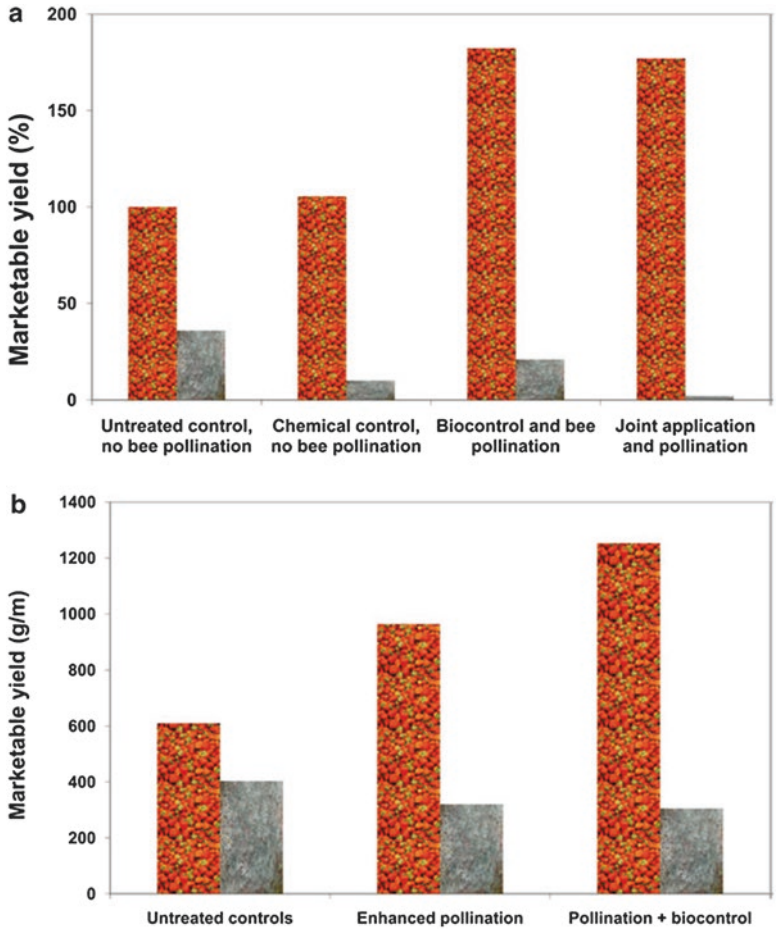

Fig. 2 Overview of the marketable yield (red bars) and mouldy berries (grey bars) per 1 m of strawberry row. (**a**) compares marketable yield from the different treatment groups relative to the untreated control group (control yield = 100%) [Data from 2008 on 4 farms, each with 4 replicates]. (**b**) shows the yield on an organic strawberry farm in 2008. Compared treatments are untreated control (no disease control and only natural pollination), enhanced pollination (no disease control and increased pollination by honey bees) and enhanced pollination combined with bee-vectored biocontrol. Taken from Hokkanen et al. (2012)

50 ± 10% for T2. The total yields (calculated as % preharvest yield x % post-harvest yield) differed significantly between T3-T4 and T1-T2, being 47 ± 10% for T3 and 56 ± 10% for T4, compared to 24 ± 14% for T1 and 25 ± 8% for T2. The authors also found that the foraging activity of the bees was not affected by the powder formulation, which is an important condition for effective use of entomovectoring. A third three-year study using Prestop-Mix was conducted in Estonia by Karise et al. (2016), investigating the potential of bumble bees of *B. terrestris* to vector the powder under open field conditions and suppress *B. cinerea* infections in open field

Fig. 3 Experimental set up of the greenhouse experiment conducted by Mommaerts et al. (2011), showing the four different treatments. Taken from Smagghe et al. (2012)

strawberries. The authors reported a significant reduction in grey mould infections during the first 2 years, but not in the third year, when the weather conditions were very favourable for *Botrytis* development, which resulted in a high disease level. Similar to the study of Shafir et al. (2006), the vectored BCO was not able to suppress the high levels of disease pressure under these very rainy conditions.

3 Entomovectoring Against *Botrytis cinerea* in Raspberry

Just like strawberries, raspberries can also suffer from yield loss caused by *B. cinerea*. Spraying applications are often not efficient due to the short lived flowers, as this makes it difficult or even impossible to time the applications so they can protect all flowers. Yu and Sutton (1997) investigated if *Gliocladium roseum* could be vectored by honey bees (*A.* mellifera) and bumble bees (*B. impatiens*) in open fields, to investigate if alternatives are available to replace spraying applications.

Field tests were conducted using two cultivars of raspberry, being the summer-bearing "Boyne" and fall-bearing "Redwing", during the summer of 1993 and 1994. In both years, crops were divided into four treatments, being (T1) control, (T2) *G. roseum* spray application, (T3) *G. roseum* honey bee-vectored and (T4) *G. roseum* bumble bee-vectored. To assess treatment effects on the incidence of *B. cinerea* in the flowers, 16 flowers were taken from each plot and divided into 4

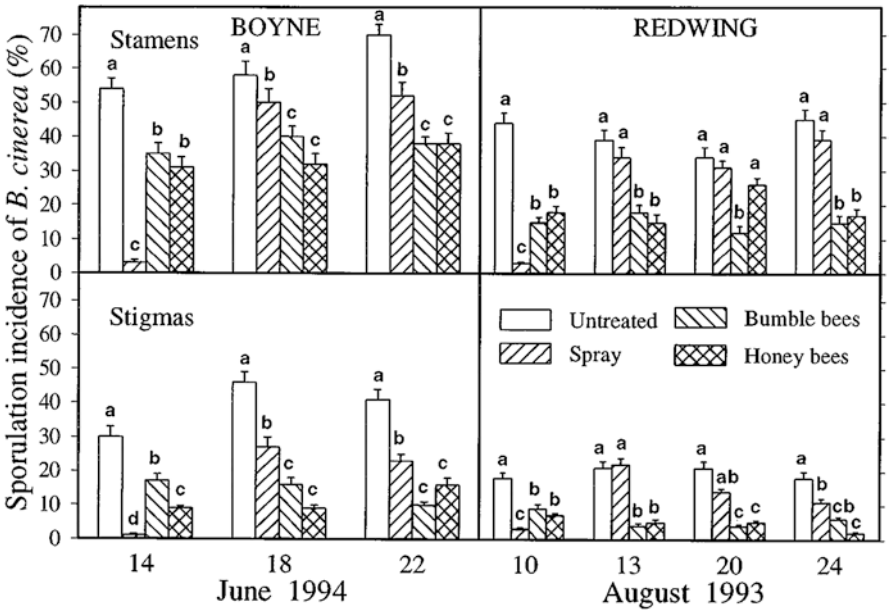

Fig. 4 Sporulation incidence of *Botrytis cinerea* on stamens and stigmas in flowers of raspberry cv. Boyne and cv. Redwing in the different treatments. Data bars are pooled means of means for flowers that were challenge-inoculated with 0, 10^3, 10^4 and 10^5 conidia of *B. cinerea* per ml of water plus surfactant. Observations assigned with a different letter are significantly different. Taken from Yu and Sutton (1997)

different groups which were sprayed with *B. cinerea* at concentrations of 0, 10^3, 10^4 or 10^5 conidia/ml.

The results of Yu and Sutton (1997) are shown in Fig. 4. Honey bee-vectored and bumble bee-vectored *G. roseum* seemed capable to suppress *B. cinerea* in both stamens and stigmas. Only on the first day after applying the pathogen (14th of June for "Boyne" and 10th of August for "Redwing"), spray applications resulted in a higher level of control. It should be noted that by looking at the results of each group separately, it was revealed that *G. roseum* vectored by honey bees and bumble bees was not able to control *B. cinerea* when a concentration of 10^5 conidia/ml was applied. To assess treatment effects on the incidence of grey mould on the fruits, 36 ripe berries were picked at random and incubated to check for the presence of the fungus (Yu and Sutton (1997). The application of *G. roseum* resulted in a significant decline of grey mould fruit rot in the cultivar "Boyne" in June 1994. There was a reduction from 90% in the control group to 41% for spray, 67% for bumble bees and 68% for honey bees. During the trails in June 1993 there was no significant reduction compared to the control group, which had an incidence of 65%. For the cultivar "Redwing", neither of the trials in 1993 or 1994 found a significant reduction of the incidence compared to the control groups (50% incidence in 1993 and 60% in 1994). While stamens and stigmas were continuously protected by the bee-vectored

G. roseum, control of fruit rot seemed to be inconsistent. Yu and Sutton (1997) attributed this to the fact that, despite the fact that *B. cinerea* often infects the fruits in an indirect way through the flowers, there is evidence that the conidia can also infect the ripe fruit surface, resulting in a grey mould infection on fruits that grew from protected flowers.

4 Entomovectoring for Biological Control in Sweet Pepper

Tarnished plant bug (*Lygus lineolaris*, TPB) and western flower thrips (*Frankliniella occidentalis*, WFT) are two pest species found on greenhouse crops, including sweet peppers. While biological control measures can in some cases be efficient to fight pests as WFT, chemical insecticides are required for effective control on sweet peppers. TPB is also difficult to be kept under control with BCOs, making chemical pesticides the main, only control strategy (Al-mazra'awi et al. 2006). The first study on entomovectoring against these pest species in sweet pepper was conducted by Al-mazra'awi et al. (2006) and focused on the BCO *Beauveria bassiana*, a fungus that is active against both TPB and WFT. The bumble bee *B. impatiens* was selected as the vector to transfer the BCO to the sweet peppers and worker bumble bees were loaded using a slightly modified model of the Peng dispenser. The trials took place in a greenhouse using a randomized block design with each trial being replicated over time. The four treatments were (T1) bee-vectored *B. bassiana* + TPB, (T2) bee-vectored *B. bassiana* + WFT, (T3) bee-vectored heat-inactivated *B. bassiana* + TPB + WFT, and (T4) TPB+ WFT, without the presence of bumble bees or BCO.

The authors found that 90% of the flowers showed detectable amounts of *B. bassiana*, demonstrating a successful transfer from the dispenser to the target crops. The BCO was also recovered on the leaves of the crops. TPB and WFT were sampled on two different dates and mortality was assessed. During the first sampling, TPB individuals in treatment (T1) displayed a mortality of 33.6 ± 6.6% (with 90.0 ± 3.1% mycosed) compared to 9.2 ± 2.7% (6.0 ± 4.4%) mortality (mycosed) for treatment (T3) and 14.8 ± 4.1% (0%) for treatment (T4). For the second sampling, this was 45.0 ± 3.9% (91.0 ± 3.0%) for treatment (T1), 15.3 ± 3.2% (14.5 ± 7.5%) for treatment (T3) and 9.0 ± 1.9% (1.7 ± 1.7%) for treatment (T4). For both samplings, the mortality in treatment (T1) was significantly higher compared to the others, demonstrating a significant effect of the vectored *B. bassiana* on the mortality of the TPB. The same significant difference between the viable *B. bassiana* treatment and the controls was found for WFT. Treatment (T2) showed a mortality of 39.5 ± 11.8% (34.1 ± 6.9%) on the first sampling date and 34.1 ± 6.9% on the second sampling date. In comparison, treatment (T3) showed a mortality of 3.4 ± 2.6% and 3.1 ± 2.6%, respectively, whereas treatment (T4) had a mortality rate of 2.2 ± 2.2% and 0.5 ± 0.5%, respectively. The percentage of individuals showing mycosis was not reported for the WFT adults.

Kapongo et al. (2008a) investigated the optimal concentration for the vectored BCO powder containing *B. bassiana* ("BotaniGard 22WP") to control TPB and green peach aphid (GPA) (*Myzus persicae*) on greenhouse sweet pepper, using bumble bees of *B. impatiens* as a vector. The experiment consisted of a randomized block design with 5 treatments, being (T1) low concentration of *B. bassiana*, (T2) middle concentration of *B. bassiana*, (T3) high concentration of *B. bassiana*, (T4) heat inactivated *B. bassiana*, and (T5) control treatment without bumble bees. For TPB, no mortality was found in treatment (T4) and (T5). Treatment (T1) resulted in the killing of 33.0 ± 5.0% of the adults, which was significantly lower compared to treatment (T2) and (T3), which had a mortality of 69.7 ± 3.6% and 67.1 ± 5.2%, respectively. Treatment (T2) and (T3) did not differ significantly from each other. For GPA, the same pattern was observed. Mortality in treatment (T1) (21.5 ± 3.5%) did differ significantly from the percentage found in treatment (T2) (33.5 ± 3.3%) and treatment (T3) (29.5 ± 5.3%), but no difference was found between the latter two. Treatment (T4) and (T5) both showed no mortality. Based on the data obtained, it looks that both the medium concentration (6.24×10^{10}) and high concentration (2×10^{11}) are able to affect the populations of both pest species.

A third experiment aimed to confirm the potential to co-vector *B. bassiana* and *Clonostachys rosea* using bumble bees of *Bombus impatiens* to control TPB and grey mould (*B.* cinerea) in sweet pepper simultaneously (Kapongo et al. 2008b). The experiment consisted of three treatments: (T1) mixed formulation of *B. bassiana* and *C. rosea*, (T2) heat-inactivated inoculum, and (T3) control treatment without inoculum or bumble bees. Plants were manually inoculated with *B. cinerea*. Treatment (T1) caused a mortality of 72.5 ± 1.4% of the adult TPB, which was significantly higher compared to the control treatments. The mortality in treatment (T2) was 10.8 ± 2.2%, and in treatment (T3) 10.8 ± 1.2%; the control groups showed no significant difference with (T2). In treatment (T1), grey mould on sweet pepper was suppressed by 58.9% in the flowers and by 46.8% on the leaves.

5 Entomovectoring for Biological Control in Tomato Plants

Another greenhouse crop which is grown around the world is tomatoes. Two pest species are frequently found on greenhouse tomatoes, being the greenhouse whitefly (*Trialeurodes vaporariorum*) and the two-spotted spider mite (*Tetranychus urticae*) (Lange and Bronson 1981). So far, no research has been done on the possibility to suppress two-spotted spider mite using entomovectoring, but two studies investigated the effect of bumble bee-vectored BCOs to control greenhouse whitefly (GWF) in tomato greenhouses. The first study by Kapongo et al. (2008a) vectored *B. bassiana* under different concentrations using bumble bees of *B. impatiens,* with the same design as described above for sweet pepper. The effects of different concentrations of *B. bassiana* were investigated by checking the mortality percentage of adult greenhouse whiteflies in each treatment. Both control treatments (no inoculum and heat inactivated inoculum) did not cause any mortality among the

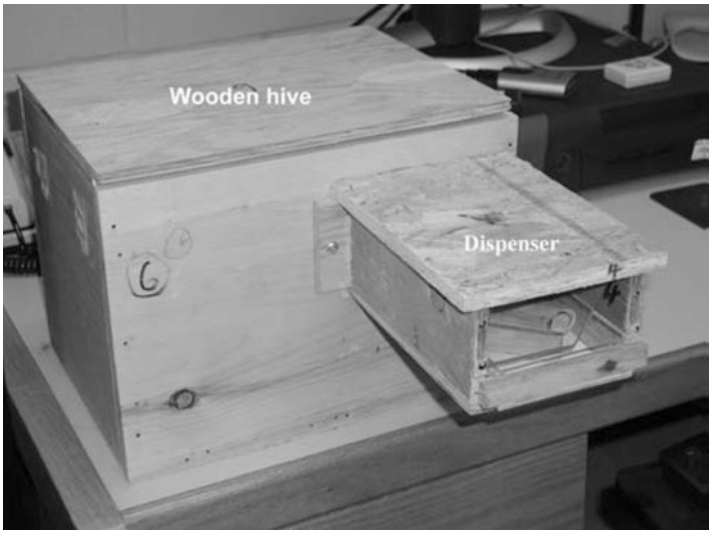

Fig. 5 Dispenser used by Kapongo et al. (2008a) to load *Bombus impatiens* with *Beauveria bassiana*. Taken from Kapongo et al. (2008a)

whiteflies. The low, middle and high concentration of *B. bassiana* resulted in 17.9 ± 2.1%, 53.9 ± 3.4% and 55.9 ± 4.2% mortality, respectively. The authors reported a significant difference between the low concentration and the middle or high concentration, but no significant difference between the middle and high concentration (Fig. 5).

A second study investigated the effect of the vectoring of a mix of *B. bassiana* and *C. rosea* to suppress both greenhouse whiteflies and grey mould at the same time (Kapongo et al. 2008b). The setup was identical as described above for the experiment with sweet pepper. Greenhouse whitefly adults in the *B. bassianae* + *C. rosea* treatment showed a significantly higher mortality percentage (59.1 ± 2.5%) compared to the ones in the heat-inactivated treatment and the control group (18.8 ± 6.6% and 20 ± 2.5%, respectively).

6 Entomovectoring Against Plant Pathogens in Blueberries

Among all diseases associated with blueberries, *Monilinia vaccinii-corymbosi* has de greatest economic impact on the industry (Scherm et al. 2001). *M. vaccinii-corymbosi* is a pathogenic fungus which infects open blueberry flowers and causes mummy berry disease, resulting in a yield decrease in blueberry fields. Since blueberries are dependent on sufficient pollination to ensure adequate fruit set, commercial blueberry producers often use supplemental bees to increase their yield (Dedej et al. 2004). However, pollinators are also the main vectors of the *M. vaccinii-corymbosi* conidia, leaving the growers with a dilemma as increasing pollination is

also likely to increase the incidence of mummy berry disease. In search of a solution for this dilemma, Scherm et al. (2004) investigated if the biofungicide "Serenade", a commercial formulation of *Bacillus subtilis*, was able to control flower infections when it was applied directly to the stigmas of open flowers. During tests in the lab, flowers were treated manually, but this would be unable to achieve in the field. In search of an alternative way to apply the "Serenade", Dedej et al. (2004) used honey bees to deliver Serenade to the stigmas of rabbiteye blueberry bushes and suppress *M. vaccinii-corymbosi*. Honey bees were loaded with "Serenade" using the Gross dispenser and delivered the powder to plants under open field conditions. Treatments consisted of vectoring "Serenade" using different bee densities in the first year (0 bees, 1600 bees or 6400 bees) of the study. During the second and third year, additional treatments were added using the same bee densities, but no "Serenade" to vector. To assess the effect of the vectored "Serenade", 30 fruit clusters were selected and bisected to assess the presence of mycelia or pseudosclerotia of *M. vaccinii-corymbosi*. It was found that disease levels increased with bee density and were lower when "Serenade" was vectored. Disease incidence in treatments with 6400 bees and no "Serenade" was highest among all treatments (21.1% in 2002 and 66.5% in 2003, compared to 14.2% and 30.5% for the control treatment in 2002 and 2003, respectively). Treatments with 6400 bees including "Serenade", resulted in that the disease incidence dropped to 6.6% and 43.5% in 2002 and 2003, respectively. These results demonstrate that increasing the number of honey bees to improve pollination may increase the risk of spreading mummy berry disease in the field. Like strawberries and raspberries, blueberries can also suffer from grey mould caused by *B. cinerea*. Reeh et al. (2014) investigated the effect of bumble bee-vectored *Clonostachys rosea* (the commercial form "Origro's Endophyte") on the development of grey mould on lowbush blueberries under open field conditions. They found a significant reduction of the percentage of blossoms infected with *B. cinerea*, but total percentage of infected blossoms still remained high (up to 90% in some cases). The results demonstrated that entomovectoring alone might not be able to provide an economic advantage for blueberry growers suffering from grey mould, but it might be able to be effective when used as part of an integrated pest management plan.

7 Entomovectoring Against Pathogens and Pests in Sunflowers

Sunflower growers often suffer economic losses by pest species such as the banded sunflower moth (BSM) (*Cochylis hospes*). While chemical pesticides may control the damage afflicted by this species, it can also be detrimental for the honey bee populations visiting the sunflowers. As honey bees of *A. mellifera* are the main pollinator of sunflowers (Sosa 1988), alternatives were needed for an efficient control of the BSM that would not affect the honey bee populations that pollinated them.

Jyoti and Brewer (1999) vectored *Bacillus thuringiensis* var. *kurstaki*, a BCO registered for use on sunflower to control BSM, with the use of honey bees under open field conditions. The potency of honey bee-vectored *B. thuringiensis* was compared with a spray application using a set-up with 3 treatments: (T1) sunflowers with bee-vectored BCA, (T2) sunflowers with spray application of the BCA, and (T3) control treatment. The experiment was performed twice, once in 1996 and once in 1997. Three sunflower heads were collected per sampling sites which radiated outward 7.6 m, 15.2 m and 22.8 m from the centre of each block and each assigned to a different treatment. The flower heads were infested with 50 BSM eggs and collected at physiological maturity. A first sample of 100 seeds was taken from each flower to determine the percentage of seeds damaged by BSM. A second sample of 100 seeds was taken to determine the weight of the seeds and seed oil concentration.

The authors found that bee-vectored *B. thuringiensis* resulted in a significantly lower amount of damaged seeds (1996: 12.1 ± 0.2%; 1997: 12.2 ± 0.4%) compared to the control group (1996: 21.1 ± 0.2%; 1997: 22.3 ± 0.4%). In 1997, there was also a significant difference between bee-vectoring and spray application, with bee-vectored *B. thuringiensis* resulting in a lower percentage of damaged seeds, but in 1996 no significant difference was found. The seed set (percentage of filled seeds) was also significantly higher in the bee-vectored treatment compared to the other two during both years. Seed oil content and seed yield always differed significantly between the bee-vectored treatment and the control group, indicating an overall positive effect of the presence of honey bees and the vectored *B. thuringiensis*. In most cases, bee-vectored control agent was also more effective compared to the spray application. These results demonstrate the positive influence of honey bees on sunflowers, both in the presence and absence of vectored *B. thuringiensis*. A second study conducted by Escande et al. (2002) in Argentina focused on entomovectoring to fight the plant pathogen *Sclerotinia sclerotiorum*, which causes sunflower head rot. The authors found that using honey bees to vector a mix containing various strains of *Trichoderma* sp. could significantly reduce head rot incidence in sunflowers. When combining the treatment with a resistant genotype of sunflowers, reductions of 90–23% were found. On top of that, the experiments were conducted under conditions where the incidence of *Sclerotinia sclerotiorum* could be as high as 86%, while under natural conditions the disease was found not to exceed 68%. Again however, no data was collected on the yield obtained in the presence or absence of honey bee-vectored *S. sclerotiorum* for determining the economic value of using entomovectoring to protect sunflowers against pathogens and pest species.

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A Case Study: Use of Prestop® Mix Biofungicide in Entomovectoring on Apple Against Storage Rot Diseases

Marja-Leena Lahdenperä

1 Background of *Gliocladium catenulatum* Strain J1446

Gliocladium catenulatum strain J1446 is a wild-type fungal antagonist isolated from Finnish field soil. Because of changes in the taxonomy of fungi, the current official name is now *Clonostachys rosea*. However, the old name *Gliocladium catenulatum* is still used in all documents and labels concerning products based on this antagonist.

G. catenulatum J1446 has efficacy against many plant pathogenic fungi especially on several greenhouse crops. It is effective not only against soil and root pathogens but also against certain foliar diseases like grey mould. Extensive research on *G. catenulatum* has resulted in the commercialization of the antagonist. Verdera Oy has developed two *Gliocladium*-based biofungicides, Prestop® and Prestop® Mix that are today widely used in greenhouses in Europe and North America.

The efficacy of *Gliocladium* against grey mould was first shown as spraying treatments, but at that time such application could not be widely used because the treatment costs were too high. This was the reason for replacing spray application with an entomovectoring method. When only flowers are treated, lower application rates become possible. The Prestop® Mix formulation, originally developed for soil treatment, suits for entomovectoring, too. This is because Prestop® Mix powder adheres well to the hairy surface of pollinators and because the product is not hygroscopic, so, it can be applied in open field in moist conditions without becoming lumpy.

In this bee-assisted biocontrol method the outlet of the honeybee hive is equipped with a microbe dispenser including an inoculum tray, on which the Prestop® Mix product is applied. When bees leave the hive and cross the inoculum field, the

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biofungicide powder adheres to their fur and legs and is transported to flowers during the pollination. The grey mould pathogen infects the developing fruit through the wilting flowers. Therefore it is particularly important that the *G. catenulatum* antagonist colonizes the flower organs in advance. Earlier studies have shown that *G. catenulatum* persists up to 4 weeks on aerial surfaces of plants in outdoor conditions (Lahdenperä and Korteniemi 2005). In the case of grey mould in strawberry flowers, colonization of stamens seems to be the major mode of action in the control mechanism of *G. catenulatum* J1446 (Lahdenperä 2006). Being the first colonizer *Gliocladium* prevents pathogens from getting space and nutrients to cause the disease. Therefore Prestop® Mix biofungicide treatments protect the plant from fungal attack, and due to this maintain the grey mould level below economic thresholds and increase the marketable yield of berry crops.

2 Introduction

The successful control of grey mould on berry plants using Prestop® Mix and honeybees suggests that the same method might be used also for other crops against various pathogens infecting via blossoms. E.g. apples occasionally suffer from core rot caused by fungi that infect through flowers. So, apple seemed to be a suitable target for Prestop® Mix. Besides, there were no earlier attempts to test pollinator-assisted delivery of the biofungicide on apple. In addition, it is very difficult to get any appropriate control against this disease using chemical fungicides. In this case study, Prestop® Mix combined with the entomovectoring technique is used in a commercial apple orchard for the control of core rot disease.

Core rot is a storage disease, which infects apple and other fruit crops through flowers. Typically, the symptoms of the disease develop during storage period and the damage of the disease becomes visible only after storing. However, occasionally the symptoms can appear already at harvest time. According to Finnish growers, certain apple varieties, such as ‘Rubinola’, ‘Gala Schnitzel’ (Fig. 1) and ‘Santana’ have turned out to be highly sensitive to core rot, and the main pathogens causing the disease are *Fusarium* and *Botrytis*, at least in Finnish conditions.

3 Material & Methods

3.1 General Issues

The combined use of Prestop® Mix and honeybees was tested on apple for the management of core rot during 2013–15. Field trials were conducted by Verdera Oy (the manufacturer of Prestop® Mix) in collaboration with the advisory service group Pro Agria Ålands Hushållningssällskap (Pernilla Gabrielsson as a contact person) and Peter Sundin’s commercial apple orchard in the Åland Islands, which is the most

Fig. 1 Most often, core rot infected apples look healthy from the top, but the core and the adjacent tissues are damaged. In the picture core rot symptoms caused by *Fusarium avenaceum* on a susceptible variety 'Gala Schnitzel'

important apple growing area in Finland. The testing was carried out as demonstration trials in practical conditions. Field trials were continued as storage tests. The orchard was managed by conventional methods, i.e. chemical pesticides were used according to normal practice when needed. This means that simultaneously with the biological control (Prestop® Mix/entomovectoring), chemical products against other pests like apple scab were applied.

3.2 Field Trials

Two field trials were carried out in a commercial orchard in practical circumstances in the growing seasons of 2013 and 2014. Because the efficacy of Prestop® Mix vectored by honeybees was tested as demonstration trials, the experimental arrangements were very simple. Thus, there was only one area where honeybee-assisted biocontrol was used, and a similar area which served as an untreated reference. These two apple areas, separated by a small forest, were located so far apart that bees delivering *Gliocladium* were not likely to fly from the treated area to untreated trees. The distance between the two trial locations was 500 meters.

In the first trial the winter variety 'Rubinola' was used as the test crop and in the second one the winter variety 'Zari'. They both are susceptible to core rot of apple.

Two bee hives equipped with a BeeTreat® microbe dispenser produced by Aasatek Oy were placed at the edge of the apple field. The hives located about 50 meters from the 'Rubinola' test tree rows, whereas in the case of 'Zari' the bee hives located very close to the other end of the test tree rows (at only 10 meters distance). The other end of the rows was at a distance of 100 meters.

In both trials the microbe tray of the dispenser was filled every morning with a couple of spoons (5–10 g) of Prestop® Mix powder. The dosing of the biofungicide continued over the whole flowering period. The total application rate was around 400 g/ha. The harvest of both 'Rubinola' and 'Zari' was carried out in October.

3.3 Storage Trials

In order to study the effect of the biological control on pathogens, both field trials continued as storage tests (2013–14 and 2014–15). The similar procedure for the two storage trials was used for both storage periods. In October externally healthy apples (4 × 5kg boxes) were harvested for storing until February. After storing the apples 2–4 months, evaluations of the quality and disease damages of stored apples were done as shown in Tables 2-3. The quality was assessed by grouping the apples in three categories: (1) 1st class yield, (2) affected by core rot (*Fusarium* and *Botrytis*) and (3) other damages mostly caused by unidentified pathogens, insect damage etc.

To be able to make observations on the internal core rot symptoms, the apples were cut in half. The core rot damage was visually determined as *Fusarium* or *Botrytis*.

In addition, several more accurate pathogen identifications were done from apples infected by internal rot. The isolations in the laboratory of Verdera Oy were done using a standard potato-dextrose agar-plate technique and microscoping.

3.4 Analysis of Flowers

Apple flower samples for microbial analysis were taken from the second field trial (2014). The samples were collected at full bloom, 10 days after the beginning of the delivery of the biofungicide. Flowers were sampled for a laboratory analysis to assess the colonization by *G. catenulatum* after entomovectoring. Flower samples of treated trees were taken at three distances from the hive, from two trees per distance and ten flowers/tree, sixty flowers in total. To ascertain that Prestop® Mix had not been carried by bees to the untreated reference area, ten random flower samples were collected also from trees grown in the area where entomovectoring of Prestop® Mix had not been used. – Flowers were collected at the fully open stage and they were packed in small plastic tubes, one flower per tube. For the transport from the orchard to the laboratory of Verdera Oy, the sample tubes were packed in a polystyrene box with an ice pack to maintain the flowers fresh.

In order to make observations of the colonization of *G. catenulatum*, fifteen stamens from each flower were plated onto water agar. In addition, pistils, petals and calyx were placed onto potato-dextrose agar. After 8 days incubation at room temperature, observations of *G. catenulatum* were done using a stereomicroscope. Simultaneously also the agents of core rot, *Fusarium avenaceum* and *Botrytis cinerea* were detected (Fig. 2).

Fig. 2 Stamens and other organs of apple flowers were plated onto agar medium for microbial analysis to observe the colonization of *Gliocladium catenulatum* and the occurrence of core rot pathogens

Table 1 The colonization of *Gliocladium catenulatum* in apple flowers after the application of Prestop® Mix/entomovectoring and the effect of the biocontrol method on the occurrence of *Fusarium avenaceum* in stamens

	Gliocladium colonized flowers	*Fusarium* in stamens	
Treatment/Distance from the hives	%	%	Relative
Untreated (500 m)	0	26.0	100
Prestop Mix (10 m)	75	4.0	15
Prestop Mix (50 m)	60	7.3	28
Prestop Mix (100 m)	50	10.7	41

4 Results

4.1 Analysis of Flowers

The analysis of apple flowers from treated trees revealed that the delivery of Prestop® Mix with the help of honeybees was successful. The microbial examination showed that 50–75% of the flowers originating from trees treated with Prestop® Mix were colonized by *Gliocladium catenulatum.* The percentage of colonization seemed to depend on the distance from the hive (Table 1). The antagonist was observed in all flower organs, i.e. stamens, pistils, petals and calyx. Instead, no *G. catenulatum* was detected in apple flowers collected from the untreated area.

In the flower analysis also the occurrence of the pathogens was recorded. *Botrytis cinerea* was not at all found on the stamens, whereas *Fusarium avenaceum* occurred quite abundantly (Table 1). The percentage of *F. avenaceum* in the stamens was the highest in the untreated reference flowers and the lowest in the flowers near the hive in the treated area. When going further away from the hives, the amount of *Fusarium* increased, but was nevertheless remarkably lower than in the untreated reference (Figs. 3 and 4).

Fig. 3 A stamen of apple flower totally colonized by *Gliocladium catenulatum* antagonist, which inhibits the pathogens from infecting the developing fruit

Fig. 4 Wilting petals and pistils of apple flowers. On the left *Fusarium* in untreated reference and on the right the colonization and hyperparasitism by *Gliocladium catenulatum* after the application of Prestop® Mix by entomovectoring

4.2 Storage Trials

After *Gliocladium* treatments during the flowering period, apples of the first storage trials were better preserved than fruits from the untreated reference. As seen in Table 2, the first class yield after 3 months' storage (in January) was higher and there was less core rot after the application of Prestop® Mix by entomovectoring than in the untreated reference. Visual observations of internal rot showed that nearly 2/3 was due to *Botrytis* and only 1/3 due to *Fusarium*. The proportion of apples in the category 'other damages' was remarkably reduced due to biocontrol.

Table 2 The effect of Prestop® Mix/entomovectoring on the quality of apples after 3 month (January 2014) and 4 month storage (February 2014). The first storage trial

	Percentages (%) of apples after storage			
	Entomovectored Prestop® Mix		Untreated reference	
Observations	3 months	4 months	3 months	4 months
First class	76	72	66	59
Core rot (*Botrytis* and *Fusarium*)	3	2	4	9
Other damages	22	26	30	32

Table 3 The effect of Prestop® Mix/entomovectoring on the quality of apples after 2 month (December 2014) and 4 month storage (February 2015). The second storage trial

	Percentages (%) of apples after storage			
	Entomovectored Prestop® Mix		Untreated reference	
Observations	2 months	4 months	2 months	4 months
First class	88	73	87	67
Core rot (*Botrytis* and *Fusarium*)	3	18	0	14
Other damages	10	10	13	23

Damages in question were mainly caused by unidentified diseases. One month later in February, the results of the evaluation of the apples were parallel to those of the previous month, but this time the differences between apples from treated and untreated trees were even somewhat greater.

Although the visual evaluation of apples demonstrated that *Botrytis* was even more abundant than *Fusarium*, the more accurate agar-plate examination of stored apples showed that at least in this apple material *Fusarium avenaceum* was the main pathogen damaging the core and the surrounding tissues. However, the isolation tests from apples with core rot symptoms showed the presence of *Botrytis* as well.

The storage results of the second-year apple trial (Table 3) were very similar to those of the first year. In December after 2 month storage, the first class yield was consistently higher and the amount of apples in the category ‘other damages’ was lower after the application of Prestop® Mix by entomovectoring. Again, in the later quality evaluation (after 4 month storage period in February) even better control efficacy was observed. In all, the differences were nevertheless smaller compared to those of the previous year, obviously owing to a lower disease pressure in the dry summer 2014.

Pathogen isolations from infected apples in the category ‘other damages’ demonstrated the presence of *Gloeosporium*, *Monilia* and *Penicillium*, which are also causal organisms for storage diseases. The proportion of apples in this category was reduced when Prestop® Mix was used, which means that the biofungicide vectored by honeybees controls or at least suppresses several storage diseases on apple (Fig. 5).

Fig. 5 Apples infected by various storage diseases. Damaged apples from the 1st trial after 4 month storing

5 Discussion

The entomovectoring technique combining Prestop® Mix and honeybees has been commercially used for the control of grey mould (*Botrytis cinerea*) on strawberry and raspberry already several years in Finland. Moreover, the method is accepted in Belgium, Denmark, Estonia and Sweden for berry crops. In this case study the biofungicide vectored by honeybees was tested on apple. Field and storage trials during 2013–2015 in a commercial orchard in Finland demonstrated that this precision control method works also on apple against core rot disease caused by *Fusarium avenaceum* and *B. cinerea*. Besides, there was a clear indication that the biofungicide simultaneously controls or at least suppresses also certain other storage diseases of apple – not only core rot.

Field and storage trials in practical circumstances showed that Prestop® Mix can be effectively transmitted to apple flowers by honeybees and thereby it controls diseases that initiate already at the time of flowering. Based on the 2 year experimental work in Finland TUKES (the Finnish Safety and Chemicals Agency) accepted the Minor Use application of this new biological control method for the management of core rot in 2015. One year later (2016) bee-assisted Prestop® Mix was accepted also in Sweden (UPMA Minor Use) for the control of fungal diseases on apple and other fruit trees (pear, cherry and plum).

The opinion of the apple grower, who was responsible for the test application treatments, found it easy to dose the Prestop® Mix powder onto the inoculum tray of the dispenser attached to the bee hive. It is also important to notice that the biocontrol method worked well although the test farm uses conventional methods, which

means that the normal chemical pesticide program was applied on the experimental area, too. Therefore, it can be indirectly concluded that the *G. catenulatum* fungus, the active ingredient of Prestop® Mix, has not been affected harmfully by chemical substances. Also earlier tests in the laboratory of Verdera Oy showed a fairly good compatibility between *G. catenulatum* and numerous chemical pesticides. Accordingly, this biological control is compatible with chemical treatments and can be used in integrated production as well. Besides, chemical pesticides are usually sprayed early in the morning or late in the evening when bees are inside the hive and not flying and spreading *Gliocladium* (Hokkanen et al. 2015).

Observations of the apple flower analysis supported the good results obtained from the storage trials showing decreased occurrence of core rot. The studies showed that honeybees had successfully carried Prestop® Mix powder to apple flowers and that the *G. catenulatum* fungus is a highly efficious colonizer of the florasphere. The antagonist was detected in all flower organs.

Until now, the efficacy of Prestop® Mix vectored by bees has been proved against grey mould on berry crops and against storage rot on apple, but it is very obvious that this biocontrol method has potential against many other flower-transmitted diseases, too. Thus, the new control system could be applied on various crops that need pollination by bees and suffer from post-harvest diseases infecting through flowers. As an example of such new targets, *Monilia laxa* causing blossom wilt and brown rot on stone fruits can be mentioned.

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Threat of *Drosophila suzukii* as an Invasive Species and the Potential of Entomovectoring

Clauvis N. T. Taning and Guy Smagghe

1 Introduction

Drosophila suzukii Matsumura (Diptera: Drosophilidae), also commonly referred to as spotted wing drosophila (SWD) and native to Southeast Asia (Kanzawa 1939; Tan et al. 1949), is a polyphagous invasive pest in America and Europe (Lee et al. 2011; Kinjo et al. 2014; Deprá et al. 2014). From its early detection in 2008, in California (USA), Spain and Italy (Europe), *D. suzukii* has rapidly spread through these two continents with the aid of global trading and absence of niche competitors (Hauser 2011; Calabria et al. 2012; Cini et al. 2012; Rota-Stabelli et al. 2013; Cini et al. 2014; Wiman et al. 2014; Asplen et al. 2015). Contrary to other closely related *Drosophila* species that would preferentially infest over-ripened and damaged fruits, and thus are not considered serious pests (Lee et al. 2011), *D. suzukii* has the ability to bore holes into the skin of maturing and undamaged healthy fruits using its serrated ovipositor and oviposits into them. The oviposition wounds caused by *D. suzukii* flies very often provide access points to other insects and undesirable secondary infections by pathogens, including fungi, yeasts and bacteria, hence, causing additional losses (Hamby et al. 2012; Ioriatti et al. 2015). All these together make *D. suzukii* a pest of great concern to maturing and ripening fruits (Mitsui et al. 2006; Calabria et al. 2012). A wide range of different soft and stone fruits including strawberry, raspberry, plums, blueberry and grapes are potential targets under *D. suzukii*'s damage range (Dreves et al. 2009; Cini et al. 2012; Bellamy et al. 2013). The damage caused by *D. suzukii* has been reported to reach up to 80% crop loss (Dreves et al. 2009; Walsh et al. 2011; Goodhue et al. 2011). Furthermore, the management of *D. suzukii* is primarily challenging because the fly can continuously

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infest various fruits available throughout the year (Lee et al. 2011), it can survive in a wide range of different climatic conditions in which their natural predators can sometimes not keep up (Chabert et al. 2012) and it also has a very short generation time (Kanzawa 1939; Lee et al. 2011; Wiman et al. 2014). Limited knowledge on how to effectively control this pest and the zero tolerance attitude for infested fruit bound for the fresh market or various export markets, has motivated the priority for more research into possible control options for this pest.

Entomovector technology, which utilizes insects as vectors of biological control agents for targeted precision biocontrol towards plant pests and diseases (Hokkanen and Menzler-Hokkanen 2007; Mommaerts and Smagghe 2011; Menzler-Hokkanen et al. 2013), presents an intriguing management option for the control of *D. suzukii* in an integrated pest management (IPM) system. Multiple studies have reported on the success of exploiting both honey bees and bumblebees to vector different entomopathogenic control agents into flowers to control pest insects which either feed on, or inhabit, the flowers (Gross et al. 1994; Butt et al. 1998; Carreck et al. 2007; Albano et al. 2009). However, the success of entomovectoring in the management of *D. suzukii* will be based on mutual and suited interactions between the appropriate components of vector, control agent, formulation and dispenser, and it needs to be safe for the environment and human health.

This chapter presents the threat of the occurrence of *D. suzukii* in Europe, and places this in context to the possible effects that it might have on entomovectoring. Insights into the possibility of exploiting entomovectoring as a management option for the biocontrol of *D. suzukii* are also discussed.

2 Threat of *Drosophila suzukii* to Fruit Production

Contrary to most other Drosophilidae, with the exemption of *D. subpulchrella, D. suzukii* is able to lay eggs in healthy, unwounded fruit and not only on damaged or overripe fruits, thanks to the serrated female ovipositor (Fig. 1) (Sasaki and Sato 1995; Cini et al. 2012; Bellamy et al. 2013). Hence, ripening fruits are preferred over overripe ones (Mitsui et al. 2006).

Although most of the damage caused by *D. suzukii* is largely due to the larvae feeding on fruit flesh, the insertion of its prominent ovipositor into the skin of the fruit can cause physical damage to the fruit. This in turn provides access to secondary infections of pathogens such as, yeasts, filamentous fungi and bacteria, which may cause faster deterioration and further losses (Hamby et al. 2012; Ioriatti et al. 2015) (Fig. 2).

Additional costs associated with the field management of *D. suzukii* are mostly related to increased production costs (monitoring and chemical input costs, increased labour and fruit selection, reduction of the fruit shelf life, storage costs) and to the decrease of foreign market appeal for fruit production from contaminated areas (Goodhue et al. 2011). Nevertheless, the oviposition habit itself is not enough to explain the dramatic impact of *D. suzukii* on fruit production. In the next sections

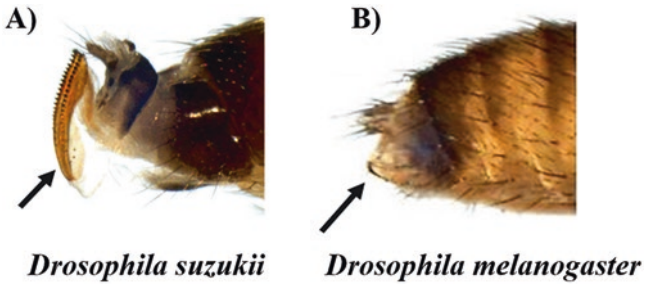

Fig. 1 Fly ovipositor. (**A**) Arrow indicates the serrated hook-like ovipositor of *D. suzukii* used in boring into unwounded ripening fruits on the fields (Photograph by Martin Hauser, California Department of Food and Agriculture). (**B**) Arrow indicates the shorter ovipositor of *D. melanogaster* used in boring into overripe and decaying fruits

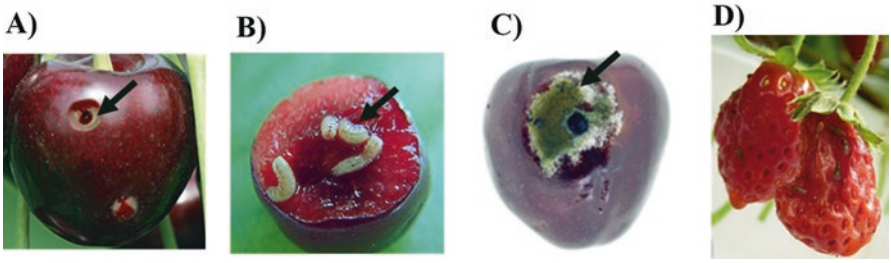

Fig. 2 Indirect and direct damages caused by *D. suzukii*. (**A**) Arrow indicates an oviposition spot created by the serrated hook-like ovipositor of *D. suzukii* on a healthy cherry. (**B**) Arrow indicates larvae feeding inside a cherry. (**C**) Arrow indicates fungi growing around an oviposition spot (Photograph by Martin Hauser, California Department of Food and Agriculture). (**D**) Deterioration and softening of strawberries following infestation with *D. suzukii*

the main characteristics making *D. suzukii* a threat of high concern for the European fruit production sector are discussed.

2.1 High Fecundity in D. suzukii

Mating in *D. suzukii* optimally occurs from the first days of life and females start to lay eggs already from the second day from emergence. Females are known to typically lay 1–3 eggs per fruit in up to 7–16 fruits per day, depending on the temperature (Kinjo et al. 2014). Since they are capable of ovipositing for 10–59 days, they can lay up to a total of 600 eggs during their lifetime (around 400 eggs on average). The eggs hatch within 2–72 h after being laid inside the fruits, and larvae mature (inside the fruit) in 3–13 days. *D. suzukii* pupae reside for 3–15 days either inside or less frequently outside the fruit. Depending on the temperature, a minimum of 10 days is required from the time the egg is oviposited to adult emergence. This very

short generation time exhibited by *D. suzukii* has a huge impact on fruit production. It implies that *D. suzukii* can complete several generations in a single cropping cycle and up to 7–15 generations in a year, depending on specific climatic conditions, thus allowing an explosive population growth [life-cycle details can be found in Kanzawa (1939); Mitsui et al. (2006); Walsh et al. (2011); Tochen et al. (2014); Wiman et al. (2014)].

2.2 D. suzukii *is Tolerant to a Wide Range of Climatic Conditions*

The ability to survive and reproduce in a wide range of different climatic conditions is obviously a relevant factor for pest insects. Limiting temperatures for *D. suzukii* reproduction have been reported to be between 10 and 32 °C for oviposition and up to 30 °C for male fertility (Sakai et al. 2005). Its development and peak activity is around 20–25 °C (Kanzawa 1939; Tochen et al. 2014). *D. suzukii* can also be described as being both heat tolerant (viable *D. suzukii* populations can resist hot summers in Spain) and cold tolerant (*D. suzukii* is present in cold areas, such as mountain regions in Japan and Alpine areas). Adult *D. suzukii* are particularly tolerant to cold compared to other drosophilids (Sasaki and Sato 1995; Mitsui et al. 2010) and mated females in reproductive diapause have been reported to be the *D. suzukii* stage that overwinters (Kanzawa 1939; Mitsui et al. 2010; Walsh et al. 2011). Whether the observed tolerance is physiological or mediated by behavioral adaptation is still unclear. However, several authors have suggested that *D. suzukii* survival under harsh conditions might be increased by acclimatization (Walsh et al. 2011), altitudinal migration (Mitsui et al. 2010), and/or overwintering in manmade habitats or other sheltered sites (Kimura 2004).

2.3 D. suzukii *has a Broad Host Range*

D. suzukii has a large host range, infesting both cultivated and wild soft-skinned fruits on host plants in both native and invaded areas, with berries being the preferred hosts (Table 1). Despite laboratory tests indicating that *D. suzukii* has a lower oviposition susceptibility and developmental rate on grapes compared to berries and cherry (Lee et al. 2011), reports from observations in vineyards in Northern Italy have clearly indicated that *V. vinifera* can become a field host (particularly with soft skinned varieties being more impacted) (Griffo et al. 2012). This could indicate that *D. suzukii* host preference is highly dependent upon the local abundance of hosts. *D. suzukii* can also be flexible with its host choice. This is demonstrated by its ability to develop on tomato under controlled laboratory conditions. However, tomato has not been so far recorded as its host in the field, even though *D. suzukii* adults have

Table 1 List of *D. suzukii* host plants grouped based on botanical family

Family name	Host plants[a]	References
Rosaceae	*Fragaria ananassa* (strawberry), *Rubus idaeus* (raspberry), *Rubus fruticosus*, *Rubus laciniatus*, *Rubus armeniacus* and other *Rubus* species and hybrids of the blackberry group, *Rubus ursinus* (marionberry), *Prunus avium* (sweet cherry), *Prunus armeniaca* (apricot), *Prunus persica* (peach), *Prunus domestica* (plum), *Eriobotrya japonica* (loquat)	Kanzawa (1939); Bolda et al. (2010); Grassi et al. (2011); Seljak (2011); Walsh et al. (2011); Klick et al. (2016); Kenis et al. (2016); Mazzi et al. (2017)
Ericaceae	*Vaccinium* species and hybrids of the blueberry group	Hampton et al. (2014)
Grossulariaceae	*Ribes* species including the cultivated currants	Cini et al. (2012)
Moraceae	*Ficus carica* (fig), *Morus* spp. (mulberry)	Lee et al. (2011); Cini et al. (2012)
Rhamnaceae	*Rhamnus alpina* ssp. fallax, *Rhamnus frangula* (buckthorn)	Asplen et al. (2015); Kenis et al. (2016)
Cornaceae	*Cornus* spp. (dogwood)	Kenis et al. (2016); Pelton et al. (2016)
Actinidiaceae	*Actinidia arguta* (hardy kiwi)	Kinjo et al. (2014)
Ebenaceae	*Diospyros kaki* (persimmon)	Kanzawa (1939)
Myrtaceae	*Eugenia uniflora* (Surinam cherry)	Cini et al. (2012); Lee et al. (2015)
Rutaceae	*Murraya paniculata* (orange jasmine)	Mann et al. (2011); Lee et al. (2015)
Myricaceae	*Myrica rubra* (Chinese bayberry)	Cini et al. (2012); Asplen et al. (2015)
Caprifoliaceae	*Lonicera spp.* (honeysuckle)	Lee et al. (2011); Cini et al. (2012)
Elaeagnaceae	*Elaeagnus* spp. (silverberry or oleaster)	Cini et al. (2012); Kinjo et al. (2013); Asplen et al. (2015),
Adoxaceae	*Sambucus nigra* (black elder)	Lee et al. (2011); Cini et al. (2012); Lee et al. (2015)
Vitaceae	*Vitis vinifera* (common grape vine), *Vitis labrusca* (fox grape)	Cini et al. (2012); Van Timmeren et al. (2013)

[a]Non-exhaustive and tentative host list, since some information is not well documented

been trapped in France in tomato crop fields (EPPO website). In addition to cultivated fruits, many wild, ornamental, and uncultivated plants can serve as potentially important hosts (Lee et al. 2015; Klick et al. 2016).

Despite its relatively recent detection in Europe, *D. suzukii* has already caused severe yield losses in several small fruit crops grown across southern Europe, such as sweet cherries, strawberries, raspberries, blackberries, and blueberries. Extreme damage has been reported for locations in Northern Italy (Trentino) and in France, with up to 100% damage reported on cranberries, strawberries, and sweet cherries (Cini et al. 2012; Warlop et al. 2013). The first evaluation of the economic impact in

Europe was presented by De Ros et al. (2013), although the study only focused on Trento Province, Italy. It was estimated in the study that 400-ha of soft fruit production areas faced losses of around 500,000 € in 2010, and three million € in 2011. Although the level of these economic impacts recorded in Trentino can be ascribed to high levels of blueberry production, this estimate is also somewhat conservative in that it did not consider the costs of control strategies and other societal consequences resulting from increased chemical inputs. In France, *D. suzukii* has also been reported on apples and peaches, although without economically significant damage (Warlop et al. 2013).

The wide host range of *D. suzukii* represents a pest management constraint in many affected regions. This is not only because *D. suzukii* can cause damage to many species, but also because populations can survive almost everywhere, alternating hosts with different ripening times through the year, both cultivated and wild. Crop plants usually cultivated in high density monoculture, allow rapid and impressive population growth, while wild hosts and ornamental plants may serve as refuges from management treatments, and provide later re-infestation sources and overwintering habitats observed (Klick et al. 2016). The ability to damage thick ripening fruits and the wide host range, gives to *D. suzukii* a wide but at the same time specialized ecological niche. Nevertheless, the overlap of niches and the possibility of competition with other drosophilids needs to be investigated.

2.4 D. suzukii *has a High Potential for Dispersal*

The rapid spread of *D. suzukii* in invaded countries and its presence on several continents, as well as remote islands [e.g. Hawaii; Kaneshiro (1983)], confirms its high dispersal potential (Hauser 2011; Calabria et al. 2012). Similar to many other invasive species (Westphal et al. 2008), passive diffusion due to global trade is most likely the main cause of the spread of *D. suzukii*. Before larval activity, the intact and healthy appearance of fruits infested with *D. suzukii* is likely to masked the damage caused to the fruit. This will lead to the risk of infestation remaining undetected and thus an increase in the risk of passive dissemination of *D. suzukii* (Calabria et al. 2012).

3 Rapid Worldwide Spread of *D. suzukii*

D. suzukii was initially described for the first time in 1916, in Japan, where it was reported to attack cherries, however, it is still uncertain whether it is native to this region or was introduced (Kanzawa 1939). The presence of *D. suzukii* has also been reported in the eastern part of China (Peng 1937), Taiwan (Lin et al. 1977), North and South Korea (Chung 1955, Kang and Moon 1968), Pakistan (ud Din et al. 2005), Myanmar (Toda 1991), Thailand (Okada 1976), the Russian Far East

(Sidorenko 1992) and India (Kashmir region, (Parshad and Duggal 1965), where it was described as the *D. suzukii* subspecies *indicus* (Parshad and Paika 1964). *D. suzukii* is currently spreading in many areas, such as the USA (West and East coast), Canada, Brazil (Deprá et al. 2014), Mexico and Europe [a history of the introduction in North America is reviewed by Hauser (2011)]. A key feature of the rapid spread of *D. suzukii* was the initial lack of regulation over the spread of any *Drosophila* species.

D. suzukii is rapidly spreading across Europe (Fig. 3). First reports of its presence in Europe were in autumn 2008 in Spain (Rasquera Province) (Calabria et al. 2012), although a later proposal suggested that southern France was the first propagation center (Cini et al. 2014). Moreover, malaise traps deployed in Tuscany (San Giuliano Terme, Pisa, Italy) in 2008 caught *D. suzukii* adults simultaneously with those deployed in Spain (Raspi et al. 2011). By 2009, in other regions of Spain, (Bellaterra, near Barcelona), France (Montpellier and Maritimes Alpes) and Italy (Trentino) (Grassi et al. 2009; Calabria et al. 2012), *D. suzukii* adults were trapped and recorded. In Trentino, first oviposition on wild hosts (*Vaccinium*, *Fragaria* and *Rubus* spp.) and economically important damage on several cultivated berries species were reported (Grassi et al. 2009; Sarto and Royo 2011). By 2010–2011, the range of *D. suzukii* was further enlarged. In Italy it was reported in several other regions: Piedmont, Aosta Valley, Lombardy, Veneto, Emilia Romagna, Liguria, Marche and Campania (Franchi and Barani 2011; Pansa et al. 2011; Süss and Costanzi 2010; Griffo et al. 2012; Baser et al. 2015; Mazzetto et al. 2015) and in France it was found from Corsica up to Ile de France. Then, many other European countries made their first record: Switzerland (Baroffio and Fischer 2011; Baroffio et al. 2014), Slovenia (Seljak 2011), Croatia (Milek et al. 2011), Portugal (Rota-Stabelli et al. 2013), Austria (Lethmayer 2011), Germany (Vogt et al. 2012; Vogt

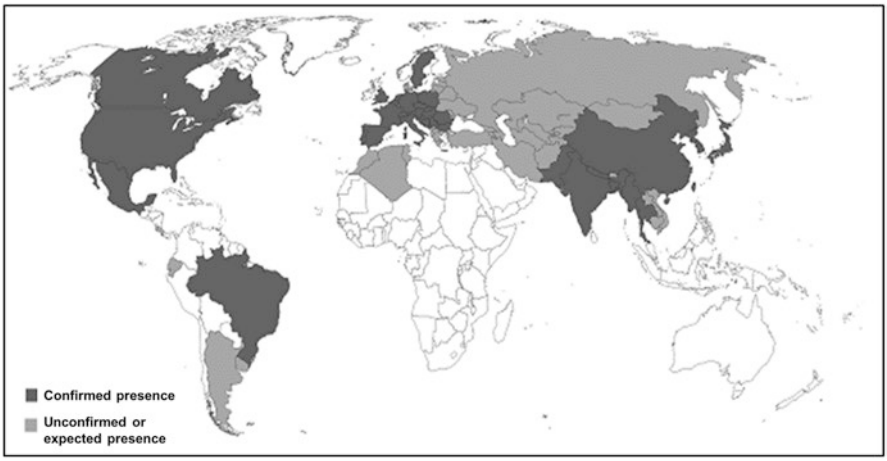

Fig. 3 Current worldwide *D. suzukii* distribution map (Asplen et al. 2015). It is worthwhile to note that the lack of reports from several areas is probably due to a lack of monitoring rather than to an actual absence of *D. suzukii*

2014; Briem et al. 2015), Belgium (Mortelmans et al. 2012; Belien et al. 2013), The Netherlands (Helsen et al. 2013), United Kingdom (EPPO 2012), Hungary (Kiss et al. 2014; Kiss et al. 2016), Poland (Łabanowska and Piotrowski 2015), Greece (Papachristos et al. 2013), Romania (Chireceanu et al. 2015), Bulgaria (EPPO 2015), Serbia (Toševski et al. 2014), Bosnia and Herzegovina (Zovko 2014) and Czech Republic (Brezıková et al. 2014). This reflects the distribution of *D. suzukii* in Europe.

D. suzikii seems to be spreading rapidly and all of continental Europe is at risk for invasion (Fig. 3). It is important to note that the lack of reports from several areas is probably due to a lack of monitoring rather than to an actual absence of *D. suzukii*. Thus, the history of reports might reflect differences in the sampling effort and/or problems of awareness rather than the true distribution of *D. suzukii*. Considering the reports together with the outputs of available degree-day phenological models (Damus 2009; Coop 2010) and analysis of the distribution of *D. suzukii* host plants (EPPO website), it is very likely that *D. suzukii* will spread all over Europe. Ecological simulations have indicated that the northern humid areas are more suitable ecosystems for *D. suzukii* compared to the Mediterranean drier environments, especially because desiccation seems to be a limiting factor for drosophilids (Walsh et al. 2011). Taking the current climate changes into account, even Scandinavian countries cannot be considered out of reach from the risk of *D. suzukii* invasion. On a wider geographic perspective, according to the biology of *D. suzukii*, global expansion in regions with climatic conditions spanning from subtropical to continental is highly likely to happen (Walsh et al. 2011). Furthermore, the occurrence of niche shifts, as was observed for other pests (e.g. *Zaprionus indianus* Gupta, Da Mata et al. 2010), should not be excluded (Calabria et al. 2012), suggesting that *D. suzukii* could become a global problem for fruit production.

4 Potential of Entomovectoring in the Management of *D. suzukii*

The success of entomovectoring in the management of *D. suzukii* will depend on mutual and suited interactions between the appropriate components of vector, control agent, formulation and dispenser, and it needs to be safe for the environment and human health. A typical scenario will be the delivery of the microbial control agent (MCA) to the flowers by the vector (e.g. honey bee or bumble bee), which will in turn lead to the protection of the resulting fruit against *D. suzukii* coming to feed on the ripening fruits (Fig. 4). In this scenario, the MCA has to be able to survive long enough in the flower to the maturation of the fruit and subsequently to the ripening of the fruit. The choice for an MCA which can survive on flower dwelling insect pest prior to fruit maturation could be a good option.

The potential MCA of choice to be used in the control of *D. suzukii* will need to fulfil the criteria as defined for agents against postharvest diseases by Droby et al.

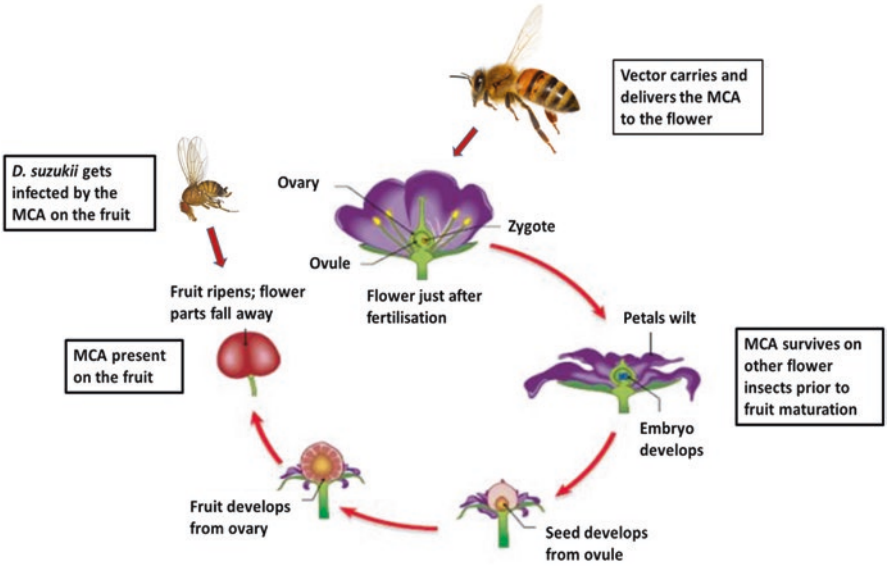

Fig. 4 Illustration of the management of *D. suzukii* through entomovectoring. In this scenario, the vector delivers the MCA to the flower during pollination. The MCA then survives by feeding on other flower dwelling insects until fruit maturation and ripening. *D. suzukii* attacking the fruits are exposed to the MCA, which subsequently leads to mortality in *D. suzukii*

(2009) and Sharma et al. (2009): (a) effective at low concentrations, (b) not fastidious in its nutrient requirements, (c) genetically stable, (d) able to survive adverse environmental conditions, (e) non-pathogenic to the host, (f) resistant to pesticides, (g) preparable in a form that can effectively be stored and disseminated and (h) not detrimental to human health. In addition to these criteria three extra characteristics should be included for a suitable MCA, namely (i) safe for the vector and the crop, (j) effective against aerial and/or foliar plant insect pests, and (k) able to survive and grow under conditions present in the flower.

Metarhizium anisopliae, an entomopathogenic fungus has been observed to infect over 200 insect pest species (Cloyd 1999). *M. anisopliae* and its related species have been tested as biological insecticides against a number of pests such as termites, thrips, pollen beetle, cabbage seedpod weevil, sweet potato weevil and fruit flies (Butt et al. 1998; Carreck et al. 2007; Reddy et al. 2014; Quesada-Moraga et al. 2006; Yousef et al. 2015; Yousef et al. 2017). *M. anisopliae* could be exploited as a possible MCA for the management of *D. suzukii*. *M. anisopliae* does not infect humans or other animals and is therefore considered safe as an insecticide. Vectoring of *M. anisopliae* on oil seed rape and canola has been demonstrated to cause high mortality in some insect pests, including larvae/adults of *Meligethes aeneus* Fabricius (Coleoptera: Nitidulidae) *and Ceutorhynchus assimilis* Dejean (Coleoptera: Curculionidae) (Butt et al. 1998; Carreck et al. 2007). *M. anisopliae* typically causes the diseases known as ‘green muscardine disease’ (due to the green

color of its spores) in insects. When the mitotic (asexual) spores (called conidia) of the fungus come into contact with the body of an insect host, they germinate and the hyphae that emerge penetrate the cuticle. Then, the fungus develops inside the insect body eventually killing it only after a few days. It is very likely that the lethal effect is aided by the production of insecticidal cyclic peptides (destruxins). Most insect species living close to the soil have evolved natural defenses against entomopathogenic fungi such as, *M. anisopliae*. To overcome the insect host defenses, this fungus is locked in an evolutionary battle, which has resulted to a large number of different isolates (or strains) that are adapted to certain groups of insects (Freimoser et al. 2003). This implies that screenings will need to be performed to select isolates with insecticidal activities against *D. suzukii*, prior to any field trials. In a recent study, Yousef et al. (2016) reported on the effectiveness of *Metarhizium brunneum* Petch (Hypocreales: Clavicipitaceae) and its crude extract in the control of *D. suzukii*. The study evaluated the use of two *M. brunneum* strains, EAMa 01/58-Su and EAMb 09/01-Su, and their extracts for the respective development of lure-and-infect and lure-and-kill devices for the control of *D. suzukii* (Fig. 5). The EAMa 01/58-Su strain designed for a lure-and-infect strategy, caused 62.2% mortality in adult *D. suzukii* (survival time of 3.6 days). Furthermore, the evaluation of horizontal transmission and sublethal reproductive effects of the fungal strain showed 48.0% mortality in untreated males after mating with fungus-treated females, whereas only 24.0% of untreated females were killed after mating with treated males, thereby revealing the horizontal transmission potential of the strain. These results show the high potential of using *M. brunneum* as an MCA in entomovectoring, contributing to an IPM program for the control of *D. suzukii*.

Another MCA which could be used in the management of *D. suzukii* is the entomopathogenic fungus, *Beauveria bassiana*. It is known to attack a broad range of insects, acting as a parasite on various arthropod species (McNeiL Jr. 2005; Barbarin et al. 2012). Studies with honey bees vectoring *B. bassiana* GHA in canola showed 22–56% mortality in *Lygus lineolaris* (Palisot de Beauvois) (Hemiptera: Miridae) (Al Mazra'awi et al. 2006). *B. bassiana* causes white muscardine (due to the white

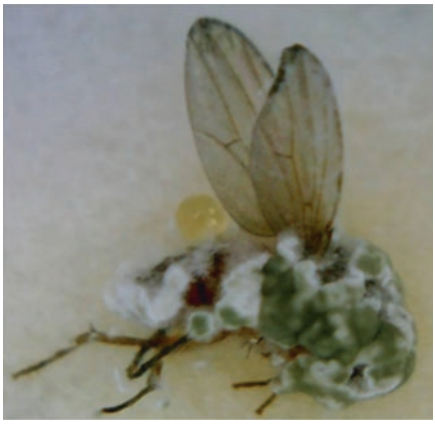

Fig. 5 *D. suzukii* adult with *M. brunneum* EAMa01/58-Su strain fungal outgrowth (from Yousef et al. 2016)

color of its spores) disease in insects, using a similar mechanism as describe for *M. anisopliae*. When the microscopic spores of the fungus come into contact with an insect host body, they germinate, penetrate the cuticle, and grow inside, killing the insect within a matter of days. New spores are then produce from a white mold which emerges from the cadaver. Since various isolates of *B. bassiana* differ in their host range and the factors responsible for host susceptibility are unknown, further research will have to be done to select an appropriate isolate to be used in the management of *D. suzukii.* A preliminary screening of some isolates of *B. bassiana* showed up to 44% mortality in *D. suzukii* (Cuthbertson et al. 2014). Another example of a possible MCA is *Isaria fumosorosea.* Cuthbertson and Audsley (2016) demonstrated the efficacy of *I. fumosorosea* against *D. suzukii* by immersing blueberries in suspensions of these fungi pre- and post-infestation. *I. fumosorosea* caused >40% mortality in adult flies within 7 days of fly contact with the fungi.

Once appropriate MCAs against *D. suzukii* are identified and tested, the next crucial step will be the development of appropriate carriers in which the MCA will be transported by the vector. An appropriate carrier will need to fulfil three criteria (Kevan et al. 2008): (a) No effect on the life span of the MCA. A good example is reported by Hjeljord et al. (2000), where the germination of *Trichoderma* spp. and *B. bassiana* spores were significantly slower when formulated with talc; (b) Safe for the vector. A good example is reported by Israel and Boland (1993), where talc irritated honey bees causing them to groom, whereas with flours as carrier, grooming decreased by 50% (Kevan et al. 2008). Similarly, Pettis et al. (2004) reported that minerals such as talc adversely affected the honey bee brood; (c) Enhance the transport capacity of the vector. In this context, Al-Mazra'awi et al. (2007) showed that direct honey bee load increased with decreasing carrier particle size and moisture content. A start point to the carriers for the management of *D. suzukii* could be adaptations from existing carriers. So far, known carrier substances are corn flour (Shipp et al. 2006), corn meal (Peng et al. 1992), bentonite (Kevan et al. 2008) and polystyrene beads (Butt et al. 1998). Despite the high efficiency of the latter carrier, these beads are prohibitively expensive for commercial formulations, whereas flours and meals have the advantage to be easily available and inexpensive, safe and food grade qualified. These carrier options could be used as basis for the evaluation of identified MCAs against *D. suzukii*, while research continues for the identification of better carriers.

It is evident that success in dissemination and deposition of the MCA is crucial in an entomovector strategy. Therefore it is of paramount importance that the most efficient vector should be selected, and this selection depends on the species, the crop visitation rate by the vector, and the deposition capacity of the MCA by the vector to the target. Honey bees and solitary mason bees are used to vector MCAs onto crops under field conditions. Besides the carrier substance and selection of an appropriate MCA against *D. suzukii*, all of the other components of an entomovectoring system (such as, the selection of the vector, vector safety, transport of MCA, dispenser design and safety of the control agent to the environment and human health) will probably be the same as reported in other cases (Kevan et al. 2008; Mommaerts and Smagghe 2011). These indicate the feasibility for the development

of an entomovectoring system, where bee-mediated dissemination of entomopathogenic MCAs could be exploited to target fruit pests, such as *D. suzukii*, within an IPM system that aims to enhance biological control and minimize insecticide use.

5 Effects of the Occurrence of *D. suzukii* on Entomovectoring

The control of *D. suzukii* populations in the field mainly relies on the use of chemical pesticides (Beers et al. 2011; Bruck et al. 2011; Whitener and Beers 2015; Andreazza et al. 2017), a practice with serious drawbacks such as indiscriminate killing of different insect species (including bees) and its use close to harvest which could lead to a risk of high residues left on fruits. The particular preference of *D. suzukii* for ripening fruit presents timing difficulties with respect to pollinator protection and pre-harvest intervals. This implies that the most effective time for applying chemical controls against *D. suzukii* is when the fruit is ripe or very nearly ripe, necessitating chemicals with a shorter pre-harvest interval. Therefore, growers of bee-pollinated crops may need to remove their bees slightly earlier than optimum to spray late-flowering fruit, before *D. suzukii* infestation, if bee kills are to be minimized.

The fast spread and establishment of *D. suzukii* in Europe will result to an increase in the use of chemical pesticides to manage this invasive pest. Certain pyrethrins and spinosad are among the authorized active materials for *D. suzukii* control (Diepenbrock et al. 2016). Increased pesticide usage to control *D. suzukii* will inevitably lead to an increase in bee mortality. Considering that bees are currently the only actively exploited vectors in the delivery of MCAs in entomovectoring, this will significantly impact efforts in promoting entomovectoring as an alternative to the use of chemical pesticides.

6 Conclusions

The rapid spread of *D. suzukii* poses a challenge to fruit production in Western countries. The biology of *D. suzukii* clearly indicates that an effective control effort requires an area wide IPM program. In order to accomplish this, research needs to address *D. suzukii* basic biology, the development of management tools, the transfer of knowledge and technology to users and, finally, the implementation of the IPM program also at a cultural and societal level. While short term solutions to limit the current dramatic damage are strongly needed, only long-term and environmentally friendly management approaches will allow a sustainable control of this pest. To this aim, research into entomovectoring as a possible biocontrol option, should be carried out to shed light on many knowledge gaps that are still present.

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The Potential of Bee Vectoring on Coffee in Brazil

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1 Introduction

Coffee is already part of people's routine life. Around 2.58 billion cups of coffee are daily consumed (Bacon 2005). Most of the coffee produced in the world comes from smallholding, and it is considered the main source of economic resources for many poor families that lives in rural communities (FAO 2015). Nearly five hundred million people are involved on coffee trade, right from the plantation of coffee to final consumption (DaMatta et al. 2007).

Despite high production and demand from consumers, coffee production around the world is strongly affected by disease and pest attacks. Actually, this is considered as one of the primary factors that lead to coffee yield reduction in the main coffee-producing countries (Oliveira et al. 2014). For instance in Brazil, the world's largest producer of Arabica coffee, annual losses due to pests and diseases are around 0.4 million tons (Oliveira et al. 2014). To compensate losses and to raise agricultural production and productivity, many farmers increase the use of chemical inputs (Wilson and Tisdell 2001). However, this can result in direct and indirect economic losses related to obtaining and using pesticides which can harm the human health and natural enviroment.

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The deleterious effects of pesticides on human and environmental health, including wild pollinators, have been discussed in the scientific literature (Fischer and Moriarty 2011; Janssen 2011), in relation to the development of resistance to major coffee diseases and pests like leaf rust (caused by *Hemileia vastatrix* Berk. and Br) (Silva et al. 2006) and the Coffee Berry Borer, *Hypothenemus hampei* (Ferrari) (Coleoptera: Curculionidae: Scolytinae) (Brun et al. 1989). Henry and Feola (2013), studying pesticide use among smallholder coffee producers in Jamaica, found that the majority of farmers suffer from at least one health symptom associated with pesticide handling, because safety practices were scarcely adopted. According to them, there was also the risk that other household members and the wider local community be exposed to pesticides. Despite that, the cost related to chemical control associated with this type of management makes clear the need of a new concept in agriculture involving a severe reduction in the use of chemical inputs (Nicolopoulou-Stamati et al. 2016), especially to control pests and diseases in coffee agrosystem. The implementation of environmentally friendly practices which are safer for the environment and human health and biodiversity, and capable of increasing crop yields quantity and quality is necessary to ensure long-term food security and profitability for coffee production.

In the early 1990s, a novel sustainable method for pest control using bees as a vector of microbial control agents against agricultural pests and diseases was proposed (Peng et al. 1992). The Bee vectoring Technology (BVT) used managed bees to deliver microbial control agents to plants against plant pathogens and insect pests of crops (Peng et al. 1992; Kevan et al. 2008). Bee vectoring technology has several advantages over spraying, it requires low amounts of inoculum, decrease the need of external inputs, it reduces the cost and labor-intensive and minimizes nontarget organisms exposure (Kevan et al. 2003). This technology combines two complementary ecosystem services, pollination, and pest control, and it might increase the potential for 'win–win' scenarios contribute to increasing crop yields, and ensure environmental safety. The most studies about bee vectoring focus in pest and disease that affects the flowers and leaves, few studies reveal the potential of these approaches in pest and disease that affects the fruits directly. Therefore, based in some studies results and a couple of information we believe that BVT can be a technique that contributes to pollination and at the same time with pest control in coffee crops as demonstrated in other crops. In this chapter, we discuss the potential use of managed bees as vectors of microbial agents to coffee berry borer control and challenges.

2 Bee Vectoring Technology (BVT)

Bee vectoring is a technology that uses managed pollinating bees to disperse beneficial microbial agents to flowering plants for the control of insect pests and suppression of plant diseases (Peng et al. 1992; Kevan et al. 2008). This approach is possible due to the interaction between the following components: the crop, the pest (weed,

disease, or herbivore), the pollinator (vector), the biocontrol agents, the powdery product, the dispensers, and the security for the environment and the human health (Kevan et al. 2008). The vector is the bee species that has a high rate of flower visitation and deposition capacity of the microbial control agent (MCA) on the target crop. The selection of MCA depends on target crop pest or disease, and it must be safe for bees and the environment. In general, the powdery MCA formulations of a commercial product is often used in BVT approach (Mommaerts and Smagghe 2011). The powdery MCA formulations are often mixed with a carrier or diluted to reduce concentration and maximize the contact with MCA and bee bodies (Kevan et al. 2008; Al-Mazra'awi et al. 2007). Designed dispensers fitted in front of the beehives make possible the contact between bees and MCA. Thus, when bees pass through the control agent provided in dispensers fitted in the beehive entrance, they pick up the inoculum of microbial agents control (fungi, bacteria, and viruses) on their bodies and hairs. Then, when bees visit flowers to collect nectar and pollen and during self-grooming on the leaves of plants, they deposit the inoculum powder on the flowers and leaves of the target crops (Kevan et al. 2008).

Some studies report the success of bee vector technology (Carreck et al. 2007; Mommaerts et al. 2010). Hokkanen et al. (2015) conducted a study in five countries on the management of strawberry grey mold caused by *B. cinerea* with the biocontrol fungus, *Gliocladium catenulatum* vectored by honey bees or bumble bees targeting strawberry cultivation in open fields. By the results, under heavy disease pressure bee vectoring provided on average a 47% disease reduction, which was a similar result to multiple fungicide sprays. However, under light disease pressure, biocontrol decreased grey mold by an average of 66%, which was more efficient than fungicide sprays. Other studies found similar results, where the use of bees as vectors of MCA was effective against pest or disease in many crops (Kovach et al. 2000; Maccagnani et al. 2005; Shafir et al. 2006).

3 Coffee Market

Coffee is considered the second most important commodity in the world after oil (Daviron and Ponte 2005). Brazil is the most significant world producer and international trade of coffee, followed by Vietnam and Colombia (FAO 2015). According to the ICO 2016 report, the total consumption of all importing countries was estimated at 104.9 million bags (60 kilograms or 132.276 pounds of coffee). The world consumption in 2015 suggests a steady increase to 152.1 million bags (ICO 2016). The average annual growth rate remains at a healthy 2% over since 2014, highlighted by an increase in consumption in exporting countries. The world's largest consumers are the European Union and the United States, both demanding around 42 and 24.4 million bags, respectively. The European Union shows an average consumption growth of 0.8% per year since 2012, but the USA continues to show an even more significant increase in coffee consumption by an estimated average rate of 3.2 % (ICO 2016).

4 Coffee Botany

All natural *Coffea* species are native to tropical and subtropical Africa. The genus *Coffea* is a member of the family Rubiaceae (Davis et al. 2006). Three species of *Coffea* are most commercially traded, *Coffea arabica*, *Coffea canephora* (commonly known as "*robusta*" coffee), *Coffea liberica* (liberica) and var. *dewevrei* (excelsa) (Davis et al. 2006, Ngo et al. 2011; FAO 2015). *Coffea arabica* is responsible for approximately 60% of the global coffee production, while the other 40% *Coffea canephora* (FAO 2015). *Coffea liberica* and other forms represent an irrelevant proportion of the entire global production (Donald 2004).

The *C. arabica* species is native to southwestern Ethiopia. Production is successful at elevation ranging of 900–1500 m (Davis et al. 2006). *C. canephora* originated in the lowlands of equatorial Africa where it grows naturally between (50–)250–1500 m (Davis et al. 2006; DaMatta et al. 2007).

Arabica coffee typically presents one main trunk, and Robusta coffee is typically multi-trunked (Vieira 2008). In both species, the trunks develop above the soil and the plant produces horizontal plagiotropic branches, on which blooming and production occur (Fig. 1) (Vieira 2008). The flowers are produced in inflorescences on the axes of plagiotropic branches (Vieira 2008). The flowers of both species are hermaphrodite, and they have five white petals, an elongated corolla tube (Klein et al. 2003b). There are five stamens, two-branched stigma, and an inferior ovary of

Fig. 1 Coffee crop in Chapada Diamantina-Brazil (Photo: Helione Barreira)

two chambers and one ovule per chamber (Klein et al. 2003b). *Coffea arabica* is allotetraploid, self-fertile and this species does not need cross-pollination. On the other hand, Robusta coffee is diploid and self-sterile (requires cross-pollination). The flower opens in the morning and the stigma is already receptive when anthesis occurs (Free 1993; Klein et al. 2003b). After that, the pollen starts shedding (Ngo et al. 2011).

The flowering phenology and the number of plants blooming per year are influenced by precipitation and region's latitude (Vieira 2008). The flowering period is stimulated by first rainfall events in the seasons followed by a dry period, and it may result in more than one bloom (Alvim 1985; Vieira 2008). In Brazil blooming occurs during the spring (e.g., from September to December in the main Chapada Diamantina coffee production areas) (Fig. 2).

The fruit of coffee is an ellipsoid drupe, their size vary with the cultivar or variety planted and cultivation conditions (Vieira 2008). In arabica coffee, ripe fruits are red or yellow (Fig. 3), in robusta plants, more hues occur (Vieira 2008). Robusta less susceptible to attacks by pests and disease, produces more berries, and the quality of the beverage is lower when compared to Arabica. (Willson 1999; DaMatta et al. 2007; Reiger 2006; Ngo et al. 2011).

Fig. 2 The flowering of *C. arabica* in Chapada Diamantina-Brazil (Photo: Acário Cordeiro)

Fig. 3 *Coffea arabica* ripe and green coffee berries in Chapada Diamantina-Brazil (Photo: Catalina Angel)

5 Coffee Pollination

Robusta coffee is self-incompatible, and *C. arabica* is self-fertile and many studies have recorded that wild and managed bees play an important role in pollination of both species (Fig. 4) (Willmer and Stone 1989; Badilla and Ramírez 1991; Raw and Free 1977; Klein et al. 2003a, b; Ricketts 2004; Ngo et al. 2011; Saturni et al. 2016; Nunes 2017; Hipólito et al. 2018). Honey bees and stingless bees are the most abundant flower visitors during mass-flowering (Willmer and Stone 1989; Ngo et al. 2011). Krishnan et al. (2012) have conducted an experiment to compare the contribution of self, wind and insect pollination to fruit set in *C. canephora*. This author's reported that the number of flowers that development in fruits was highest when hand cross-pollinated (44%), followed by open- (insect and wind combined; 33%) and wind- (22.1%) pollination treatments. The flowers from open-pollinated treatments received almost the double of pollen grains than wind-pollinated flowers. The pollination provided by bees increased fruit production by 50% in *C. canephora*. In India, Boreux and collaborators (2013) found that bees contributed significantly to coffee production by increasing the number of berries produced in *C. canephora*. However, this is related to the initial flower number. The visitation by bees can increase berry production by more than 25%. According to Classen et al. (2014), bees contribute significantly increased fruit weight of coffee by an average of 7.4% in *C. arabica*. Bagging experiments conducted by Nunes (2017) with *C. arabica* in Brazil show that the rates of pollen deposition on stigmas and growth of the pollen tube were higher when the flower was visited by *Apis mellifera scutellata* Lepeletier, 1836 than those by spontaneous self-pollination. Thus, a single visit from *A.*

Fig. 4 Honey bee, *Apis mellifera scutellata Lepeletier, 1836*, visiting *Coffea arabica* flower during the blooming (Photo: Helione Barreira)

mellifera contributes to fruit development with weight, height, and width more regular. A recent study on coffee farms of *C. arabica* in Chapada Diamantina shows similar results, coffee flower visitors improved the yield on average 30% (Hipólito et al. 2018). All results reveal the importance of pollination services providing by management and wild bees to increase the yields.

6 Coffee Pests

The natural characteristic perennial coffee plant (*Coffea* spp.) facilitates attacks by some insects and diseases (Barrera 2008). The coffee root, trunk, foliage, and berry are susceptible to attack both in plantation and post-harvesting. In most cases, the pests weaken the plant, reducing yield or affecting the quality of grains (Barrera 2008).

Among the pest that attacks coffee plants, the coffee berry borer (*Hypothenemus hampei* Ferrari (Coleoptera: Curculionidae: Scolytinae) is the worst pest threatening coffee crop throughout the worldwide (Barrera 2008; Vega et al. 2015). *Hypothenemus hampei* originating from Africa now is considered cosmopolitan (Barrera 2008). This beetle causes direct damage to the coffee because attacking berries in all development phases, especially those with more than 20% dry matter (Damon 2000). Fruits attacked show a little hole in its apical portion, located at the center or ring of the berry's ostiole (Barrera 2008; Vega et al. 2015). Usually, injured fruits fall and rot prematurely. All these damages lead to a reduction in yield and affecting bean quality (Barrera 2008). In Brazil, this pest causes annual losses around at US$215–358 million (Oliveira et al. 2013; Infante et al. 2013; Vega et al. 2015).

For some years, the synthetic insecticide Endosulfan ($C_9H_6Cl_6O_3S$) was used in many countries against CBB. The application of the CBB population's levels up to 80% (Aristizábal et al. 2016). Despite the ease of application in the field and insecticidal efficacy, the misuse of Endosulfan resulted in indirect economic losses, leading to social and environmental consequences (Lubick 2010; Infante 2018). Due to

the effects of pesticides on human and environmental health, some countries have banned the use of endosulfan (Janssen 2011). Since Brazil forbade the use of Endosulfan, the infestation levels of CBB have reached alarming levels (Brazil 2015). Alternatively, another insecticides such as pirimiphos-methyl, fenitrothion, chlorpyrifos, and fenthion, have been used with success against the CBB (Bustillo-Pardey 2002). A variety of strategies have been proposed to reduce the infestation levels of CBB (Vega et al. 2015; Infante 2018). Many studies revealed the efficacy of adopting the Integrated Pest Management (IPM) methods to control CBB (Aristizábal 2005; Benavides et al. 2012). Infante (2018) summarized several additional methods.

Among the techniques used in IPM, the biological control with the entomopathogenic fungus *Beauveria bassiana* plays a major role in controlling CBB. This fungus is considered a natural controller of CBB because it is found infecting the *H. hampei* in all coffee plantations where CBB has arrived (Benavides et al. 2012). *Beauveria bassiana* is considered as an environmentally safe bioinsecticide, no deleterious effects on humans and the environment and has a low impact on non-target organisms including CBB natural enemies (Zimmermann 2007; Aristizábal et al. 2016). This fungus attack their host insects usually percutaneously (Zimmermann 2007).

The use of *B. bassiana* for CBB control is carried out through one or more flood applications of large numbers of aerial conidia in dry or liquid formulation (Mascarin and Jaronski 2016). The inundative application is performed using traditional spray methods and recently by autoinoculation traps (Mota et al. 2017). Despite that the autoinoculation trap provided high levels of *H. hampei* mortality in the field, the traps only attract a small amount portion of the insects in the field (Mota et al. 2017; Infante 2018). The efficacy of autoinoculation traps at long-term control of *H. hampei* and the cost-benefit of this strategy need to be investigated.

In inundative applications of *B. bassiana* by spray application, a high concentration of conidia ranging from 1×10^{11} to 1×10^{12} conidia/ha in aqueous suspension has been used (Benavides et al. 2012; Mascarin and Jaronski 2016; Nakai and Lacey 2017). As summarized for Nakai and Lacey (2017) the mortalities rates of CBB by spray application of *B. bassiana* in fields trials ranged from 10% to 90%. A variety of factors influence the sucess of *B. bassiana* against CBB, such as the temperature, altitude, humidity, formulation, application equipment, strain, concentration, virulence, infestation level and location of CBB (inside or outside of fruit) (Mascarin and Jaronski 2016; Nakai and Lacey 2017).

According to Vega et al. (2015) most of the studies about mortality rates of CBB by spray application of *B. bassiana* in fields trials do not include the cost-benefit analysis. In the field, high concentrations of *B. bassiana* are spread by spray application, and this increases the cost of CBB control (Benavides et al. 2012). Besides that, spray applications cause negative impact on conidia viability of the microbial control agent (Nilsson and Gripwall 1999). This method requires ready access and much water throughout the plantation, labor work, and machinery (Vega et al.

2015). Thus, it is essential to develop cost-effective and low impact practices for *B. bassiana* field application on coffee. Below we discuss how the adoption of the Bee Vectoring Technology can improve the efficacy to delivery *B. bassiana* spores against CBB, and improve the initial, maturation and harvest fruit set.

7 Can Pollinators Help to Control Diseases/Pests on Coffee?

The close relationship between coffee and bees has been described above. Among coffee flower visitors, the honeybee is the most frequently reported one in the literature as an important pollinator for *C. arabica* and *C. canephora*. Overall, the terms "increased production", "the most dominant visitor", "the most frequent flower visitor", "the primary pollinators" and "important pollinator" are frequently cited in studies that investigated the role of honey bees to improve coffee yields in many sites around the world (Roubik 2002a, 2002b; Ricketts 2004; Veddeler et al. 2006; Bos et al. 2007; Vergara et al. 2008; Ngo et al. 2011). Raw and Free (1977) reported that coffee brushes caged with honeybees showed higher yields of berries in *C. arabica*. In *C. canephora*, Klein et al. (2003a) and Krishnan et al. 2012 concluded that *Apis* spp., not only *A. mellifera*, are the most common visitors to coffee flowers.

As mentioned above, honeybees visit the coffee crop efficiently. Their interactions with the coffee plant covers one crucial assumption for BVT success: the close vector-plant interactions. Moreover, honeybees have a large foraging range (up to 3 km in radius) facilitating the spreading of biocontrol agents in large areas (Mommaerts and Smagghe 2011; Abou-Shaara 2014). Thus, honeybees have the needed requirements to be employed as a vector for disseminating microbial agents control on coffee crops.

Honey bees have been used in many studies to investigate their ability to disseminate some microbial control agents in both greenhouse conditions and open field cultivation (Peng et al. 1992; Butt et al. 1998; Carreck et al. 2007; Johnson et al. 1993). In the study of Dedej et al. (2004) using honey bees as a vector of the bacterium *Bacillus subtilis* against mummy berry disease incidence in flower infection by *Monilinia vaccinii-corymbosa*, they found that bee-vectored agent Serenade reduced the incidence of mummy berry disease. Combining the results available in the literature on the success of honey bees for coffee pollination and vectoring of other crops we believe that the honey bee has a high potential as a vector of microbial agents control against the pest and disease in coffee.

BVT also requires that microbial control agents need to be safe to pollinator/vector. Regarding coffee pests, the entomopathogenic fungus *Beauveria bassiana* can be used for dissemination by honeybees for coffee berry borer control. Several studies reported the effects of *B. bassiana* on *A. mellifera* (Alves et al. 1996; Al-Mazra'awi et al. 2007; Meikle et al. 2008). These effects are conditioned to conidia concentration of *B. bassiana*, the strain and the types of exposition (Al-Mazra'awi et al. 2007; Potrich et al. 2018). It is necessary to quantify the effects of this fungus on the honeybees before the trial to better understand the optimal

concentration of *B. bassiana* that poses the least for this vector and causes high mortality of CBB.

Some studies demonstrate the efficacy of honeybees to vectoring *B. bassiana* (at rates 1 $\times 10^9$ conidia/g) against some pests. The potential of dissemination of *B. bassiana* by honeybees for control of Tarnished Plant Bug *Lygus lineolaris* (Al-Mazra'awi et al. 2007; Palisot de Beauvois) on canola was investigated by Al-Mazra'awi et al. (2006). The bees effectively vectored the inoculum from the hives to the crop and these results indicated that bees might provide a novel means for applying *B. bassiana* to manage *L. lineolaris* in canola. According to these authors, the benefits are better pollination, reduction in pest pressure of *L. lineolaris*, and reduced reliance on insecticides.

The results mentioned above show the capacity of honey bees to spread the fungus *B. bassiana* to many crops around the world against some pests. These results refer to pests and diseases that attack the flowers. It is surprising that one fundamental question remains unanswered: how bees can help control a pest that has a cryptic life? The process that leads to bee-vectoring *B. bassiana* to infecting the CBB remains unclear. Almost all life cycle of the *H. hampei* occurs inside of the coffee berry which difficult their control (Barrera 2008). In the field, post-harvest fallen berries in the ground are a source of new infestations because they are reservoirs for adult insects and larvae (Castaño et al. 2005; Benavides et al. 2012). Few months after plants are blooming, when conditions are appropriate occurs the massive adult emergence of the old coffee berries (Barrera 2008). Those adults mate with their siblings and fly, repeating the entire cycle (Benavides et al. 2012). According to Cure et al. (1998), control measures need to be carried out between the end of harvesting and the appearance of the first fruits of the early maturation of the crop. Generally, *B. bassiana* is applied when female *H. hampei* are just starting to penetrate the berries at the beginning of the year or in fallen berries on the ground (Damon 2000; Aristizábal et al. 2016). According to Alves (1998), insect vectors are essential to inoculation and infection of others insects that live in sheltered places as CBB, because the former insect is capable of dispersing the fungus across the farm.

Bees spread the inoculum on the flowers and leaves of the crop, maybe in the soil too. A coffee stand has one or more blooming periods, and sometimes the vectorization of the fungus by bees can happen more than once. These repeated applications might increase the natural population of *B. bassiana* in an agrosystem. The adults of CBB are infected by bee-vectored *B. bassiana* through: (1) fallen berries contaminated with bee-vectored biocontrol agent; (2) Other insects visiting the coffee plants and then disseminating *B. bassiana* between host insects; (3) Alternative hosts may be infected and produce spores that also infect the CBB; (4) by wind currents.

Ureña and Chuncho (2008) investigated the ability of honey bees to deliver *B. bassiana* to coffee crops targeting *H. hampei*. Their results show that honey bees vectoring of *B. bassiana* spores can provide a coffee berry protection against berry borer infestation in coffee fields. The inoculum of *B. bassiana* used in the experiment had a concentration at 6.5×10^{10} colony forming units (CFU) per gram. The average percentages of infested berries with *B. bassiana* infection in field trial increased after the fungal dispersion by bees, but the inoculum distribution was not

Fig. 5 The inoculum dispenser (see Peng et al. 1992). The dispenser is loaded with inoculum (*Beauveria bassiana* + Vectorite) and attached to the beehive in field trial in coffee crop (Chapada Diamantina-Brazil) (Photo: Juliana Macedo)

homogeneous. Ureña and Chuncho (2008) also found that when bees vectored the fung, the average number of infested berries with *B. bassiana* infection was 43%, exceeding that provided by spray fungal suspension (23–30%). In some sample plots, when bees vectored the fung, the population of *B. bassiana* increased and reduced the population of coffee berry borer. Moreover, the bees vectored *B. bassiana* spores at a distance up to 200 m from the hives. According to Ureña and Chuncho (2008), an apiary of 4 bee hives can cover 12.5 ha of coffee homogeneously.

In Brazil (Macedo, personal communication, December 25, 2017), some experiments are developing (Fig. 4). Honey bee hives, *A. mellifera scutellata,* were used during the experiment. Dispensers similar to those used by Peng et al. (1992) were used in field trials (Fig. 5). Preliminary results show that honey bees can deliver *B. bassiana* spores to coffee at a distance up to 350 meters from hives (Fig. 6). The low percentage of fungal colony forming units (CFU) of *B. bassiana* were observed on the leaves and flowers sampled in a field trial. The low amount of conidia on coffee flowers and leaves (Fig. 7) might be attributed to rain during the field trial. Overall, the results show that that microbial biocontrol can be vectored at long distances by bees into coffee fields and the dissemination of *B. basssiana* spores by bees during blooming can contribute to the regulation of CBB populations. As a result, rise the CBB control, and increase the fruit set by pollination service, and protect the coffee berries during maturation/ripening yield.

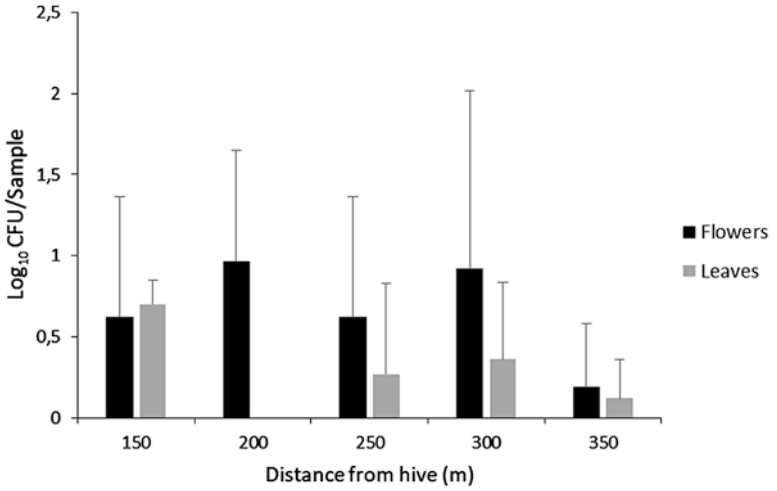

Fig. 6 Graph of the concentration of conidia of *Beauveria bassiana* as $\log_{10}$ CFU/ml of field collected samples of coffee flowers and leaves versus the distance of beehives in fields trials in Brazil

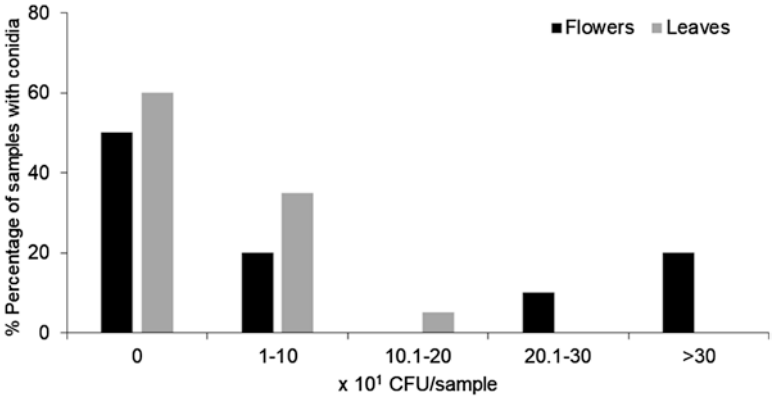

Fig. 7 Frequency of distribution of sampled flowers and leaves with the concentration of conidia of *Beauveria bassiana* in fields trials in Brazil

8 Challenges

Although the evidence compiled in this chapter points out that using BVT on coffee can be considered a suitable management method in pest control, there are many gaps to explore in this field of science in order to better understand the multitrophic relationships (between agent and vectors), and dynamics of this practice.

As mentioned above, the use of bees as a vector of biocontrol agents for crop protection is possible. The bees can disseminate the inoculum to flowers and

leaves, but we need to assess the distribution, deposition of bee-vectored *B. bassiana* in coffee plants a long time. This helps to estimate the persistence and recovery of this fungus in the coffee plants and environment. The development of research is essential to study the efficacy of mixing strains of *B. bassiana* with other agents (e.g. *Metarhizium anisopliae*) for bee-vectoring and their effects on the health of bees. This can help improve CBB control.

Coffee plantations around the world are grown under a wide range of conditions (e.g., shade levels and sun light). These conditions can affect the viability of the control agents used (positively or negatively), as well as the foraging of bees, and this topic need further investigation. In unshaded coffee production, sunlight and warmth affect the post-application persistence of *Beauveria bassiana*. Spore shelf-life and longevity need to be improved to enhance their persistence in the field. The implementation of BVT in different spatial and temporal scales, as well as landscapes effects on the effectiveness of BVT use, and different management techniques, also need to be investigated. The evidence compiled in this chapter points out that BVT can be considered a suitable management method to coffee IPM.

9 Final Considerations

The impacts of intensive agriculture are clear. Thus, a new approach like BVT is necessary. BVT increases pest control efficiency and crop productivity. This approach should be employed as a part of the Integrated Pest Management (IPM) combined with other non-chemical pest control methods for coffee berry borer control. Some years ago, BVT could be considered only as a management perspective, but nowadays BVT is a reliable method for pest control in some crops (apple, strawberry, canola), and has a great potential for coffee plantations.

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Using Bumblebees (*Bombus terrestris*) as Bioagent Vectors to Control Sclerotinia Head Rot on Sunflower in Serbia

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1 Introduction

Sunflower (*Helianthus annuus* L.) is an important agricultural crop grown for vegetable oil, confection, bird seed and for improved seed stock. The estimated global value of the sunflower crop is US$20 billion (Seiler et al. 2017). Sunflower yield can be severely reduced by various pests and diseases against which numerous chemical pesticides are routinely applied. Biological control of pests and diseases of the sunflower head have met with success, but biological control technology has not been widely incorporated into production systems, even for organic/pesticide-free operations or the production of high value-added seeds (e.g. hybrid seeds, cultivar certification).

Sunflower is often labeled as environmental-friendly as a consequence of no irrigation and limited amounts of N fertilizers and pesticides used (Debaeke et al. 2017). Biotic stresses like fungal diseases are among the most important causes preventing the fulfillment of the potential sunflower seed yield, although a relatively small number can cause severe damage to the crop. A commonly occurring necrotrophic fungus *Sclerotinia sclerotiorum* (Lib.) de Bary, is a potential constraint for successful production of sunflower. The fungus is known to cause soil-borne root rot and air-borne rot of upper parts of the sunflower plant. Disease incidence and severity is highly related to weather conditions. Environmental factors are particulary important for the development of the fungal fruiting body and the dissemination of ascospores

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during periods when sunflower is in a critical growth stage. Symptoms are usually in the form of sunken, pale lesions where the fungus forms large melanized sclerotia. These features are useful diagnostic tools for disease detection (Bolton et al. 2006). There are still no efficient control methods against this pathogen. The most effective management integrates several practices. Cultural practices such as crop rotation have limited effect due to the pathogen's large number of hosts, while influencing crop microclimate can be disruptive for *S. sclerotiorum* development (Gulya et al. 1997). Plant resistance to this disease has not been observed. However, sunflower genotypes significantly differ in the level of partial resistance (Škorić et al. 2012). Application of fungicides does not offer complete control, leaving the sunflower white rot problem unsolved and open for further research (Leite 2014).

Nowadays, food production demands more environmentally friendly methods, and biocontrol agents are less harmful to the environment as well as for the health of farmers and plants. Biological pesticides (biopesticides) or biological control agents (bioagents) can be defined as microbial, viral, and fungal organisms, as well as entomophagous nematodes, plant-derived pesticides (botanicals), insect pheromones, and genes used to transform crops to express resistance to insect, fungal, herbicide, and viral attacks (Menn 2003). Bioagents can be transferred to crops by different means, however, in recent decades, entomovectors are gaining popularity. An entomovector is a species of insect, usually a pollinator, which is used to transfer the biological agent from a specific place to its target, mainly plant flowers.

The most commonly used control methods of sunflower diseases that have been used so far are fungicide applications in combination with host resistance to major pathogens. The main biocontrol mechanisms are mycoparasitism and antibiosis, when contact with pathogen occurs (Howel 2003). Another quite effective mechanism is competition for soil nutrients and space without confrontation with the pathogen. There are numerous fungal species in the soil and organic matter that are confirmed antagonists of common soil-borne pathogens. Commercial biocontrol products based on *Trichoderma harzianum* Rifai and *T. viride* Pers. have been tested and registered on sunflower against *Alternaria* sp., *Sclerotinia sclerotiorum, S. minor*, *Macrophomina phaseolina*, *Fusarium* spp., *Pythium* sp., *Rhizoctonia solani*, *Phytopthora* sp. and *Botrytis cinerea* (Whipps and Lumsden 2001; Desai et al. 2002). Aditionally, *Trichoderma* spp., *Coniothyrium minitans*, *Trichothecium roseum* and *Talaromyces flavus* have been reported as potentially effective fungal organisms for biocontrol of cosmopolitan and destructive pathogens such as *S. sclerotiorum* (Huang and Erickson 2008; de Vrije et al. 2001). *Gliocladium virens* and *G. roseum* (syn. *Clonostachys rosea*) have been reported as moderate antagonists for *S. sclerotiorum* (Budge et al. 1995; Jones and Stewart 2000). It is a well-known fact that pollinators can be used as vectors in the translocation and dissemination of bioagents in various crops and agricultural environments. Taking all the mentioned facts into consideration, the biocontrol of *S. sclerotiorum* using *Clonostachys rosea* f. *rosea* (Link) Schroers as a bioagent seems possible with the use of appropriate pollinator vectors.

Sunflower is known as a melliferous plant worldwide, while in Serbia it is one of the most important honeybee pastures. The significance of pollinators for sunflower

seed production greatly increased since the discovery of cytoplasmic male sterility and the first male fertility restorer genes, which allowed sunflower hybrids to replace varieties in production (Terzić et al. 2017). Pollen transfer became necessary for the production of hybrid seed so that more attention was directed to all factors that affect plant-pollinator interactions and pollinator performance.

The first experiments with pollinators used as bioagent vectors commenced at the beginning of the 1990's when Peng et al. (1992) deployed honeybees (*Apis mellifera* L.) to transfer the beneficial fungus *C. rosea* to strawberry flowers against grey mould (*Botrytis cinerea* Pers.). The results of the experiment were promising, and a new branch of entomology has started to develop with the aim of improving the techniques of efficiently transferring the bioagent from its source to its target and selecting the most appropriate insect vectors for each plant species or crop.

Even though practically any insect species can be used as a bioagent vector, flower-visiting and pollen grazing species are preferred for their tendency of visiting flowers. Moreover, social or gregarious insects are favored because of their habits to gather and return to their nesting sites. The most promising species turned out to be social insects like honeybees (Peng et al. 1992; Kovach et al. 2000; Hokkanen et al. 2015), which are already domesticated and used for honey production and pollination, bumblebees, (Kovach et al. 2000; Reeh 2012) which have been commercially used for pollination, especially in greenhouses, and even solitary bees like mason bees (Maccagnani et al. 2006; Maccagnani et al. 2009; Biddinger et al. 2009).

The proper choice of entomovectors is, of course, of crucial importance and it depends not only on the crop on which the biocontrol will be applied but also on the environment, a period of the year and many other factors that could influence the efficacy of the vectoring outcome. It is important to bear into consideration that honeybees' activity is highly dependent on weather conditions and workers do not fly in low temperatures and during rainy days (Mommaerts and Smagghe 2011). Honeybees have nonetheless shown increased aggressiveness when used in confined spaces such as small isolation cages, thus making them less suitable for biovectoring in these environments (Terzić pers.obs.). Studies performed on mason bees are thus far concentrated on their use in orchards (Maccagnani et al. 2009; Biddinger et al. 2009), and there is no information on their possible use in other crops or environments. The tame nature of mason bees (*Osmia bicornis* (L.) and *O. cornuta* (Latr.)) in confined spaces like pollination isolation cages would make them good candidates for bioagents in greenhouses (Franeta and Milovac pers. obs.); however, particular attention should be devoted to the design of dispensers for this pollinator group.

Bumblebees, on the other hand, are often commercially produced for pollination in confined environments like greenhouses and are more adapted to these conditions, especially to the high-temperature variations that can occur in closed environments. However, most trials using bumblebees as bioagent vectors were performed in greenhouses (Mommaerts and Smagghe 2011), so that very little information of their usefulness in open spaces is available.

Pollination by insects, especially by managed pollinators like honeybees (*A. mellifera*), improves sunflower yields in both quantity and quality, even in self-compatible

cultivars (Terzić et al. 2017), while managed pollinators can be used to disseminate biological control agents from their hives to flowering heads where pest and disease control can be achieved (Mommaerts and Smagghe 2011). Therefore, experiments were initiated to test the possibility that the technology could be practical for seed production. The trials consisted of growing sunflowers under field-like conditions, artificially inoculating the blooming heads with ascospores of *Sclerotinia sclerotiorum* while they were being visited and pollinated by managed bumblebees (*Bombus terrestris* (L.)) colonies in domiciles equipped with dispensers containing (or not, for the controls) the anti-head rot biological control agent *Chlonostachys rosea* formulated with diluents/carrier which they delivered to the blooming heads. We hypothesized that the incidence of sunflower head rot (caused by *S. sclerotiorum*) would be reduced by the presence of *C. rosea* delivered by the managed pollinators.

2 Materials and Methods

2.1 Plant Material and Field Design

For this study, the plant material was a commercially available sunflower hybrid "Oskar" created by the Institute of Field and Vegetable Crops, Novi Sad (IFVCNS). It belongs to the group of middle-late maturity sunflower hybrids and has high yielding potential. It is also adaptable to different agro-ecological conditions and possesses a high degree of tolerance to major diseases and parasites in the region, such as stem canker and broomrape.

Seeds were treated with Apron XL fungicide (active ingridient - Metalaxyl-M) as a common seed treatment against downy mildew and hand sown with row spacing of 70 cm and plant spacing of 30 cm. Herbicides Dual Gold 960 EC (Syngenta) (1 l/ha) and Girasolin (2 l/ha) (Agromarket) were applied after sowing and before emergence. No pesticides were sprayed during the flowering time. The final plant population of 47.500 plants/ha (19.200 plants/ac; ha = ac∗2.47) was established by manually thinning the sunflowers at the V3 to V4 growth stage of three to four true leaves (Schneiter and Miller 1981).

2.2 Climatic Conditions

The trial was set up at the IFVCNS trial site in Rimski šančevi near Novi Sad in 2016 and 2017. The plots were not irrigated in 2016 while the precipitation in the flowering stage from July 26th to August 3rd was 9.2 mm, with temperatures reaching 32.7 and 33.4 °C on July 24th and August 1st. The period between two

inoculations was characterized with daily maximum temperatures ranging from 28 to 33 °C and relative air humidity between 69 and 86%.

In 2017, the plots were irrigated three times per week using sprinklers starting from June 13th to August 4th with 25 liters per square meter delivered for 2 h. The precipitation in the period from July 10th to 30th when flowering occurred was 10.6 mm with temperatures reaching 37.8 and 35.4 °C on July 11th and 25th. The period between two inoculations was characterized with daily maximum temperatures ranging from 25 to 28 °C and relative air humidity between 52 and 64%.

2.3 Trial Setup

The trial was setup with two isolation cages placed over the plants in the budding phase for direct comparison of treated versus control plants. Each isolation cage was 12 m long and 4 m wide, with a total area of 48 m^2 containing 5 rows with 40 plants (200 plants per cage). The plants grown out of the isolation cages were used for the evaluation of spontaneous occurrence of white head rot (Fig. 1).

2.4 Disease Establishment – Inoculation Methods

All plants in each cage were inoculated. Inoculations were conducted over multiple days, so that every head was inoculated twice – once at approximately R5.5 (40–60% of the disk flowers blooming or already bloomed) and once at approximately R5.9 (50–90% of the disk flowers blooming or already bloomed). Inoculations were conducted after 7 PM in both years in order to minimize the adverse effects of heat on inoculation success (Table 1).

Spore solutions of *S. sclerotiorum* were prepared by the IFVCNS plant pathology laboratory according to the standard procedure used for plant resistance tests.

Fig. 1 Trial site with sunflower hybrid “Oskar” and isolation cages for bioagent testing

Table 1 Timetable of sunflower development phases corresponding to hive placement, *S. sclerotiorum* inoculation and disease evaluation in 2016 and 2017

Activity	Sunflower development phase[a]	2016	2017
Sowing		5/17	4/27
Hives placed	R5.1 start of flowering	7/21	7/7
1st inoculation	R5.4–5.6 40–50% flowering	7/26	7/14
2nd inoculation	R5.5–5.9 50–90% flowering	8/1	7/18
	R6 end of flowering	8/8	7/25
Fungal presence sampling		8/26	7/31
Disease evaluation 1	R6-R7 ray flowers wilting	8/15	8/7
Disease evaluation 2	R7-R8 grain filling	8/23	8/18
Disease evaluation 3	R9 physiological maturity	9/13	8/30

[a]https://www.sunflowernsa.com/uploads/10/stagesofsunflowerdevelopment.pdf

Fig. 2 Representative sunflower heads for phases of disease assessments

Spore solutions were prepared by adding laboratory-grown ascospores of *S. sclerotiorum* to non-chlorinated water with an addition of one to two drops of Tween® 20. Hand-held spray bottles were calibrated to determine the amount of liquid released through each spray of the bottle, and the spore solution was adjusted so that each squirt of the spray bottle delivered 5000 spores. At each inoculation, 15,000 spores were applied to the front of each head (each head received approximately 30,000 spores over two inoculations).

Head rot disease assessments were made in the field three times: when all plants had reached the R6 growth stage, and none exceeded R7 (end of flowering), when all plants reached the R7 growth stage and none exceeded R8 (seed filling), and when all plants had reached the R9 growth stage (full maturity) (Fig. 2). For each plant, the percent of the head exhibiting *Sclerotinia* head rot was estimated visually. Incidence was determined as a number of infected in comparison to the total number of plants, while the disease severity was determined on a 0–5 scale:

0 = no Sclerotinia head rot,
1 = 1–25% of head exhibiting symptoms of Sclerotinia head rot,
2 = 26–50% of head exhibiting symptoms of Sclerotinia head rot,
3 = 51–75% of head exhibiting symptoms of Sclerotinia head rot,

4 = 76–99% of head exhibiting symptoms of Sclerotinia head rot, and
5 = 100% of head exhibiting Sclerotinia head rot.

2.5 Bumblebee Hive Installation & Monitoring

The managed pollinator vectors were supplied by Koppert biological systems in hives equipped with empty dispensers. A base was built to support hives which were located in the center of the cage at approximately 0.5 m in height. The hives were placed on top of a support base, and a waterproof cover for shade and protection from rain was placed over the hives. They were introduced into the isolation cages (one per cage) at the start of flowering. In 2016, the hives were placed in the isolation cages with no modifications; while in 2017, they were modified in order to facilitate bee orientation towards the exit hole. Rectangles were cut from the cardboard above the entry area of the tray and above the tray towards the exit hole.

Disposable trays (Vectorpak™) with formulated *C. rosea* (BVT-CR7) Vectorite™ were placed into one hive in one isolation cage and into one hive outside the isolation cages, the same day the hives were placed in the field on July 21st 2016 and July 7th 2017 (R4-R5.1). Trays were replaced on July 25th, 28th and August 3rd in 2016 and July 11th and 14th in 2017 (Fig. 3)

Hives were monitored each week for (1) quantity of BVT-CR7 powder in tray, and (2) bee health and activity.

Fig. 3 Vectorpak tray placement and hive position in the isolation cage

2.6 Bee Vectoring Technology (BVT) System

The bumblebee hives were supplied in a BeeVector hive (Koppert Natupol type) without the Vectorpak trays. The hives were placed in the field at the start of flowering. The hive for the treated plants was supplied with BVT-CR7 bioagent containing *C. rosea* spores, while the control hive did not contain the bioagent. The Vectorpak tray with the bioagent was replaced every 3–4 days to ensure sufficient quantity and quality of spores having in mind the variations in air temperature and humidity. The vector trays were weighed before and after each removal to check the quantity of Vectorite +BVT-CR7 powder that was taken out of the trays by the bumblebees.

In 2017, the cartridges weight decreased from 48.3 to 45.1 g after the first change (July 11th) and from 48.2 to 44.8 in the second change (July 14th). At the last check when the plants reached R9 stage of physiological maturity (August 18th), no live bumblebees were present in the hives and the surface of the cartridges was still covered with a thin bioagent layer.

2.7 Evaluation of Fungal Presence on Disk Flowers

Samples were taken during the grain filling stage, 2 weeks after the end of flowering in 2016 and 1 week after flowering in 2017 by extracting five disk flowers from each of the sampled inflorescences. Samples were taken from three inner rows of both treated and control isolation cages. Sampled disk flowers, as well as sampled plants, were equally distanced from each other. Immediately after sampling, the disk flowers were placed on potato dextrose medium (PDA) and incubated for 7 days at room temperature. Microscopic evaluation of fungal presence on disk flowers was done according to Leslie and Summerell (2006) and Watanabe (2010).

2.8 Seed Yield and Quality Traits

Seeds were harvested at physiological maturity (R-9) with four replications per cage in sampling, each consisting of 20 plants bagged at R7 stage. The bagged heads were clipped and thrashed, the seeds were cleaned and quality parameters and yield were measured. Samples were taken from the three center rows, excluding the first and the last plant in a row. Besides yield, seed quality traits were determined including 1000 seed weight, hectoliter seed weight, seed germination after 4 days (germination energy) and after 10 days (percentage of germination). Descriptive statistics were used to summarize the data as inferential statistics were not applicable for the trial in 2017 due to a high frequency of outliers.

3 Results and Discussion

3.1 Bumblebee Activity

Bumblebee activity was monitored for three consecutive days by counting the number of workers exiting or entering both of the available openings. The activity was observed for half an hour on each of the hives in the period between 13 h and 14:30 h. Bumblebees were most active between 06 and 19 h so that the time of observation was selected to be approximately in between that period. Hives were not disturbed before evaluation.

The temperature did not vary considerably during all 3 days of observation in 2016, and was in the range of 24–27 °C. Rainfall was registered on the first day with a total of 0.4 l/m^2, and on the second with 15.3 l/m^2 but in early morning. In 2017, the air temperature was in the range of 30–33 °C. There was no rain during the monitoring period, but the field was irrigated on July 17th, 19th and 21st with 25 l/m^2.

On the first day of evaluation in 2016, several fold higher activity was observed in the hive outside the cages (hive Nr. 3) in comparison to the hive without bioagent in the control cage (hive Nr. 2), and more than 4-fold in comparison to the hive in the cage with bioagent (hive Nr. 1) (Fig. 4). This result strongly suggests that the bioagent does affect the behavior of bumblebees and that if possible, the workers will avoid going through the bioagent powder. That is also confirmed for hive Nr. 2, without bioagent, where the activity was the same on both openings. Lower activity in the hives Nr. 1 and Nr. 2, may be due to the smaller area for flight and foraging, and a small number of plants still in flower in the cages. No insecticides were used in the surrounding area, and the hives seemed to contain colonies of the same size when they were supplied.

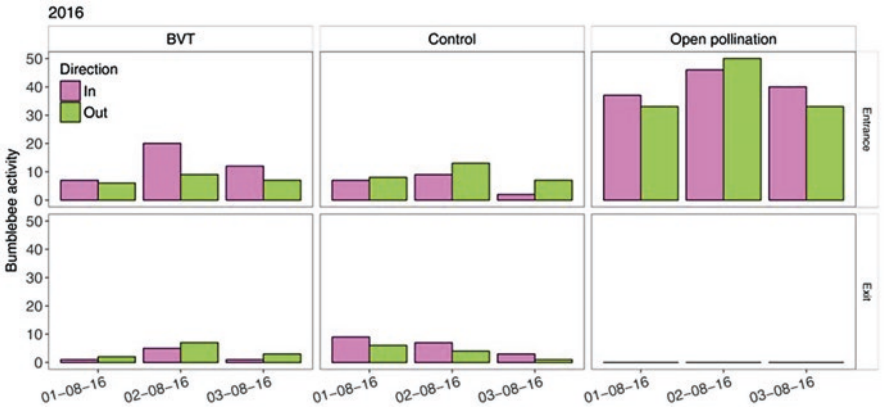

Fig. 4 Bumblebee activity in 2016 on treated (13:30–14 h), control (14–14:30 h) and open pollinated plants (13–13:30 h) given separately for each of the evaluation days as the total counted bumblebees per direction and hive opening

During the second day a similar activity pattern was observed. The bees from hive Nr. 3 again showed the highest activity, but only used the entrance opening. The exit opening was not obstructed, yet it was not used by the foraging workers. Activity in hives Nr. 1 and Nr. 2 was higher than the previous day, but again the movement of bumblebees was not as expected. Most of them used the entrance, avoiding the bioagent, while some even entered through the exit opening in the hive Nr. 1 with bioagent (Fig. 4). While monitoring the activity, on several occasions the bumblebees were seen approaching the hive, but instead of entering they continued foraging. Some of them had visible pollen loads. One was spotted entering through the exit opening in hive Nr. 1 and then turning back and entering through the entrance.

On day three, a decrease in bumblebees' activity was observed. Flowering was almost over, so that the availability of pollen decreased. Bumblebees were again seen approaching the hive Nr. 1 and continued to forage without entering. This behavior was observed seven times during the observation period. Three times bumblebees entered through the exit but returned back and entered through the entrance in hive 1. Activity was again much higher both in and out through the entrances than exits in both hives with bioagent (Fig. 4).

Due to the use of the entrance hole both for getting in and out of the hives in 2016, a flap excluder was placed in the left-hand side hole in 2017 so that bumblebees were forced to exit only using the path over the cartridge (Fig. 5). However, the behavior of bumblebees was different in each of the hives in regard to how much they used the exit hole as an entrance.

On the first day in 2017, the total activity was similar in all three hives (Fig. 6). Due to the flap excluder, no bumblebees exited through the entrance hole. Strict usage of the exit and entrance hole was only registered in hive Nr. 2 in all 3 days of observation. No insecticides were used in the surrounding area and similar to 2016, the hives seemed to contain colonies of the same size when they were supplied.

During the second day, the activity was increased in hives Nr. 2 and Nr. 3 but the flap excluder was difficult for bumblebees to use especially in hive Nr. 1. On several occasions, the bumblebees were seen approaching the entrance, trying to push the flap but eventually entered through the exit hole (Fig. 6). Some bumblebees did not enter the hives but found their way between the cardboard and the plastic hive.

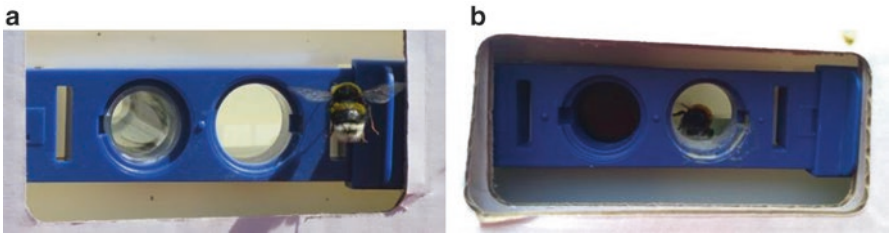

Fig. 5 Opening on the bumblebee hives showing (**a**) entrance without the flap excluder and (**b**) with installed flap excluder

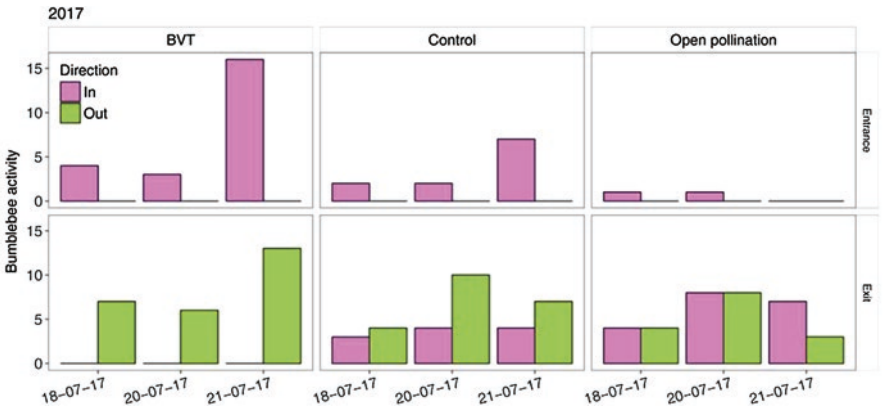

Fig. 6 Bumblebee activity in 2017 on treated (13:30–14 h), control (14–14:30 h) and open pollinated plants (13–13:30 h) given separately for each of the evaluation days as the total counted bumblebees per direction and hive opening

During the 30 min observation, there were on average five bumblebees entering and exiting between the cardboard and the plastic in all hives. Day three showed a decrease in activity except in hive Nr. 2. The workers activity was again higher through the exit hole, except in hive Nr. 2 (Fig. 6).

The choice of adequate pollinator is important as breeding programs also involve growing plants in small isolation cages not suitable for typical honeybee hives so that either hand pollination or other pollinators like bumblebees are used (Terzić et al. 2010). Overall, the pollinator activity in the current trial was significantly lower in 2017 than in 2016, possibly influenced by higher temperatures reaching 33 °C in 2017. Another possible factor is the lack of recommended 2 days acclimatization of bumblebee's to the environment/situation as the hives arrived in both years at the start of flowering and bumblebees immediately started foraging. Efficient bioagent delivery can thus be accomplished with bumblebees as vectors, but with care for timely hive delivery and proper placement, so that pollinator activity is highest at the peak of the target crop flowering.

3.2 Disease Assessments

White head rot was observed in both experimental years and in both control and BVT treated plants. There were no visible white head rot symptoms on the plants out of the isolation cages suggesting absence of *Sclerotinia* ascospores and unfavorable environmental conditions. Disease incidence, as well as severity increased from the first towards the third disease assessment in both 2016 and 2017 (Tables 2 and 3).

Table 2 Incidence and severity of Sclerotinia head rot on sunflower in 2016

Cage 1 BVT-CR7	**Total number of plants**	**Plants with symptoms**	**0 healthy plants**	**1 (<25%)**	**2 (26–50%)**	**3 (51–75%)**	**4 (76–99%)**	**5 (100%)**
8/15	162	7	155	7	0	0	0	0
8/23	162	31	131	29	1	0	1	0
9/13	162	139	23	20	29	37	52	1
Cage 2 control	**Total number of plants**	**Plants with symptoms**	**0 healthy plants**	**1 (<25%)**	**2 (26–50%)**	**3 (51–75%)**	**4 (76–99%)**	**5 (100%)**
8/15	168	19	149	18	1	0	0	0
8/23	168	62	106	58	2	1	1	0
9/13	167	151	16	18	34	39	59	1

Table 3 Incidence and severity of Sclerotinia head rot on sunflower in 2017

Cage 1 BVT-CR7	**Total number of plants**	**Plants with symptoms**	**0 healthy plants**	**1 (<25%)**	**2 (26–50%)**	**3 (51–75%)**	**4 (76–99%)**	**5 (100%)**
8/7	187	0	187	0	0	0	0	0
8/18	187	14	173	10	4	0	0	0
8/30	187	54	133	35	14	2	3	0
Cage 2 control	**Total number of plants**	**Plants with symptoms**	**0 healthy plants**	**1 (<25%)**	**2 (26–50%)**	**3 (51–75%)**	**4 (76–99%)**	**5 (100%)**
8/7	195	0	195	0	0	0	0	0
8/18	195	30	165	25	5	0	0	0
8/30	195	89	106	52	16	16	5	0

Higher disease severity was registered in 2016 due to lower temperatures and higher relative air humidity which favored the development of white rot (Tables 2 and 3). The disease incidence was more than 50% lower in treated plants in the second evaluation during grain filling, but the favorable conditions for white rot increased the incidence in 2016 in the stage of physiological maturity, while in 2017 with lower pathogen pressure, the incidence was lower (from 46 to 29%) (Fig. 7).

The difference in disease incidence was highly significant between successive evaluations. Mean disease incidence was 2, 13 and 57% when the biocontrol agent was present compared to 6, 26 and 68% in the controls.

3.3 Fungal Presence

Floret samples collected at the end of the flowering period confirmed the presence of *C. rosea* in treated plants in 2016 (7.4%) while in 2017 the fungus was not found in the treated plants (Table 4).

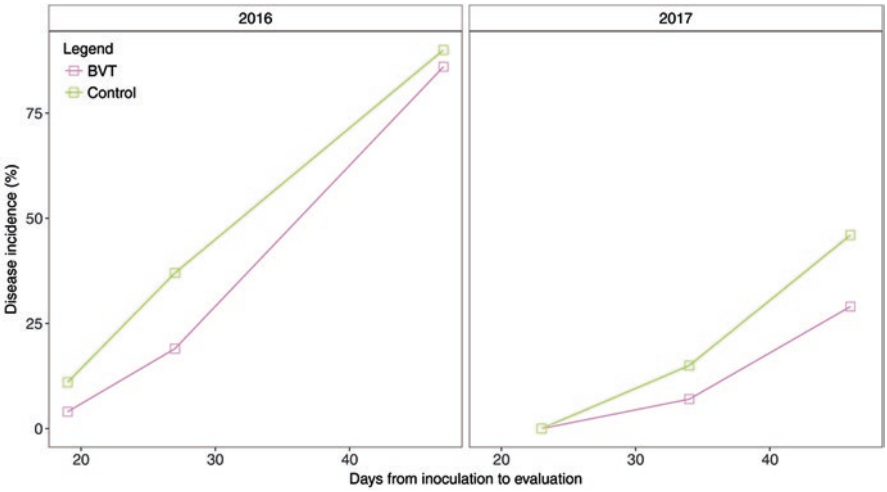

Fig. 7 Incidence of Sclerotinia head rot on BVT-CR7 treated and control sunflower in 2016 and 2017

Table 4 Fungal presence on (a) control and (b) bioagent treated sunflower plants

(a) Control plants					
2016	Identified species/genus	%	2017	Identified species/genus	%
1	*Alternaria* sp.	52.9	1	*Alternaria* sp.	82.7
2	*S. sclerotiorum*	32.4	2	*S. sclerotiorum*	13.5
3	*Fusarium sporotrichioides*	8.8	3	*Epicoccum* sp.	1.9
4	*Penicillium* sp.	4.4			
5	*F. tricinctum*	1.5			
(b) Bioagent treated plants					
2016	Identified species/genus	%	2017	Identified species/genus	%
1	*Alternaria* sp.	50.0	1	*Alternaria* sp.	84.9
2	*S. sclerotiorum*	30.9	2	*S. sclerotiorum*	9.4
3	*Clonostachys rosea*	7.4	3	*Epicoccum* sp.	1.9
4	*F. sporotrichioides*	4.4	4	*F. semitectum*	1.9
5	*Fusarium* sp.	2.9	5	*F. tricinctum*	1.9
6	*Aspergillus* sp.	2.9			
7	*Penicillium* sp.	1.5			

Similar Sclerotinia presence on the florets of sunflower inflorescence in the control and treated plants in 2016 was a result of favorable environmental conditions for the development of this aggressive pathogen. In 2017, 13 days after the second inoculation, the presence of the pathogen was 30% lower than in the control plants, due to both unfavorable conditions for Sclerotinia development including higher air temperature and lower humidity, and *C. rosea* activity.

3.4 Seed Yield and Quality

The results showed that even though the bioagent was not delivered at the intended quantity, due to a technical issue with the excluder tube on the hive, the damage from Sclerotinia head rot in 2016 was significantly lower in the treated plants (Table 5). Sclerotinia presence was not significantly lower, but the negative effect on the plant was reduced by the bioagent treatment. The yield of the treated plants was more than 25% higher than in the control (Fig. 8).

The yield of the treated plants was lower in 2017 than in 2016, most likely due to significantly lower pollinator activity in both isolation cages (Figs. 4 and 6). Even though the infection was less present in the treated cage (Tables 2 and 3), it did not result with increased yield (Table 5) which may be explained by a lower number of active bumblebee workers. Except for 1000 seeds weight in 2017, all analyzed seed quality traits were improved for the seed produced by treated plants in both years.

4 Conclusions

In countries like Serbia with relatively hot summers and low rainfall, environmental conditions for infection with *S. sclerotiorum* occur relatively rarely during sunflower flowering period. Application of the bioagent is, therefore, more likely in specific growing environments with increased air humidity and lower temperatures. The efficiency of traditional fungicides strongly depends on the proper timing of application and disease pressure. Both vectors and bioagent need to be readily

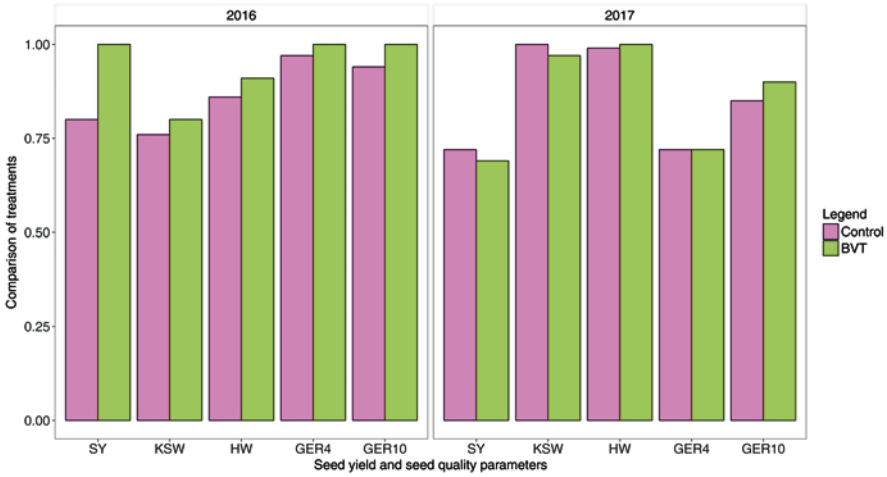

Fig. 8 Sunflower seed yield (SY), 1000 seed weight (KSW), hectoliter seed weight (HW), and germination after 4 (GER4) and 10 days (GER 10) compared between BVT treated and control plants

Table 5 Seed yield from 20 sampled heads and quality parameters of the CR7 treated and control sunflower plants in 2016 and 2017

Treatment	Year	Yield (kg)	1000 seed weight (g)	Hectoliter mass (kg/hl)	Germination energy (%)	Germination rate (%)	Pollinator activity
Treated	2016	1.17	49	41	91	95	80
Control	2016	0.93	47	39	87	89	76
Treated	2017	0.81	60	45	66	85	49
Control	2017	0.84	62	44	65	80	43

available at the site, but with honeybees as most frequent pollinators of sunflower, it may be feasible. The cost of application and willingness of seed producers to accept new technologies also needs to be taken into account especially because of the necessary choice between the application of bioagents and traditional fungicide treatments.

The sampling of florets in the first trial year showed a relatively small presence of *C. rosea* in the total number of isolated fungal colonies, while in the second trial year no *C. rosea* colonies were isolated. The percentage of *C. rosea* colonies may have been higher at the time of flowering, prolonging the incubation time for *S. sclerotiorum,* thus protecting the plant, but such hypothesis should be investigated by monitoring the fungal presence from the start of the bioagent application until harvest. The evaluated method of biocontrol could possibly be further improved by investigating potential trade-offs between biocontrol and pollination services most important in hybrid sunflower seed production.

S. sclerotiorum is one of the most aggressive sunflower pathogens, and still remains a challenge for biocontrol. The results obtained in this trial are promising, as they indicate that bumblebees can be used as efficient vectors of *C. rosea* to sunflower plants for the suppression of sclerotinia head rot. Still, more work is needed in order to improve the efficacy of the bioagent application.

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Advances in the Implementation of Apivectoring Technology in Colombia: Strawberry Case (*Fragaria x ananassa*)

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1 Introduction

In Colombia, a vast strawberry export potential has been detected by the country to keep the offer throughout the year (Casierra and Salamanca 2008). That is why the nation has led sectoral studies, which have allowed to determine the limiting factors for this crop, and standing out the high disease incidence is among those (Asohofrucol 2013). *Botrytis cinerea Pers.:Fr*, cause of the grey mold, is one of the most limiting pathogens in strawberry (*Fragaria x ananassa*), principally due to its great inoculum production capacity as well as its genetic diversity, which allow it to be adapted to different environmental conditions (Fernández-Ortuño et al. 2015). It is complex to calculate the losses since *B. cinerea* affects different production stages, including post-harvest and marketing (Steiger 2007). This pathogen is able to infect a broad range of plant species, and flowers are mainly appealing because of their nutrients.

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For instance, pollen and nectar are full of proteins, sugars, minerals and amino acids. These stimulate the conidia germination as well as increase the fungus growth rates (Huang and Kokko 1999; Ngugi and Scherm 2006; Reich et al. 2015). Thin-walled, waxy cuticle petals are also infected by this pathogenic fungus (Huang and Kokko 1999; Ngugi and Scherm 2006; Gossen and Swartz 2008; Reich et al. 2015).

Disease control has historically been dependent on the application of chemical pesticides. The most common management practices consist of alternating mixtures of fungicides with different modes of action (Fernández-Ortuño et al. 2015). According to the Fungicide Resistance Action Committee (FRAC), the intensive use of chemical fungicides has led to the development of fungicide resistance with the pathogen (Fernández-Ortuño et al. 2015). Moreover, it might cause low crop production and malformations due to a decrease in the germination rate of the pollen (Kovach et al. 2000). This outlook matches with the international demanding tendencies, that revolve safety and traceability requirements around clean production systems.

There is a need of valuing alternate control methods that should lead to international quality standards. It eases the access to high-value markets. The usage of bio-pesticides has the potential to help in counteracting deficiencies, regular failures, and concerns related to control practices. It often involves cultural and sanitation measures, microclimate regulation, and a great dependence on fungicides (Yu and Sutton 1997). Apivectoring Technology leverages that potential, namely the distribution of bio-pesticides as microbiological control agents with the usage of bees as vectors. Its aim leads to disease control and a synergistic effect in fruits output and quality. It is due to a higher pollination rate (Kevan et al. 2003; Mommaerts and Smagghe 2011; Smagghe et al. 2012; Hokkanen et al. 2015). This technology was developed by following a holistic approach in which the interaction between the bio-pesticide organisms, the pathogen to be controlled, the type of crop to be applied, and the interaction between the vector and the crop plants are taken into account. The bio-pesticide concentration carried by each bee and its efficiency to be settled out in the plant, in addition to the safety for human beings, insects and environment, must be kept in mind (Kevan et al. 2003; Mommaerts and Smagghe 2011; Smagghe et al. 2012; Hokkanen et al. 2015).

Just over 40 associated studies, done from the first strawberry research in the 90s, reported an increase between 26–40% in the fresh fruit weight and a disease elimination between 48–64% (Peng et al. 1992). Most studies agree on the Apivectoring Technology implementation and its outstanding pathogen control provide that precautionary approach. It is often concluded that Apivectoring Technology is such an useful tool in the setting of an integrated pest and disease management (IPDM) (Yu and Sutton 1997; Kevan et al. 2003; Al Mazra'awi et al. 2006; Kapongo et al. 2008; Mommaerts and Smagghe 2011; Reeh 2012; Shipp et al. 2012; Smagghe et al. 2012; Smagghe et al. 2013; Hokkanen et al. 2015). On the other hand, it creates a management option to promote product safety, therefore contributing to the health of producers and consumers (Kovach et al. 2000).

For the first time in Colombia, this research evaluated the effect in the control of *B. cinerea* by using honey bees *Apis mellifera* L. to distribute *Trichoderma harzia-*

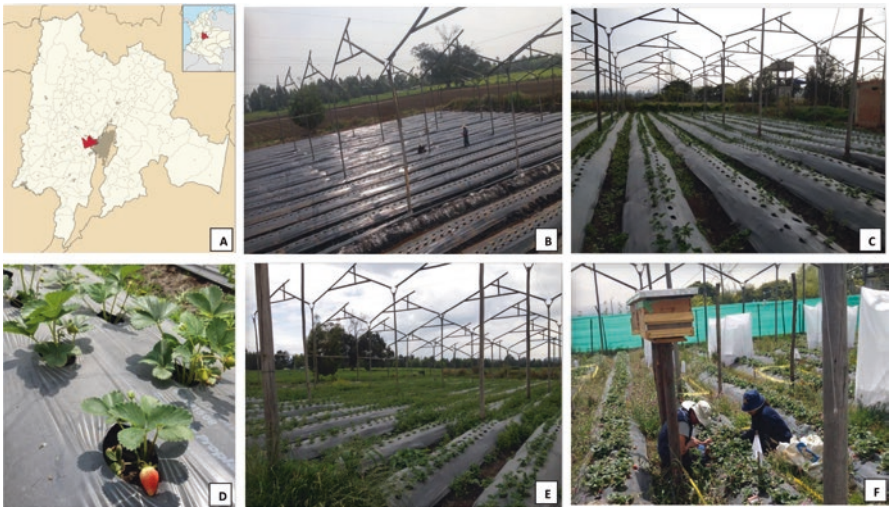

Fig. 1 Location and overview of the experiment at the farm in the municipality of Mosquera located in the Western Savanna Province of Colombia at 2516 meters above sea level and an average temperature of 12–14 °C, and that has a field size of 2500 m^2. (**A**) Geographical location: department of Cundinamarca, Western Savanna province, Mosquera municipality. (**B**) Planting process, January, 2016. (**C**) First flowering, April 2016. (**D** and **E**). Start of fruit production, June 2016. (**F**) Start of the experiment, sampling, hives (*A. mellifera*) with inoculum devices November, 2016

num in an experimental plot of strawberry in the Andean conditions (*Fragaria x ananassa*). Figure 1 provides an overview of the experiments at the farm in Mosquera located in the Western Savanna Province of Colombia at 2516 meters above sea level and an average temperature of 12–14 °C, and that has a field size of 2500 m^2.

1.1 Development and Perspectives of Apivectoring Technology

Apivectoring Technology derives from the early 90s. It can be seen as the use of bees as vectors of a bio-pesticide or biological control agent against a disease and/or pest in a crop. Peng et al. (1992) developed the first research looking for the disease control, the production increase, and the improvement in the strawberry quality because of the cross-pollination effect. Researchers used *Clonostachys rosea* Schroers, (synonym: *Gliocladium roseum*) vectored by *A. mellifera* for the control of *B. cinerea* in strawberry crops (*Fragaria x ananassa* DUCH.). As most important result of this research, there was a 60% elimination of the disease. Several projects have been implemented from then onwards. The development of different inoculum dispenser models and the assessment of different pollinators have become a result, as follows: *A. mellifera* (Johnson et al. 1993; Maccagnani et al. 1999; Bosch and

Vicens 2000; Kovach et al. 2000; Escande et al. 2002; Dedej et al. 2004; Maccagnani et al. 2006; Shafir et al. 2006; Albano et al. 2009), *Bombus impatiens* Cresson (Hymenoptera: Apidae) and *Bombus terrestris* L. (Hymenoptera: Apidae) (Kovach et al. 2000; Maccagnani et al. 2005; Kapongo et al. 2008; Albano et al. 2009; Reeh 2012; Shipp et al. 2012; Smagghe et al. 2013; Karise et al. 2016a), and *Osmia cornuta* Latreille (Hymenoptera: Megachilidae) (Maccagnani et al. 2006; Maccagnani et al. 2009). Likewise, different bio-pesticides have been tested, creating commercial patents and developments (Sutton et al. 1997; Ngugi et al. 2005; Kapongo et al. 2008). Mommaerts et al. (2011) widened the definition of Apivectoring by coining the term Entomovectoring as the combination of techniques using insects as vectors of biological control agents. Its purpose focuses on making a monitoring, precision control for pests and diseases. There are studies known in which some predators are used as vectors (Zhu and Kim 2012). However, the use of bees is more usual. That is why the current document makes reference to the term (Kevan et al. 2003).

When thinking of the different components of Apivectoring Technology, the success for this control method in a specific species is emphasized. It depends on the interaction of 5 relevant factors: (1) Right vector selection, since it determines the effective transport of the bio-pesticide agent inside the crop as it is influenced by the plant-vector interaction, (2) Control potential by the bio-pesticide product, (3) Significance to use a carrier (vehicle) and formulation (carrier + bio-pesticide), since these carriers can improve the load and the vector transport, (4) Dispenser selection with a suitable design for the vector, and (5) Safety for vectors, producers and consumers (Kevan et al. 2003; Kevan et al. 2008; Mommaerts and Smagghe 2011; Smagghe et al. 2012).

This technology has been used in the control of different pathogens, including *B. cinerea*, source for the grey mold in strawberry, raspberry and tomato (Peng et al. 1992; Kovach et al. 2000; Shafir et al. 2006; Kapongo et al. 2008; Albano et al. 2009; Shipp et al. 2012; Hokkanen et al. 2015; Karise et al. 2016a), *Erwinia amylovora,* known as the source of the fire blight in the pear tree (Maccagnani et al. 2005; Maccagnani et al. 2006), *Monilinia vaccinii-corymbosi* that causes blueberries mummification (Dedej et al. 2004), and *Sclerotinia sclerotiorum* in sunflower and alfalfa crops (Escande et al. 2002; Sutton and Kevan 2013). Regarding pests, some reported ones as follows: cabbage looper (*Trichoplusia ni*), sunflower's congregated moth (*Cochylis hospes Walsingham*), bollworm (*Helicoverpa zea*) (Gross et al. 1994), pollen beetle (*Meligethes aeneus*) (Butt et al. 1998), among others. There are also some experiments related to weeds control. The use of *Metschnikovia reukaufii* (Ascomycetes) was tested for the control of *Asclepias syriaca* (Asc. Epiadaceae) (Kevan et al. 1989; Eisikowitch et al. 1990). Table 1 sums up results from published researches about this topic.

Table 1 Summary from published researches on Apivectoring technology

Bio-Pesticide	Pathogen/Pest	Crop	Vector	CFU/Vector	CFU/Flower	% Of injected flowers	Device	Location	References
Clonostachys rosea	*Botrytis cinerea*	Strawberry	*A. mellifera*	$3.6–32 \times 10^4$ $8.8–180 \times 10^4$	$1.5 \times 10^3–2.2 \times 10^4$ $3.0 \times 10^2–2.7 \times 10^4$	NA	One way	Ontario, 1992	Peng et al. (1992)
		Blueberry	*A. mellifera*	$1.3–81 \times 10^4$	$6.9 \times 10^2–1.6 \times 10^3$	NA	One way	Ontario, 1997	Yu and Sutton (1997)
			Bombus	$0.3–128 \times 10^4$	$4.5 \times 10^2–2.4 \times 10^3$	NA			
Pseudomonas fluorescens	*Erwinia amylovora*	Pear + apple	*A. mellifera*	$1.0 \times 10^4–1.0 \times 10^6$	NA	27–41	One way	USA, 1992	Johnson et al. (1993)
Pantoea agglomerans a.k.a. *Erwinia herbicola* + *Enetrobacter agglomerans*				NA	NA	70	One way	New Zealand, 1996, 1998, 1999, 2002	Vanneste (1996; Vanneste et al. (1999); Vanneste et al. (2002)
B. thuringiensis + nuclear Polyhedrosis virus	*Cochylis hospes*	Red clover	*A. mellifera*	NA	NA	NA	Two ways	USA, 1994	Gross et al. (1994)
	Helicoverpa zea								
Trichoderma harzianum	*Botrytis cinerea*	Strawberry	*Bombus*	4.3×10^4	NA	NA	Two ways (Op)	Italy, 1999	Maccagnani et al. (1999)
				4.35×10^2	NA	NA	Two ways (Ssp)		
				4.0×10^5	Average density	65	NA	USA, 2000	Kovach et al. (2000)
			A. mellifera	1.0×10^4	Average density	69	NA		
				$3.9 \times 10^4–1.5 \times 10^5$	NA	NA	Two ways (Triwaks)*	Israel, 2006	Shafir et al. (2006)
				$1.7–3.9 \times 10^3$	26.27 ± 87.99 $1.1 \pm 2.2 \times 10^2$	33–35	Two ways (Houle- Apis)*	Quebec, 2009	Albano et al. (2009)
			Bombus terrestris	$7.1 \times 10^4 \pm 2.1 \times 10^4$	$1.2 \times 10^3 \pm 8.9 \times 10^2$ $1.2 \times 10^2 \pm 1.9 \times 10^2$	75–100	Two ways (Houle-Bombus)*		

(continued)

Table 1 (continued)

Bio-Pesticide	Pathogen/Pest	Crop	Vector	CFU/Vector	CFU/Flower	% Of injected flowers	Device	Location	References
Metarhizium anisopliae	*Meligethes aeneus*	Canola	*A. mellifera*	NA	NA	NA	Two ways	UK 1994, 1998, UK 2007	Butt et al. (1998; Butt, et al. (1994); Carreck et al. (2007)
B. Thuringiensis	*Cochylis hospes*	Sunflower	*A. mellifera*	NA	NA	NA	Two ways	USA, 1999	Jyoti and Brewer (1999)
Trichoderma spp.	*Sclerotinia sclerotiorum*	Sunflower	*A. mellifera*	NA	1.0×10^7–1.0×10^9	NA	Two ways	Argentina, 2002	Escande et al. (2002)
Bacillus subtilis	*Monilinia vaccinii corymbosi.*	Blueberry	*A. mellifera*	5.1–6.4×10^5	$<1.0 \times 10^3$	NA	Two ways	USA, 2004	Dedej et al. (2004)
	Erwinia amylovora	Pear tree	*A. mellifera*	1.0×10^4	1.0–2.0×10^3	NA	Two ways	Italy, 2006	Maccagnani et al. (2006); Maccagnani et al. (2009)
			+ osmium	1.0×10^4–1.0×10^7	2.0×10^3–1.40×10^4	NA			
Coniothyrium minitans + *Trichoderma atroviride*	*Sclerotinia sclerotiorum*	Alfalfa	Alfalfa leaf bees	NA	NA	NA	Handmade inoculation, in petri dishes	Alberta, 2005	Li et al. (2005)
Beauveria bassiana	*Lygus lineolaris*	Canola + sweet pepper	Bombus	2.1–2.3×10^6	1.4–2.1×10^5	64–77 flowers 70–82 leaves	Two ways (hive-mounted)*	Ontario, 2006	Al Mazra'awi et al. (2006)
Metarhizium anispoliae	*Meligethes aeneus* + *Ceutorhynchys assimilis*	Canola	*A. mellifera*	NA	NA	NA	Two ways	UK 2007	Carreck et al. (2007)

Beauveria bassiana +	*Lygus lineolaris, Trialeurodes vaporariorum*	Tomato + sweet pepper	Bombus	$5.5–8.0 \times 10^5$	$1.6–1.0 \times 10^4$ Flowers $7.1 \times 10^3–5.5 \times 10^4$ leaves	76–84 flowers	Two ways	Ontario, 2008	Kapongo et al. (2008); Shipp et al. (2012)
Clonostachys rosea	*Botrytis cinerea*			$2.6–5.0 \times 10^5$	$4.3 \times 10^3–4.8 \times 10^5$ flowers $3.2–6.1 \times 10^3$ leaves	60–82 flowers 90–76 leaves			
Trichoderma atroviride + *Hypocrea parapilulifera*	*Botrytis cinerea*	Strawberry	Bombus	$43.3 \pm 1.6 \times 10^4$	$97.5 \pm 41.3–105 \pm 38$	NA	Two ways	Belgium, 2010	Mommaerts et al. (2010)
Gliocladium catenulatum	*Botrytis cinerea*	Strawberry	*Bombus*	NA	NA	NA	Two ways	Belgium, 2011	Mommaerts, Put, Vandeven and Smagghe (2011)
			Bombus	NA	NA	NA	Two ways (flying doctors)	Germany, 2013	Soboksa et al. (2014) (ISARA and Wageningen)
			A. mellifera				Two ways (Dispensador Triwaks)		
			Bombus and *A. mellifera*	NA	NA	NA	Two ways (Beetreat - A. mellifera)	Finland (2006) Estonia and Italy (2012) Slovenia and Turkey (2013–2014)	Hokkanen et al. (2015)
			Bombus terrestris	NA	12.3	NA	Two ways	Estonia, 2016	Karise et al. (2016a)

(continued)

Table 1 (continued)

Bio-Pesticide	Pathogen/Pest	Crop	Vector	CFU/Vector	CFU/Flower	% Of injected flowers	Device	Location	References
Clonostachys rosea + *Bt*	*S. Sclerotiorum*	Sunflower	*Bombus*	NA	NA	NA	Two ways	Ontario, inédito.	Sutton and Kevan (2013)
Streptomyces griseoviridis + *Gliocladium catenulatum*	*Botrytis cinerea*	Blueberry	*Bombus*	NA	NA	70–100	Two ways	USA, 2011	Smith et al. (2012)
Clonostachys rosea	*Botrytis cinerea*	Blueberry	*Bombus*	$1.0–6.5 \times 10^4$	NA	0–5	*Two ways* (Dispensador de Madera, Koppert, Dispensador PK)*	New Scotland	Reeh et al. (2014); Reeh (2012)

*Name of the device model NA: Not applicable (Information not reported by author).

1.2 Perspectives

Although models implemented have obtained promising results (Hokkanen et al. 2015), it is necessary to improve the system efficiency under adverse environmental conditions. It also needs to develop products with suitable formulations in order to get a better acquisition by the vector, widen the commercially formulated bio-pesticide pool, as well as develop products focused on the simultaneous precautionary control for more than one pathogenic organism or crop pest. It might also be possible to distribute low-risk chemicals.

It is also required to consider the option of using this technology to control pathogens that affect the leaf area. It is known that bees are capable of distributing the bio-pesticide onto leaves as well. For instance, distributed levels in leaves have been quantified in sweet pepper and tomato where 76%–90% of the samples tested had bio-pesticide spores (Al-Mazra'awi et al. 2006; Kapongo et al. 2008). In addition, other important crops have been investigated such as coffee, passion-flowers, blackberry, avocado, citrus fruits, Quito orange, peach tree, melon, watermelon, cucumber, among others.

With over 20,000 bee species worldwide (FAO 2016), a test about other bee species as vector is required. The pollination efficiency for some native species may be higher than the *A. mellifera* or *B. terrestris* ones. It is due to the closest co-evolutionary relationships with the target vegetal species, or simply a better adaptation to specific weather conditions. In Colombia, the use of other bee species different from *A. mellifera* is shown as an important option. It is due to the trend of farmers towards feeling fear of bees after the arrival of the Africanized bee hybrid in the early 80s (Sánchez et al. 2013). The aforementioned bee biotype takes up 98% of the national beekeeping nowadays (Tibatá et al. 2017).

It is also important to define standard protocols, which allow researches in different areas to be comparable. In that regard it is required to make use of techniques that allow doing a more efficient technology test. For instance, strains marked with green fluorescence protein (GFP) might be used in field distribution experiments (Reeh 2012).

Another important fact to be considered in the commercial implementation of this model is the resistance to change on behalf of the producers. Traditional agriculture limits the implementation of alternate technologies due to rooted cultural matters. In most cases these imply the application of products for the control of pests and diseases which are harmful for bees. On the other hand, the productive systems of an organic or clean type are more affordable. In either case Apivectoring Technology must be understood as part of the integrated pests and diseases management of precautionary nature. Expectations that omit technology limitations can be created, causing a counterproductive effect in its adoption by the farmers. Likewise, government support for the technology application must be asked, not only as to resources intended to its research and promotion but also in regard to legislative issues focused on the creation of clear legislations for the products commercialization and application with harmful active components for bees. Moreover, the recog-

nition to farmers who implement these kind of practices in their crops, as seen in the European case where the reform to the European Union Common Agricultural Policy (CAP) makes reference to new statutes regarding Apivectoring Technology, in the "Alternate Crops Protection in the Berries and Fruits Production" section. This was the setting in which conventional farmers promised to replace treatments with chemical pesticides with entomovectoring by receiving 500 €/ha/year, as part of an environmental support (Hokkanen et al. 2015).

In the last 27 years, over 40 publications relating to the proof that Apivectoring Technology shows great benefits not only as a sustainable environmental option but also as economically viable. However, there are also some limitations as well as its application must be framed in an integrated model for the pests and diseases management in a precautionary approach developed with prior knowledge of the conditions for all components to work appropriately. The possibility of unifying the system completely is connected to the variability of each component, which not only requires the knowledge from each one of them but also its different interactions. This would allow necessary adaptations according to specific conditions of the productive system in which the technology is implemented plus increasing its chance for success. That is why some interdisciplinary researches are needed in different topics the technology includes, which finally allows making commercial developments for itself or strengthen the existing ones.

The first case study in Colombia as presented below in which the use of *A. mellifera* as vector of *T. harzianum* for the control of *B. cinerea* was tested in an experimental strawberry crop (*Fragaria x ananassa*), Albion selection. It is located in the municipality of Mosquera, Colombia, at an altitude of 2516 meters above sea level, with an average temperature of 12–14 °C, an average precipitation between 500 and 1000 mm/year in a bimodal pattern (dry period: late December to early March plus July, August, and September). Geographic coordinates: 4° 40′ 9. 34″ North latitude. 74° 15′ 5. 07″ West longitude (Fig. 1).

This study was developed in three stages: (i) Pollinators density associated to strawberry crops was valued, before and after introducing hives in the productive system, (ii) Biocontrol products development and testing for Apivectoring Technology implementation was done, and (iii) Disease influence and its effect on the production was valued in six different agronomic models, as follows:

*1.3 Evaluation of the Foraging Preference of Africanized Bees (***Apis mellifera***) in Commercial Strawberry Crop*

One of the necessary conditions for the Apivectoring Technology operation belongs to bees that visit on the flowers of the farming crop of interest. With the purpose of valuing this condition, two field tests were developed. The first test was based on palynology techniques to establish the bee visits frequency indirectly for the recollection of this resource. The second test evaluated the flower visitor densities through direct inspection by following FAO recommendations.

Test 1. Indirect evaluation of the recollection frequency for the strawberry pollen resource by *A. mellifera* with the use of palynologic techniques.

Ten *A. mellifera* hives were installed inside a strawberry crop for this test (*Fragaria x ananassa*) located in the municipality of Sibaté (Cundinamarca), during April and May 2015. Hives were found in similar development conditions in terms of population. In the same way there were young queen bees in all cases, which were introduced inside the hives 2 months prior to the data recollection. A pollen collector device was installed in each hive from which previously standardized samples were obtained and taken to the laboratory in order to carry out a palynologic analysis. Samples were collected every 3 days and nine samplings were carried out for 90 samples during a 27-days period trial. Through the sampling period, hives were weekly fed with water/sugar syrup in a relation 1:2 in weight. Each hive received 1.5 liters of syrup. Samples' palynologic analysis was carried out with the acetolysis technique, described by Erdtman (1969), which consists of making an acid hydrolysis of the pollen material, where the intine and protoplasm of the pollen grain are degraded; only the outer wall of sporopolenin remains.

Results showed how that the most abundant botanic species were eucalyptus (*Eucalyptus globulus*), turnip (*Brassica* sp.), red clover (*Trifolium pratense*), white clover (*Trifolium repens*), passion fruit (*Passiflora tripartita*) and several Asteraceae (Fig. 2).

The strawberry pollen happened to be an unrepresentative resource of the recollected samples, and no case was among the six resources with higher abundance. In order to calculate the relative importance of the floral resource (in this case, strawberry pollen grains) inside the range of pollen of the 10 *A. mellifera* hives, the quantitative analysis was the tool used through the adaptation of the Importance Value Index (IVI) sensu Amaya et al. (2001). IVI considers the relative frequency of floral

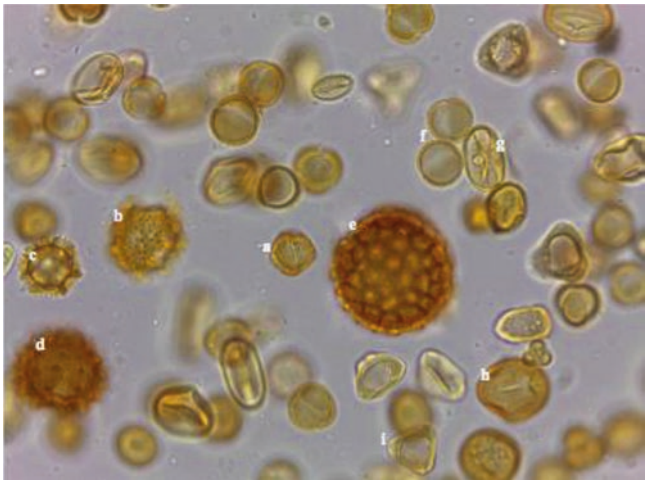

Fig. 2 Different pollen under light microscope. (**a**) *Fragaria x ananassa*, (**b**) *Asteraceae tubiliflorae*, (**c**) *Hypochaeris radicata*, (**d**) Asteraceae, (**e**) Polygonum sp., (**f**) *Brassica* sp., (**g**) *Trifolium repens*, (**h**) *Trifolium pratense*, and (**i**) *Eucalyptus globulus*

use by a species (in this case, a hive), about the total diversity of the species community (in this case, the times the strawberry pollen grain was in different samplings per hive).

According to Danka et al. (1986), the percentage of working bees that had gathered forage at a certain time for a hive of 30,000 individuals (estimation for the 10 hives of the experiment) plus a good nectar availability is around 1.2%. That is why it was considered that 360 working bees from each hive were foraging at a specific time in the strawberry crop (IVI crops were done based on this number; that is why the estimate number of abundance for field bees per hive was standardized in 360).

The modified IVI was calculated as follows:

P_{ix}: it is the frequency of the *Fragaria x ananassa* resource use (count in palynologic samples)/total frequency of the resource used by the hive X (total counts in palynologic samples).

F_{ix}: it is the number of field working bees of hive X that used the *Fragaria x ananassa* resource/total number of individuals in hive X.

M: it is the number of positive palynologic samples to the presence of *Fragaria x ananassa*/total number of samples taken from hive X.

On that basis, modified IVI calculations noted that *Fragaria x ananassa* was slightly more important for hives #9 (0.0605) and #3 (0.0572), while for #1 (0.0018), #2 (0.0036), #7 (0.0024) and #12 (0.0083) it was slightly less important (Table 2).

Working bees from some hives were more constant in their visiting to the strawberry flowers, although its relative importance was smaller (hives #1 and #2). While some others with a smaller persistence showed a low relative importance too (hives #7 and #12). No hives showed that the frequency of pollen grains in the segments placed was stable and continuous (Fig. 3).

During the tested period, results showed that all hives visited strawberry flowers to obtain pollen as food resource. However, the low resource abundance shows this pollen is unattractive and recollects itself occasionally. These results coincide with other studies in which it is concluded that the main motivation for bees to visit the

Table 2 IVI values for each *Apis mellifera* installed in the *Fragaria x ananassa* crop

Hive (code)	*Fragaria x ananassa* (freq.)	P_{ix}	F_{ix}	Use intensity ($P_{ix}F_{ix}$)	Total samples	Samples (+) to *Fragaria x ananassa*	M	IVI
1	28	0.0233	0.0778	0.00181	7	7	1	0.0018
2	37	0.0308	0.1028	0.00317	9	8	0.888	0.0036
3	147	0.1225	0.4083	0.05000	8	7	0.875	0.0572
4	99	0.0825	0.2750	0.02269	7	4	0.571	0.0397
5	102	0.0850	0.2833	0.02408	9	7	0.778	0.0310
6	71	0.0592	0.1972	0.01167	7	6	0.857	0.0136
7	24	0.0200	0.0667	0.00133	9	5	0.556	0.0024
8	107	0.0892	0.2972	0.02650	9	5	0.556	0.0477
9	140	0.1167	0.3889	0.04537	8	6	0.75	0.0605
12	30	0.0250	0.0833	0.00208	8	2	0.25	0.0083

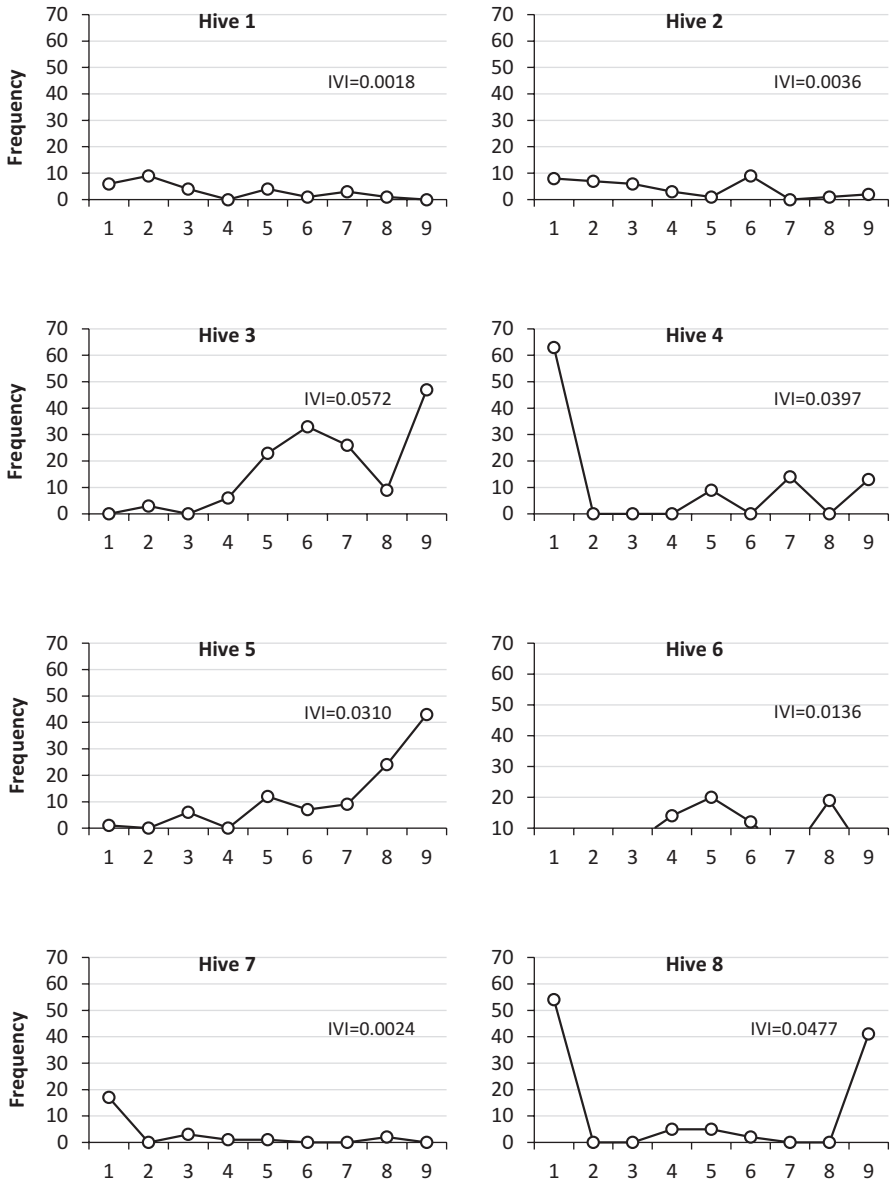

Fig. 3 Appearance frequencies of *Fragaria x anannasa* pollen grains

strawberry flowers is the nectar recollection (Goodman and Oldroyd 1988; Coffey and Breen 1997; Poveda et al. 2018). Therefore, the preference of the bees for the pollen recollection of plant species was part of the usual environment for the strawberry crops in Colombia, as is the case of eucalyptus (*E. globulus*), red clover (*T. pratense*) and white clover (*T. repens*). These three species were also reported as

resources with higher abundance in *A. mellifera* bee honeys, coming from Cundinamarca (Nates et al. 2013). This showed that these are also attractive for the nectar recollection. It must be taken into account since the strawberry crops environment shows more attractive plant species for bees in comparison with strawberry. The effects of the Apivectoring Technology can be drastically reduced on the interest crop, so a higher number of hives will be required or implement method that improve the insects visits frequency to the crop.

1.4 Evaluation of the Use of Apivectoring Technology in a Pilot Strawberry Crop

The evaluation of this technology was performed in open field. The different procedures as performed from 2015 to 2017, are described. The floral visits density was tested. A specialized product was formulated to allow an adequate bio-pesticide distribution. Then the distribution capacity of the vector was determined (flowers, leaves, and fruits), and the effectiveness of the Apivectoring Technology in *B. cinerea* control and its effect on the strawberry crop production were tested.

1.5 Floral Visitors' Density Test

Since the Apivectoring effectiveness is depending directly on the visits performed by the bee to the interest crop, a procedure was done to establish the floral visitors' density in the experimental smallholding before and after hives introduction. A 50 × 25 meters crop area, in which a 45 × 20 meters experimental area was defined. The presence of forests or bees' productive units, other crops zones and weeds areas were characteristic in a 1 km-ratio. The experimental area was divided into subareas at 5, 15, 35 and 45 meters. A transect was done in each one in which 100 flowers in anthesis were tested, and flowering was guaranteed to be over 10%. Tests were performed during 4 days, before and after the hives introduction into the experimental smallholding. The number and type of pollinators and/or floral visitors were registered, inspections were done in five moments throughout the day: 6 am, 9 am, 12 m, 3 pm and 6 pm (adapted from Protocol FAO to Detect and Assess Pollination Deficits in Crops: A Handbook for its Use. Vaissière et al. 2011). For the statistical analysis, the Mann-Whitney test was used in order to compare the appearance frequencies of the floral visitor between both tests before and after bees' introduction.

Surrounding areas characterization to the experimental smallholding showed that 80% of the tested area was grown with vegetables, mainly lettuce (*Lactuca sativa*) and potato (*Solanum tuberosum*). 5% belonged to a weed zone where Kikuyu grass prevailed (*Pennisetum clandestinum*), forage turnip (*Brassica rapa*), sow thistles (*Sonchus arvensis*) and red clover (*T. pratense*), located alongside the experi-

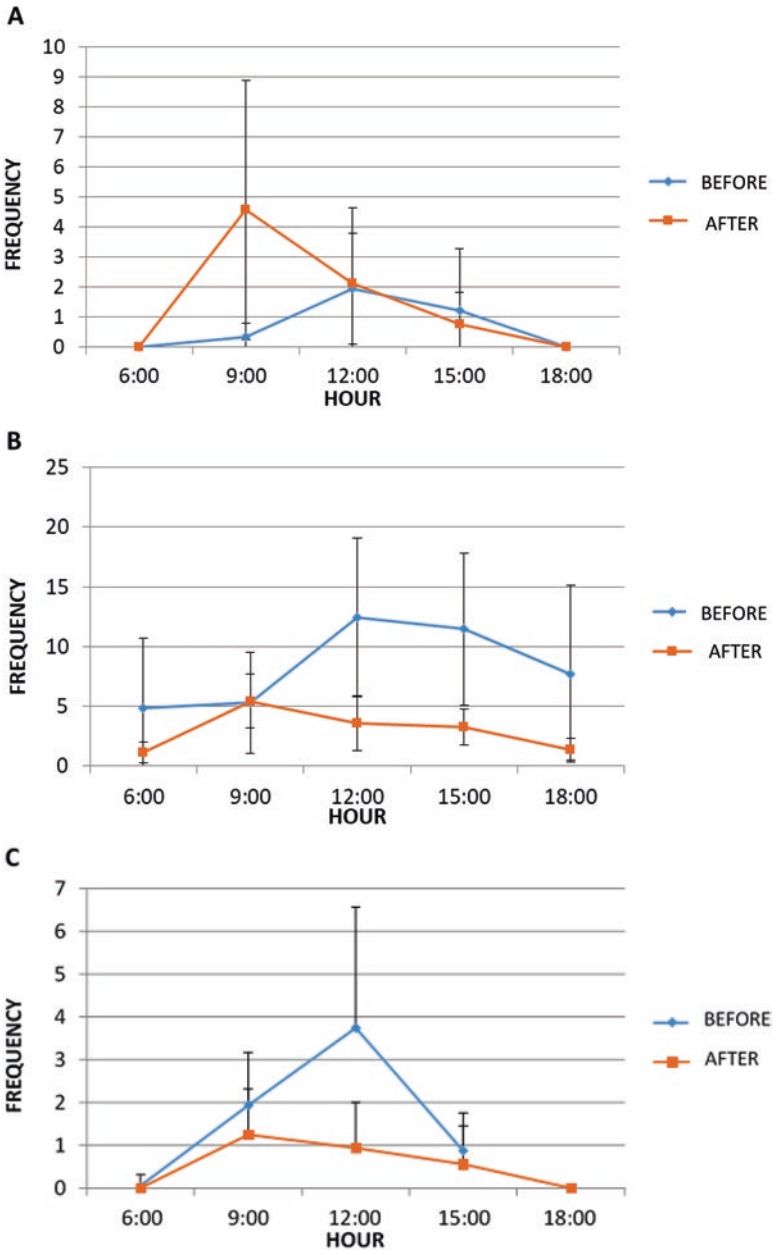

Fig. 4 Floral visitors' population dynamics by transept throughout the day, before and after hives introduction in the experimental smallholding. (**a**) *Apis mellifera*, (**b**) Diptera, (**c**) *Syrphidae*

mental smallholding. Moreover, the smallholding was demarcated by eucalyptus hedges (*Eucalyptus)* occupying 15% of the total area. There was no identification about apiary presence when testing a 1 km around the experimental smallholding.

Floral visitors' population dynamics by transect throughout the day, before and after hives introduction in the experimental smallholding is shown in Fig. 4. The figure shows the average number of individuals per transept observed during 4 days, before and after the hives introduction in the experimental smallholding.

The observation prior to hives introduction showed that Diptera often were the most usual ones with an average of 8.35 ± 6.7 individuals per transect. Although they generally perched on the flowers, there was no evidence of an active pollination process. As a result, all individuals from *Syrphidae* family were counted separately. Although they are Diptera, they showed an important pollinator activity, registering an average of 1.3 ± 1.9 individuals per transect during 4 days of observation. In both cases, their labor was focused between 9 am and 3 pm. Bees showed a great difference with only 0.68 ± 1.44 individuals per transept, only bees from *A. mellifera* species were observed and their busiest period was between 11 am and 3 pm.

When introducing hives into the crop, the frequency of floral visitors showed meaningful changes. Although Diptera kept being the most usual ones with 3.12 ± 2.31 individuals per transect, the number of individuals observed decreased 62.6% approximately ($U = 4134.5$, $p < 0.001$). This is believed to be linked with a higher competence per resources with *A. mellifera*, which population duplicated to 1.65 ± 2.92 individuals per transect, being meaningfully higher than before introducing hives ($U = 5934{,}5$, $p = 0.019$). Therefore, the highest activity period changed between 8 am and 1 pm by having hives inside the colony. That is, the most reachable resource was the strawberry flowers. That is why the foraging activity started earlier and lasted 2 more hours.

Syrphid did not show any significant differences even though they were in a lesser amount, with an average of 0.61 ± 0.94 individuals per transept when being introduced to the hives.

In strawberry crops (*Fragaria x ananassa*), the flower attraction level can change among varieties (Klatt et al. 2013; Klatt 2013), due to a differential quantities production of volatile compounds. It affects the attraction level and determines the visits rate. It also determines the pollination success as well as performance and quality (Dötterl and Vereecken 2010; Karise et al. 2016a, b). In this study the attraction level for the Albión variety over *A. mellifera* was reflected in the amount of individuals per transept, which was 0.68 ± 1.44 before the hives introduction and it increased to 1.65 ± 2.92 individuals per transect after the colonies installation. It was concluded that the Albion variety creates a good attraction level over *A. mellifera*, in contrast to other varieties such as Sonata. Karise et al. (2016a) also tested two strawberry crops, located in the south of Estonia. They counted the number of visits from bumblebees, syrphids, honeybees and Diptera, and reported 0.46 ± 0.04 *A. mellifera* individuals per transect. Overall the attraction level of Sonata variety over the pollinators tested was low, and the introduction of bees in this *B. terrestris* case did not show any meaningful change in fruits weight. That is why it was concluded that this variety does not require any cross pollination.

1.6 Formulation of a Bio-Pesticide Product That Can Be Transported by Bees

Since the Apivectoring Technology is new in Colombia, there is no evidence of validated specific bio-pesticide products. In order to define the most appropriate bio-pesticide product for the development of the study, the active ingredient content of four products was tested (AI): two commercial ones (P1 and P2) and two experimental ones (P3 and P4).

The active ingredient concentration test by product was made through the content quantifying of the colony-forming units (CFU) for *Trichoderma* spp. As gram product, through the serial dilutions method and plate count (Valencia 2010), in modified TBS selective means, made up of glucose 3 g, NH_4NO_3 1 g, KH_2PO_4 0,9 g, $MgSO_4$ 0.2 g, KCl 0.15 g, Rose bengal 0.15 g, chloramphenicol 0.25 g, streptomycin 0.05 g, benomyl 0.5 mg, captan 0.1 g, metalaxyl 0.08 g, Tween 80 at 0.01% ml, agar 18 g and water 1000 ml (Hetong et al. 2008). Pentachloronitrobenzene was not added (PCNB) because it is not in Colombia at a commercial level. This mean it was selected due to its very demanding conditions in terms of resistance to fungicides, ideal characteristic when a bio-pesticide is applied in the field in integrated production models. Crops were incubated at 26 ± 2 °C for 48 hours, then the CFU counting was done, and the test was done in duplicate.

Products composition was as follows: P1: Commercial product based on *T. harzianum* with lactose as carrier. P2: Commercial product based on *T. viride* with lactose as carrier. P3: BVT experimental product based on *T. harzianum* mixed with the Vectorite carrier, provided by Bee Vectoring Technology Company (BVT) (patent pending). P4: Corn flour experimental product based on *T. harzianum* mixed with corn flour used as carrier (Kevan et al. 2003; Mommaerts and Smagghe 2011; Smagghe et al. 2012).

In order to create the experimental products, a *T. harzianum* strain was isolated in a selective-modified TBS mode (Hetong et al. 2008), of a strawberry smallholding located in Sibate, Colombia. Afterwards, its preparation was made through *T. harzianum* spores recollection, in 15-day period crops grown in a selective modified TBS mode (Hetong et al. 2008) that were mixed with the corresponding excipient (P3: Vectorite and P4: Corn flour), until a concentration over 1×10^8 CFU/g of product was obtained. The CFU/g quantified spores of *Trichoderma* spp. In every tested product is shown in Table 3.

Table 3 *Trichoderma* spp. content per product to be distributed by bees

No.	Production	Product	CFU/g
P1	Commercial	*T. harzianum* + lactose	2.7×10^5
P2	Commercial	*T. viride* + lactose	4.8×10^5
P3	Experimental	*T. harzianum* in selective mode + BVT powder	2.0×10^9
P4	Experimental	*T. harzianum* in selective mode + corn flour	2.4×10^9

In tested conditions, products P1 (*T. harzianum*) and P2 (*T. viride* which carrier lactose) were related to commercial products got the lowest values in product CFU/g concentration, with countings lower to 1×10^6 CFU/g. These were under the expected level from the tag (1×10^8 CFU/g). This can be related with the TBS selective means, used for the counting. This means that it is mainly used in soil isolations (Hetong et al. 2008), so it contains several fungicides (benomyl 0.5 mg, captan 0.1 g, metalaxyl 0.08 g) that could have affected the *Trichoderma* spp. spores germination percentage. These findings showed that these products tested in this study require some adjustments to be used with BVT. So some other products are suggested available in the market can also have some limitations.

Experimental products P3 (*T. harzianum* plus Vectorite) and P4 (*T. harzianum* plus Corn flour) with countings 2.0×10^9 CFU/g and 2.4×10^9 CFU/g, respectively, showed the highest values in the test, due to the use of isolated modified TBS *T. harzianum* strain from a commercial strawberry crop. It was usual to have fungicide applications, so that isolation was properly adapted to the conditions of the selective means characterized for its substances content, such as benomyl, captan and metalaxyl. That is why products P3 and P4 reached concentrations over 1×10^8 CFU/g and a high spores viability in moderate toxicity conditions. This eased its field survival under integrated production models (Kevan et al. 2008; Mommaerts and Smagghe 2011; Smagghe et al. 2012).

1.7 Acquisition of Bio-Pesticide by the Vector

Once the appropriate bio-pesticide was obtained to be used within the framework of the Apivectoring Technology and given that the number of visits of *A. mellifera* doubled when introducing the hives in the experimental plot, the acquisition capacity of the vector was evaluated using a two-way device, as described below.

When the crop entered the productive stage (flowering greater than 10%), two hives were located without enclosure in the center of the plot and on a platform 1.5 m high. A period of adaptation of the hives was defined 2 months after their installation in the experimental plot. Then a two-way inoculation device was installed in the entrance of the hives; 2 days later it was verified that the bees recognized entry and exit.

To evaluate the acquisition capacity to the vector of the products added with the carriers lactose (P1 and P2), BVT powder (P3) and corn flour (P4), the acquisition of CFU/bee of the bio-pesticide was determined after passing through the device.

In order to establish the initial conditions of the test, the presently occurring charge of *Trichoderma spp*. was quantified in the vectors. Before loading the device with the bio-pesticide, samples of thirty bees were collected at the exit of each hive in groups of five individuals and the CFU count of *T. harzianum* per bee was made, using the methodologies of serial dilutions and plate count (Valencia 2010) in modified TBS (Hetong et al. 2008).

In order to monitor the carrying capacity of the bees, each device was loaded with 10 grams of the product evaluated as recommended by the BVT and proceeded again to collect thirty individuals per hive and the subsequent count of CFU/bee, which was carried out daily for a period of 5 days. In order to determine the recharge time of the device, the entire procedure was performed in duplicate. For the statistical analysis of the data obtained when carrying out the counts of *T. harzianum* per bee for each product, the non-parametric Friedman test was applied.

To evaluate the acquisition capacity to the vector of the products added with the carriers lactose (P1 and P2), BVT powder (P3) and corn flour (P4), the acquisition of CFU/bee of the bio-pesticide was determined.

When the bees fulfilled the period of adaptation in the experimental smallholding, a Peng two-track device was installed for each hive. It was verified that the bees recognized entry and exit and proceeded to perform the evaluation of acquisition of the bio-pesticide by vector.

In order to establish the initial conditions of the test, the presently occurring loading of *Trichoderma spp.* was quantified on the vectors. Before loading the device with the bio-pesticide, samples of thirty bees were collected at the exit of each hive in groups of five individuals, and the CFU count of *T. harzianum* per bee was made, using the methodologies of serial dilutions and plate count (Valencia 2010) in modified TBS (Hetong et al. 2008).

In order to monitor the carrying capacity of the bees, each device was loaded with 10 grams of the product evaluated as recommended by the BVT and proceeded again to collect thirty individuals per hive and the subsequent count of CFU/bee. It was carried out daily for a period of 5 days, in order to determine the recharge time of the device. The entire procedure was performed in duplicate. For the statistical analysis of the data obtained when carrying out the counts of *T. harzianum* per bee for each product, the non-parametric Friedman test was applied.

At the end of the sampling, the average inoculum quantity with which the vectors are loaded when passing through the device was determined (Kovach et al. 2000) as well as the recharge interval for each product.

The acquisition of the bio-pesticide on the body of the bee is given by the texture and properties of the carrier. In this case the products containing lactose, P1 and P2, had amounts lower than 70 CFU/bee. This fact together the low viability of spores in conditions of moderate toxicity by fungicides (P1 = 2.7×10^5 CFU/g and P2 = 4.8×10^5 CFU/g), evidenced the need to develop commercial products that enable the successful implementation of the Apivectoring Technology at scale in the Colombian fruit and vegetable production sector. There are examples of companies that have developed business models based on Apivectoring Technology, such as BVT (Canada) or Assatek (Finland), successfully scaling up the development of products specifically formulated to be distributed by bees, which we believe could serve as basis for future developments in Colombia too. However, complementary studies are required to make high-quality formulations and ensure control of the target disease.

The products that used Vectorite (P3) and corn flour (P4) as a carrier presented a greater acquisition on the vector with average counts of $1.2 \times 10^4 \pm 1.5 \times 10^4$ and

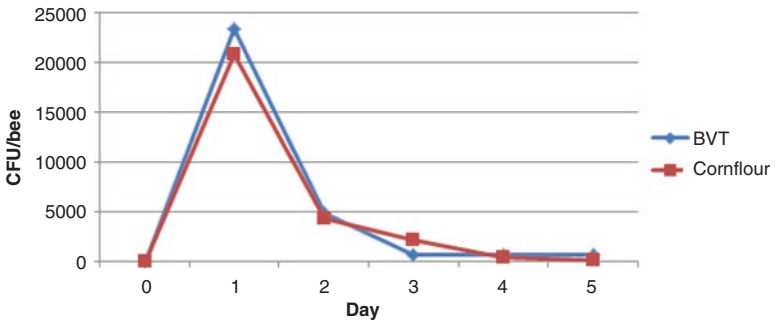

Fig. 5 Monitoring of the loading capacity of the vector with *Trichoderma harzianum* using two products with different carriers (BVT and corn flour) in a two-way device

$1.1 \times 10^4 \pm 1.2 \times 10^4$ CFU/bee, respectively, although the difference between them was not statistically significant (Friedman T2 = 0.36, p = 0.551). The monitoring of the loading level of the products that showed the greatest acquisition to vector P3 (Vectorite) and P4 (corn flour) is shown in Fig. 5.

The vector acquisition values of P3 used as Vectorite carrier (1.2×10^4 CFU/bee) and P4 in which corn flour (1.1×10^4 CFU/bee) was used as a carrier, coincided with the reports from Peng et al. (1992), Maccagnani et al. (2005) Kovach et al. (2000), Albano et al. (2009) and Shafir et al. (2006) who quantified values between 1.7×10^3 and 1.5×10^5 CFU/bee (*A. mellifera*). In addition, it was established for both products that the recharge time was 2 days, which is consistent and ideal, since the minimum required recharge time is 1 day (Mommaerts and Smagghe 2011; Smagghe et al. 2012). This eases the management, since long recharge times reduce labor and avoid excessive manipulation of hives, facilitating the implementation of the technology to commercial level.

It can be concluded that both carriers are suitable for formulations under the Apivectoring Technology. In this case, the product P3 of *T. harzianum* with Vectorite was selected because it presented a slightly higher level of acquisition and when applied in the recharge tray, it was observed that the level of compaction was lower since it did not hydrate as easily as the P4 product that used corn flour as a carrier.

1.8 Bio-Pesticide Distributed in Flowers, Fruits and Leaves

To compare the level of dispersion of the bio-pesticide in strawberry plants using the Apivectoring Technology in different agronomic models, 6 treatments were established in the experimental smallholding, as described below: T1: Absolute control: No chemical or biological product is applied for the control of *B. cinerea*, besides bees are excluded; T2: Commercial Control: synthetic fungicides are applied for the control of *B. cinerea* under a traditional model, besides bees are excluded; T3: Organic Control: products are applied for the control of *B. cinerea* in

a model of ecological agriculture, Apivectoring is included; T4: Integrated Management: Products are applied for the control of *B. cinerea*, in a model of clean agriculture, Apivectoring is included; T5: *T. harzianum* control: Only *T. harzianum* was applied at a concentration of 1×10^8 CFU/g of the product using fumigating pump at a rate of 2 g of product/liters of water, excluding bees, and T6: Apivectoring: Only Apivectoring is implemented. No other product is applied for the control of *B. cinerea*.

To carry out the exclusion of the bees in treatments one, two and five, veil meshes of 3 m × 1 m × 2 m were installed. Ten repetitions were made, for a total of 60 experimental units, in a randomized complete block design. Crop beds corresponded to the blocks. Six areas of 3 m × 0.8 m were delimited in each block, and an experimental unit of 2 m × 0.8 m was defined inside, with a distance of 2 m from each other. Likewise, the different blocks were separated leaving a bed in between to avoid drift effects when making the corresponding applications for each treatment.

The variables of interest were evaluated every 20 days during 4 months, in which a rainy season was registered comprising the months of November and December 2016 plus a dry period including the months of January and February 2017.

The number of CFU of *T. harzianum* present in flowers, leaves and fruits was quantified in each of the treatments established in the experimental plot, using the serial dilutions and plate count methodologies (Valencia 2010) in modified TBS (Hetong et al. 2008). A sample of 10 flowers, 3 leaves and 3 fruits was taken, for each experimental unit of 2 m × 0.8 m, a total of 60 experimental units were sampled (6 treatments, 10 repetitions), every 20 days from October 2016 to February 2017. The number of samples was defined taking into account that the successive samplings did not affect the physiology of the plants.

The statistical analysis of the bio-pesticide distributing capacity of the vector for flowers, leaves and fruits was carried out by implementing the Kruskal-Wallis test.

Under the study conditions, using a Peng-type two-ways dispenser that was recharged every 2 days and the P3 product based on *T. harzianum* and *Vectorite* as a carrier, each bee acquired an average charge of 1.2×10^4 CFU from the bio-pesticide, whose distribution in the crop was observed in flowers, leaves and fruits. Table 4 shows the average value of CFU/organ, obtained by treatment, during the six samplings carried out from October 2016 to February 2017.

The value by organ was averaged from the ten experimental units (10 repetitions) established in the experimental smallholding for each treatment, for sixty experimental units evaluated every 20 days for 4 months.

Table 4 Countings of *T. harzianum* in flowers, leaves, and strawberry fruits in six treatments of the experimental smallholding

	Treatment (CFU/organ)					
Organ	T1	T2	T3	T4	T5	T6
Flowers	5 ± 5	0	190 ± 110	730 ± 590	3500 ± 1700	3000 ± 1700
Leaves	17 ± 7	2 ± 2	250 ± 160	69 ± 29	320 ± 140	79 ± 34
Fruits	9 ± 9	25 ± 14	510 ± 390	42 ± 30	100 ± 43	75 ± 41

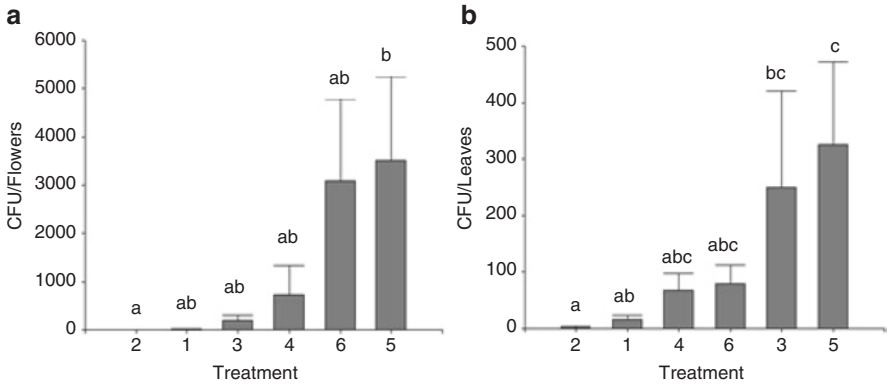

Fig. 6 Average dispersion of CFU of *Trichoderma harzianum* in strawberry plants by treatment. (**a**) Flowers, and (**b**) leaves
The presented value was averaged from the ten experimental units (10 repetitions) established in the experimental smallholding for each treatment, for sixty experimental units evaluated every 20 days for 4 months. Different letters represent statistically significant differences ($P < 0.05$)

The organs of the plant that had the highest CFU countings of the bio-pesticide distributed by the effect of the pollinators were the flowers, showing significant differences between treatments ($H = 7.36$, $p = 0.0002$), followed by the leaves in which significant differences were also observed between treatments ($H = 12.49$, $p < 0.0001$) as shown in Fig. 6. Finally, there were fruits in which no significant differences were observed between treatments ($H = 1.98$, $p = 0.0713$).

When quantifying the average per organ in the ten repetitions per treatment during the six samplings, it was observed that the Absolute control (T1) and Commercial Control treatments with fungicides in a traditional model (T2), in which Apivectoring was not included, showed average levels lower than 26 CFU/organ of *T. harzianum* distributed in the tested strawberry plants. These findings showed distribution of conidia possibly by air. Although meshes were installed to exclude vectors, they cannot prevent the entry of spores of the bio-pesticide to those treatments. These data are also similar to those reported by Kovach et al. (2000), who argue that this is due to the bio-pesticide drift generated by bees loaded with the bio-pesticide, which frequently flew close to the meshes. Another possible explanation was the conidial drift of the *T. harzianum* Control treatment, in which bio-pesticide was applied with a fumigating pump (T5), although such treatment had space left between treatments and a screen was used in the lance of the sprinkler to direct the application.

The Organic Control treatment including Apivectoring (T3) presented average *T. harzianum* countings of 2.5×10^2 CFU and 5.1×10^2 CFU in leaves and fruits. However, the level of *T. harzianum* was lower in flowers than the one registered in other organs of the same treatment with 1.9×10^2 CFU. The integrated management treatment (T4) including Apivectoring and applications of fungicides in a clean agriculture model, showed higher *T. harzianum* levels in flowers with 7.3×10^2 CFU, than in leaves and fruits with average values less than 70 CFU/organ. While

the *T. harzianum* Control treatment (T5), in which the bio-pesticide was applied with a spray pump, showed high levels up to 3.5×10^3 CFU of *T. harzianum* in all the evaluated organs. The Apivectoring treatment (T6), which did not include any application of fungicides, had a high UFC content of *T. harzianum* with values up to 4.9×10^3 in flowers, but in leaves and fruits, the average counts were lower to 1×10^2 CFU.

When observing the amount of CFU of *T. harzianum* in flowers and comparing the treatments including Apivectoring Technology T3, T4 and T6, it was observed that the Apivectoring treatment (T6) presented a higher count of bio-pesticide with a difference up to two logarithms regarding organic treatment (T3) and integrated management ones (T4).

When comparing the three treatments exposed to the visit of the inoculated bees with the bio-pesticide: Organic control (T3), Integrated management (T4) and Apivectoring (T6), a decrease in the number of *T. harzianum* spores in flowers in organic treatment (T3) and integrated management (T4), regarding Apivectoring treatment (T6) in which no applications of pesticides were made. This is explained because in the framework of the proposed management for treatments T3 and T4, pesticide applications had to be made, biological ones in T3 and synthetic ones in T4, which probably decreased the rate of visits of the vectors, affecting the amount of bio-pesticide deposited in the flowers.

However, CFU/organ countings were similar in leaves and fruits for the three treatments (T3, T4 and T6). This was possibly due to the drift effect caused by the air currents while bees fly near the plants (Kovach et al. 2000).

In the *T. harzianum* Control treatment (T5), the CFU/organ counts recorded the highest values on average. However, counts did not show significant differences specifically in flowers, regarding the Apivectoring treatment (T6) ($T5 = 3.5 \times 10^3$ CFU/flower vs. $T6 = 3.0 \times 10^3$ CFU/flower). So it can be concluded that the distribution level of the *T. harzianum* control agent reached by using bees (*A. mellifera*) as vectors in strawberry flowers, is equivalent to that obtained by using a spray pump. This behavior was similar to that described by Kovach et al. (2000) and evidences one of the advantages of the Apivectoring Technology as proposed by Kevan et al. (2003), Kevan et al. (2008), Mommaerts and Smagghe (2011) and Smagghe et al. (2012). Indeed, when implementing Apivectoring, flowers get inoculated which presents daily anthesis. In addition, each flower is subjected to several visits and the bee is able to distribute the bio-pesticide deeply in the floral whorls. It is also considered that there is an efficient, directed and constant distribution with a level of CFU/flower similar to that obtained when applying the bio-pesticide with fumigating pump, but the amount of water used and the necessary labor is reduced to perform the applications.

Specifically, for the Apivectoring treatment (T6) in flowers, a count of 3.0×10^3 CFU/flower was recorded. This value was higher than the one found by Albano et al. (2009) in their study in a similar experimental model in which the control exerted by *T. harzianum* was tested and distributed by *A. mellifera* on *B. cinerea*. They reported that between amounts of 26.3 CFU/flower and 1.1×10^2 CFU/flower, and these values differ from the results presented here, possibly because a Houle

type two-track device was used and it loaded each bee with 1.7–3.9 × 10^3 CFU. However, they are similar to that reported by Peng et al. (1992) who evaluated the acquisition of *G. roseum* by *A. mellifera* also using a Peng inoculation device, which charged each bee with 8.8–180 × 10^4 CFU of *G. roseum* under open field conditions, obtaining distribution values in flowers in a range of 3.0 × 10^2–2.7 × 10^4 CFU/flower.

In leaves, the countings of 79.2 CFU/leaf were lower than the ones observed by Al Mazra'awi et al. (2006), who evaluated *B. bassiana* distribution by *A. mellifera* in canola, obtaining bio-pesticide countings between 1.4 × 10^5–2.1 × 10^5 CFU/leaf. It is believed that this difference can be associated with a greater preference of the vector for canola plants than for strawberry plants (Peng et al. 1992; Thapa 2006). Strawberry leaf countings were also below those reported by Shipp et al. (2012) who quantified the dispersion of *Clonostachys rosea* with *B. impatiens* in tomato, registering values of 3.2 × 10^3–6.1 × 10^3 CFU/leaf. In this case the vectors were loaded with 2.6–5.0 × 10^5 CFU, so that it was possible to disperse a greater quantity of inoculum from the bio-pesticide.

It should be noted that although the level of distribution in flowers was similar between treatments T5 and T6, in the *T. harzianum* Control treatment (T5) in which the bio-pesticide was applied with a fumigating pump, an approximate amount of control product was spent of 3600 g/ha/month, while in the Apivectoring treatment (T6), approximately 2560 g/ha/month of bio-pesticide product was spent, without the need of using water. Importantly, that is a 28% reduction for product obtained under the conditions evaluated. Under seasonal production conditions, Kovach et al. (2000) reported a decrease up to 94% for product used and also a reduced use of water resources, saving up to 2000 liters of water/ha/month.

1.9 Evaluation of the Use of Apivectoring Technology in a Pilot Strawberry Crop

In the design established in the experimental smallholding in which six agronomic treatments were included: T1: Absolute control, T2: Commercial Control, T3: Organic Control, T4: Integrated Control, T5: *Trichoderma harzianum* Control, and T6: Apivectoring. Dispersion of the bio-pesticide was also evaluated, the incidence of *B. cinerea* in flowers: quantified in five plants of each experimental unit; Total fruits: quantified in five plants of each experimental unit and harvested fruits: which only includes the fruits in physiological maturity (≥70% red), quantified in all plants of each experimental unit (Flórez and Mora 2010). All treatments of the experimental smallholding were evaluated during 4 months, sampling every 20 days. The calculation of the incidence percentage was performed as described by Hoyos et al. (2011), as shown below:

$$\%\text{INCIDENCE} = \frac{\text{No.structures with } B.cinerea}{\text{No.total structures}} * 100$$

Table 5 Incidence of *Botrytis cinerea* in strawberry plants, in six treatments of the experimental smallholding

Treatments	Incidence Average (%)					
	Flowers		Total Fruits		Harvested Fruits	
T1: Absolute control	17.95	c	5.99	b	15.25	c
T2: Commercial control	14.63	Bc	3.35	Ab	8.70	b
T3: Organic control plus Apivectoring	7.36	Ab	3.22	Ab	5.89	Ab
T4: Integrated control plus Apivectoring	4.06	a	3.01	a	3.21	a
T5: *Trichoderma harzianum* pumping application	17.55	c	5.86	Ab	15.30	c
T6: Apivectoring	4.92	a	3.89	Ab	6.82	Ab

The average percentage of incidence of *B. cinerea* obtained in the ten experimental units of each treatment quantified for flowers and total fruits in five plants for each experimental unit and in fruits harvested in the total of the plants of each experimental unit, during the period of 4 months in which the trial was developed, is shown in Table 5, where a greater percentage of infection in flowers than in fruits is evidenced in all the treatments implemented.

Values were obtained by averaging the percentage of *B. cinerea* incidence in the ten experimental units evaluated for each treatment, during the six samplings carried out from October 2016 to February 2017. Different letters represent statistically significant differences.

The highest percentage of incidence occurred in the Absolute Control (T1), as *T. harzianum* Control (T5) in which CSF was applied with fumigating pump, followed by the Commercial Control (T2), these three treatments did not include Apivectoring and in general presented higher *B. cinerea* levels than compared with the treatments including it, Organic control (T3), Integrated Control (T4) and Apivectoring (T6), obtaining on average difference in percentage of incidence of the disease of 1.68% for total fruits, 7.78% for fruits harvested and 11% in flowers.

Organic Control (T3) recorded *B. cinerea* incidences of 7.36% in flowers, 3.22% in total fruits and 5.89% in harvested fruits. These values were lower than those obtained with Commercial Control (T2) in which chemical synthesis fungicides were applied in a traditional scheme, which presented 14.63% in flowers, 3.35% in total fruits and 8.70%. No statistically significant differences were observed between both treatments, which indicates the level of control of the disease in both was similar, as seen in Fig. 7.

The highest incidence of the pathogen was present in flowers (17.5%) of Absolute Control (T1), in which no type of control was applied to *B. cinerea*. This agrees with the statements by Huang and Kokko (1999), Ngugi and Scherm (2006) and Reich et al. (2015) who explained that nectar and pollen are rich in proteins, sugars, minerals and amino acids, which promote the germination of conidia and can increase growth and development of the pathogen. In addition, petals are more susceptible to the attack of microorganisms because their walls are thin and do not have a waxy cuticle (Reich et al. 2015).

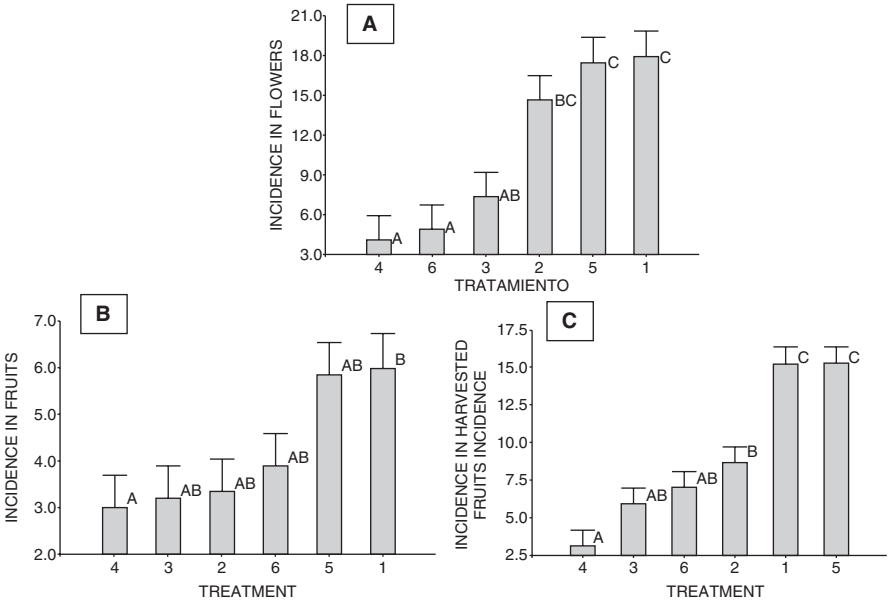

Fig. 7 *Botrytis cinerea* incidence for six treatments in experimental plot in Cundinamarca in Colombia. (**A**) Flowers, (**B**) Total Fruits, and (**C**) Harvested Fruits

In the rainy season of November and December there was a higher incidence of *B. cinerea* in all treatments compared to the dry period. Increased rainfall incremented the amount of free water on the structures of the plant, promoting the process of germination of conidia, thus generating new infections. In addition, it increased the distribution of the pathogen by the splash generated by the rain (Huang and Kokko 1999; Agrios 2005), in both periods in the Apivectoring treatment (T6) in which no other type of product was applied for the control of *B. cinerea*, more than the *T. harzianum* distributed by the bees. It obtained low percentages of incidence of the disease with values of 4.92% in flowers, 3.89%, in total fruits and 6.82% in harvested fruits. This was mainly due to the bees distributing bio-pesticide deeply in these organs, providing a protection constant throughout the flowering cycle and as the flowers enter anthesis (Kovach et al. 2000; Kevan et al. 2008; Smagghe et al. 2012; Hokkanen et al. 2015). This experiment validated the role of the bees as there was an efficient distribution of *T. harzianum* for the control of the pathogen in strawberry crops under the conditions of Colombia.

The lower percentages of incidence of *B. cinerea* were quantified for the Integrated Management (T4) treatment, which included Apivectoring in a clean agriculture scheme, with values of 4.06% in flowers, 3.01%, in total fruits and 3.21% in harvested fruits, being the management scheme that achieved the greatest decrease in percentage of organs affected by *B. cinerea*. The previous results showed that the integration of the Apivectoring Technology in the framework of an integrated management of pests and diseases is compatible both with an organic Scheme (T3) and with a clean production Scheme (T4) and allows keeping the populations

of the pathogen. It is reflected in low incidence values ≤10%, which remained stable in the evaluated period, achieving the lowest epidemics of *B. cinerea* by integrating the Apivectoring Technology into a clean production Scheme (T4). The application reduced 50% of pest and disease control products usage. In addition, 60% of the products applied were of low impact for human health, bees and the environment, regarding commercial control (T2) that represents the current practices of production, in which calendar applications of chemical control products are made. It should be mentioned that in the traditional model obtained a percentage of incidence of *B. cinerea* of 14.63% in flowers, while in fruits the incidence ranged between 3.35% and 8.70%. This difference supports the argument that the applications with the fumigating pump failed to adequately protect the flowers, especially if you take into account that around 40,000 flowers/ha enter anthesis each day (Hokkanen et al. 2015).

Regarding the application of *T. harzianum* using a fumigant pump (T5), a high amount of the bio-pesticide was recorded in the distribution test. However, the treatment presented the highest incidence of *B. cinerea*. This finding is attributed to several circumstances, the bio-pesticide calendar applications that only achieve a partial protection of the flowers (Smagghe et al. 2012; Hokkanen et al. 2015), the increase in the amount of free water in the plant due to high rainfall in the first sampling, and the pump application that requires an approximate 450 liters of additional water per ha, which is in turn promoting the germination of conidia of *B. cinerea* and therefore the development of new infections that quickly reach high levels of *B. cinerea* incidence higher than 25%, in which it was difficult to control the epidemic. It should be mentioned that the method did control the disease, since the incidence of the disease in flowers in this treatment was 34% in the first sampling. In other samples, the percentage tended to decrease reaching values close to 15% in the final sampling. However, the level of control was generally much lower regarding other treatments, in which the levels of incidence did not exceed 20% in any of the six samplings performed. This behavior coincides with the one described by Shafir et al. (2006) who argued about cases where the incidence in fruits is high, neither the management of organic type, nor the handling with products of chemical synthesis, generated an adequate control of the disease.

As previously mentioned, two pillars support Apivectoring Technology: the control of pests and/or diseases, and the increase in production due to pollination. The results obtained in this project have to be interpreted in this context.

1.10 Productive Level Test for the Six Treatments of the Experimental Smallholding

The fruits harvested in all plants of each experimental unit (10 experimental units for each treatment), were classified following national parameters through the Colombian Technical Standard NTC 4103, which is based on the evaluation of the fruits by size (diameter in mm), as seen in Table 6.

Table 6 Classification of strawberry fruits by size (NTC 4103)

Diameter (mm)	Gauge	Average Weight (g)
Greater or equal to 34	A	21.8
33–30	B	16.1
29–25	C	11.7
24–21	D	8.0
Smaller or equal to 20	E	5.3

Source: Florez and Mora (2010).

Table 7 Classification by size of strawberry fruits harvested on the experimental smallholding for six treatments

	Classification Per Gauge (%)				
Treatment	A	B	C	D	E
T1: Absolute control	6.7	17.9	33.0	26.5	15.9
T2: Commercial control	12.2	18.1	30.1	24.2	15.3
T3: Organic management plus Apivectoring	8.2	16.2	30.8	28.7	16.2
T4: Integrated management plus Apivectoring	12.3	15.9	34.1	25.1	12.6
T5: *Trichoderma harzianum* pumping application	8.4	15.8	33.0	25.3	17.5
T6: Apivectoring	12.0	16.6	33.0	24.7	13.8

The evaluation period was established in the main productive phase of the crop, which oscillated between 8 and 15 months after sowing, in order to control factors associated with the age of the plant, either the first production of superior quality or related lower qualities with the end of the productive cycle. For the statistical analysis of the data, an analysis of variance (ANOVA, $p = 0.05$) and Tukey multiple comparison test were carried out, in order to determine significant differences between treatments, in order to establish the best management scheme.

Table 7 shows the classification by size and category, for the fruits harvested in the six treatments implemented, averaging the data obtained in the ten experimental units of each treatment during the four samplings carried out in the evaluated period.

For gauge A, treatments T2, T4 and T6 had a higher percentage of strawberries of superior quality, with values close to 12%, while for treatments T1, T3 and T5, the percentage of strawberries of gauge A was below 8.4%.

For all treatments, the highest percentage distribution was located in grades B, C and D, in which no significant difference was observed.

Regarding gauge E, treatments T4 and T6 including Apivectoring,were highlighted with the lowest percentages of low quality strawberries, specifically values lower than 13.8%, while other treatments presented percentages higher than 15%, being statistically different ($Chi^2 = 32.78$, $gl = 20$, $p = 0.0357$).

There is an increase in the number of larger fruit (gauge A) for T2, T4 and T6 with 4% over the other treatments. Likewise, in T4 and T6 a decrease between 2% and 5% of the quantity of strawberries of lower quality was observed compared to the treatments that did not include Apivectoring (Fig. 8).

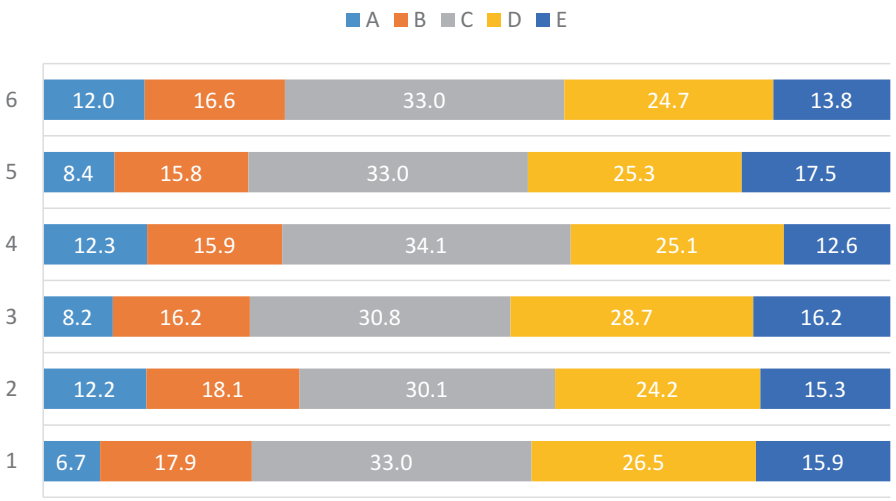

Fig. 8 Treatments distribution according to the classification by gauge described in the Colombian Technical Standard (CTS 4103)
Values were obtained by averaging the percentage of *Botrytis cinerea* incidence in ten experimental units tested for each treatment, during the six samplings carried out from October 2016 to February 2017. Different letters represent statistically significant differences ($P < 0.05$)

1.11 Commercial Potential of the Apivectoring Technology Implementation

Apivectoring technology is structured in two main components, namely the control of diseases and the increase of production due to pollination (Mommaerts and Smagghe 2011; Smagghe et al. 2012). Findings of this study showed that this technology represents a viable option to be implemented in strawberry crops (*Fragaria x ananassa*) within the framework of integrated management of pests and diseases in a clean production Scheme (T4), under the conditions of the Andean area. Under this model, control of *B. cinerea* was achieved in 77.4% of flowers and 79.0% of fruits, with incidences of only 4.06% and 3.21%, respectively. While the absolute control (T1) showed an average incidence of 17.95% in flowers and 15.25% in fruits, and this was with commercial control 14.63% and 8.70%, respectively. In addition, an increase of the productive variables was obtained, and results showed an increase of the weight of the fruit between 0.87 g and 2.35 g that was equivalent to a percentage of 6.7% and 18.28%. In comparison with the treatments that did not include bees, this means that implementing Apivectoring caused an increase of between 66.22 grams and 180.95 additional grams per kilo of strawberries produced. Likewise, an increase in diameters was obtained ranging between 2.7% and 6.8% for equatorial diameter and between 9.0% and 13.5% for polar diameter. This is directly related to a greater efficiency in the process of pollination that increased the number of fertilized ovules and therefore obtained a greater number of achenes

(Swingle 1928; Denney 1992), achieving up to 29% more as compared to the absolute control (T1) and up to 51.8% more compared to the commercial control (T2). Similar benefits in productivity and yield have been reported by other authors who have tested the technology for the control of *B. cinerea* in strawberry in other countries (Kovach et al. 2000; Soboksa et al. 2014; Hokkanen et al. 2015). It is here the first time that a study of this type has been done in Colombia, therefore this work provides the baseline for future research.

In this study, Apivectoring Technology presented positive results at the technical level that represent the possibility of obtaining higher income in the productive system. Regarding the technical results obtained, the following economic benefits could be extrapolated:

1. Increase in production due to pollination. There was a minimum increase of 0.87 g/fruit, which represents an average of 66.22 g per kilo produced. As farmers produce 43.5 tons/year (Ministry of Agriculture 2016), this turns into an increase in productivity of 2.8 tons/ha/year, which with an average price of $1.6 US (Ministry of Agriculture 2016), which in turn is representing an approximate additional gross income of $ 2987 US/ha/year.
2. Decrease in the application of pesticides. In the clean production model, the numbers of pesticide applications were reduced by 60% (and these applications were made with category III or IV products). This turns into a reduction in the cost of the applications of approximately $1405 US/ha/year, according to the costs established by the Strawberry Chain in 2016 (Ministry of Agriculture 2016).
3. Decrease in the losses by *B. cinerea*. In the case of harvested fruits, when comparing the commercial treatment with the Apivectoring one, a reduction in the losses by *Botrytis* was approximately 1.88% which is equivalent to an additional 817 kg in fruit production. With an average price of $1.06 US (Ministry of Agriculture 2016), would represent an additional income of approximately $872 US/ha/year.

According to registered values taking into account the additional income, the cost reduction and the value of the implementation of the Apivectoring Technology, it has been estimated that there is an additional utility close to $3.710 US per hectare, which is the sum of the three values mentioned above but subtracting the approximate costs for the implementation of the technology. However, it must be clarified that the values presented here are general and a deep economic analysis is required, which includes the risk factors, and quantifies the environmental benefits. Other authors have made approaches to the economic quantification of the implementation of Apivectoring Technology (Kovach et al. 2000; Hokkanen et al. 2015), although the analysis differs in scales and indicators, it is agreed that Apivectoring Technology generates the decrease of costs and the increase of the productivity of the crop.

It is clear that the Apivectoring Technology has broad advantages. However, this study also identified some limitations for the Colombian case. Since Apivectoring is an original concept in the country, there are no commercial products on the market

specifically formulated for Apivectoring. Tested commercial products showed a deficiency regarding the characteristics of the added carrier, which in this case was lactose. In addition to a low conidia viability in moderate toxicity media (2.7 and 4.8×10^5 CFU per g of product), results suggested that it is necessary to promote the development of business models in the country in order to offer producers the option of finding high quality products on the market, specially formulated to be used in the framework of the Apivectoring. In this sense, both Vectorite and corn flour as carriers presented adequate levels of acquisition using a Peng-type two-ways device. No significant differences were found in terms of inoculum acquisition by the vector with P1 = BVT powder: 1.2×10^4 CFU/bee, and P2 = corn flour: 1.1×10^4 CFU/bee, demonstrating that both carriers are suitable to make the formulation. However, the Vectorite presented less compaction in the recharge tray of the device, wherefore we believe this carrier is more suitable for the formulation of products for specific use for Apivectoring.

It is therefore necessary to identify and recognize limitations and conditions of the implementation of Apivectoring Technology. It should be applied in the framework of an integrated management of pests and diseases with a preventive approach, also involving all levels of living beings. So it requires special care in the structuring of each of its components. For example, it must take into account the interaction between the plant and the vector, the type of device, the characteristics of the vector, the climatic conditions and the particular characteristics of the productive system in which the technology is to be implemented (Kevan et al. 2003; Kevan et al. 2008; Mommaerts and Smagghe 2011; Smagghe et al. 2012).

1.12 Experience in the Transfer of Apivectoring Technology to Agricultural Producers in Colombia

For the first time in the country, the development of the project allowed the validation of the Entomovectoring Technology or Apivectoring, as a tool aiming to improve the competitiveness in a crop with export potential as is the case of strawberry (*Fragaria x ananassa*). The research managed to adjust the technology to the local conditions present in the peripheral areas of Bogota, and showed positive results on fruit quality parameters, as well as a decrease in the incidence and severity of the entomopathogenic fungus *B. cinerea*, considered as one of the main health problems for this crop. The economic impact of the technology was valued, compared to the conventional management carried out by the producers of three organizations, which allowed proposing alternatives to bring the service to a commercial phase. Regarding the scope of the results obtained, these are applicable for the use of Apivectoring Technology with bees of *A. mellifera* in strawberry crops in the open field for the control of *B. cinerea*. Hence, in the future the Apivectoring Technology should be investigated and validated for its implementation in other crops, systems under cover, other bee species or the control of other diseases.

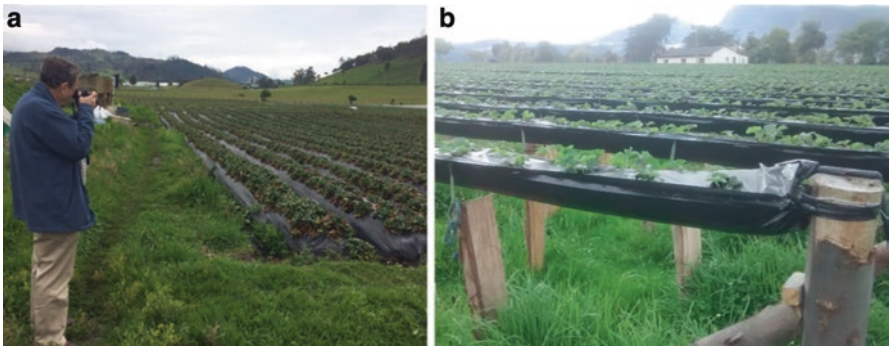

Fig. 9 (A) Crop in soil model with a conventional management. (B) Crop in a hydroponic system with conventional chemical products management

The strategy for the technology transfer with the producers was based on the implementation of the technology in three demonstrative production units with differences in the production methods. So in the first unit the production model was in soil with a conventional management, In the second productive unit the crop was under a hydroponic system with conventional chemical products management (Fig. 9). The third unit corresponded to a crop in soil with an organic management. These units worked to carry out trainings in which farmers were sensitized on the importance of bees and the advantages of the presence of insects in the crop. Given that in Colombia the predominant type of bee is the Africanized hybrid, which is characterized by a greater defensiveness, it was necessary to carry out a process of selection and genetic improvement of queens from the characteristic of meekness before the implementation of the demonstration productive units, which was carried out 3 years prior to this study including obtaining fecundated queens by artificial insemination techniques to guarantee the paternal and maternal characteristics. This activity allowed generating a greater degree of confidence with the producers and thus improving the availability to access the technology.

In total, more than 10 training activities were carried out with producers, which included Field Schools in crops, and this allowed to reach the producers of three organizations in two municipalities directly. However, it is necessary to establish a defined business model for the Apivectoring Technology in such a way that it facilitates the decision of the farmer on the adoption of the technology.

2 Final Considerations

It is necessary to understand the Apivectoring Technology, as a management tool within a structured program of pest and disease control, whether it is organic and/or traditional. In addition, it is necessary to create awareness with the farmer about the

importance of carrying out the programming of applications with all the preventive measures that guarantee not only their own welfare but also the hives.

We must combine efforts in the development of products with specific formulations to be applied with the Apivectoring Technology. These must comply with high levels of quality and control in its production process. It is also recommended to evaluate the bio-pesticide that simultaneously controls several pathogens associated with the crop, in order to offer an integrated approach solution to the farmer.

In future studies, the level of attraction for bees by the different varieties that are marketed in the country should be investigated, in order to select these ones that are appropriate to implement the Apivectoring Technology.

To achieve an adequate distribution of the product, it is necessary to consider introducing an adequate number of hives, for instance 4 per hectare. These can be located in the periphery of the crop; however, it is recommended to place them in the center of the crop for better results. In the Colombian context, it is fundamental to guarantee that the colonies of *A. mellifera* have been selected for meekness and that the personnel is adequately trained in order to minimize the risk of accidents.

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Making a Pest Beneficial: Fungus Gnats [*Bradysia impatiens* (Diptera: Sciaridea)] as Potential Vectors of Microbial Control Agents to Suppress Pathogens they Also Spread

Jean-Pierre Kapongo, Peter G. Kevan, Les Shipp, and Hisatomo Taki

1 Introduction

Pest arthropods on crops cause damage directly by feeding or oviposition or both (Pedigo and Rice 2008). They may also transmit various pathogens and cause infections (Hatcher 1995). Soil inhabiting arthropods, such as fungus gnats (Sciaridae) are no exception (Hatcher 1995; Willsey et al. 2017). During our research on the use of insects (beneficial managed pollinators) to disseminate biological control agents against pests and diseases in various cropping systems (Kevan et al. 2003, 2007, 2008; Kevan and Shipp 2017), we reasoned that insects usually designated as pests could, if dosed with microbial biological control agents, be used to the same beneficial end, as was suggested by Whipps and Budge (1993) for the spread of *Coniothyrium minitans* by a springtail (Collembola) as antagonistic to *Sclerotinia sclerotiorum.* We tested the capacity of the fungus gnat (*Bradysia impatiens* Johannsen [Diptera: Sciaridae]), normally a disperser of plant pathogens, as a potential carrier of *Chlonostachys rosea* that has proven antagonistic to a wide range of plant pathogenic fungi and to suppress both *Pythium* and *Fusarium* (Sutton, personal communication, January 2018).

Fungus gnats, *Bradysia* spp. thrive in high-moisture environments, particularly those common in greenhouses. Two species are usually recognized in association

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with greenhouse crops (*B. impatiens* and *B. coprophila* Lintner) (Gardiner et al. 1990; Harris et al. 1996). *Bradysia impatiens* typically inhabits moist, shady areas within woodlands, fields, plant nurseries, and greenhouses (Harris et al. 1996). This fungus gnat was considered a minor pest because it could be controlled by use of the heavy minerals in soil-based greenhouses and nursery potting media (Stanghellini and Rasmussen 1994; Harris et al. 1996). However, it is a serious pest in modern, soil-less greenhouses and nurseries (Lindquist et al. 1985; Harris et al. 1996; Lindquist 1997). Research on control measures for *B. impatiens* and related species has focused on insect regulators (Ludwig and Oetting 2001; Ludwig et al. 2003; Jagdale et al. 2004); biocontrol agents, either entomopathogenic nematodes (Nedstam and Burman 1990; Harris et al. 1995; Vanninen 2003; Kim et al. 2004), predatory mites (Wright and Chambers 1994; Ydergaard and Enkegaard 1997) or rove beetles (*Atheta coriaria* (Coleoptera: Staphylinidae) (Jandricic et al. 2006) or in combinations that may affect the compatibility of effectiveness issues among pest management strategies (Ludwig and Oetting 2001; Krishnayya and Grewal 2002; Jandricic et al. 2006). More recently, Cloyd and Dickinson (2005) tried unsuccessfully to control larval fungus gnats by adding diatomaceous earth to the culture media.

Adults of *B. impatiens* are generally non-feeding (Kennedy 1976) but their larvae feed on plant detritus, fungi (Kennedy 1974; Anas and Reeleder 1988a, b), as well as on living root hairs and stem tissues where they can cause severe damage, especially to seedlings (Springer and Carlton 1993; Cloyd and Dickinson 2005). Adult females can be attracted to oviposit on microbe inoculated seedlings (Braun et al. 2012b). Both, larvae and adults can transmit root and foliar pathogens, such as *Fusarium* (Leath and Newton 1969; Graham and McNeill 1972; Gillespie and Menzies 1993; El-Hamalawi and Stanghellini 2005; Hurley et al. 2007; Elmer 2008; El-Hamalawi 2008; Scarlett et al. 2014; Marin-Cruz et al. 2017), *Pythium* (Leath and Newton 1969; Graham and McNeill 1972; Gardiner et al. 1990; Goldberg and Stanghellini 1990; Jarvis et al. 1993; Stanghellini and Rasmussen 1994; El-Hamalawi 2008; Hyder et al. 2009; Braun et al. 2010, 2012a, c), *Theilaviopsis* (Goldberg and Stanghellini 1990; Stanghellini et al. 1999; El-Hamalawi 2008), *Verticillium* (Kalb and Millar 1986; Shamshad et al. 2009), *Coniothyrium minitans* (Grendene and Marciano 1999; Whipps and Budge 1993) and possibly *Sclerotinia* (Anas and Reeleder 1988a) to the plants by carrying spores on their bodies or in their guts allowing for contact or fecal infection. Trans-stadial carryover of spores is not likely (Jarvis et al. 1993; Braun et al. 2010).

The three-way interactions (plant, pathogen and vector) (Hatcher 1995) may have synergistic effects by increasing the plants' susceptibilities to pathogens (e.g. Leath and Newton 1969), adding to the attractiveness of the plants to herbivory, or even helping the plants combat damage (Arnold et al. 2008; Braun et al. 2009).

Fusarium oxysporum Schlecht. emend. Snyder & Hansen (Ascomycota) and *Pythium aphanidermatum* (Edson) Fitzp., (Oomycetes) are common microbial pathogens of many species of plants, including crops. Their spores can be dispersed in the soil, air, by rainfall, and by various arthropods (as noted above); the latter also has motile zoospores.

Chlonostachys rosea (Link) Schroers (Ascomycota) is a common soil inhabiting fungus. It has the capacity to become endophytic in plant tissue and to suppress the growth and development of various plant pathogens (Sutton et al. 2002), including *Fusarium* and *Pythium* (Sutton, personal communication, January 2018). The report that it may be entomopathogenic (Toledo et al. 2006) is probably wrong, at least in concentrations normally found in nature. It can be used as a biological control agent to suppress plant pathogenic fungi in various cropping systems and has been used successfully in crop protection through dispersal of its spores to flowers by managed pollinators (honeybees [*Apis mellifera* L.]) and bumblebees (*Bombus* spp.) (Hymenoptera: Apidae) (Peng et al. 1992; Yu and Sutton 1997).

To the best of our knowledge, the idea that a pest insect could be used in a beneficial way by applying its herbivorous and microbial-vectoring habits to the dissemination of biological control agents has been rarely considered (Whipps and Budge 1993). Our objective was to test the potential for transforming a pest (*B. impatiens*) into a beneficial organism by making it vector of the antagonistic fungus *C. rosea* while it carried the pathogenic spores of *Pythium* and *Fusarium*.

2 Material and Methods

2.1 Fungus Gnat Colony

Fungus gnats that constituted the first generation (F_1) of the cohort in the experiment were collected from the growing media of potted greenhouse tomato *Lycopersicon esculentum* Mill. (Solanaceae) (cv Rapsodie) maintained at the Harrow Research and Development Centre 'HRDC' located at Harrow, Ontario, Canada. Representative specimens of collected individuals were sent to Dr. John Huber, Canadian Forestry Service, Ottawa, Canada, who confirmed them as *B. impatiens*. The F_1 and subsequent generations were maintained according to the methods described by Taylor et al. (2007) on flakes of potato (*Solanum tubersoum* L. (Solanaceae)). Potato tubers were surface-sterilized by submerging them in 70% ethanol for 15–20 s and then in 0.025% NaOCl (sodium hypochlorite, 95%) solution for 3 min. The tubers where then rinsed with sterile distilled water + Tween 20 (Shipp et al. 2003) to eliminate all saprophyte fungi. They were then cut into flakes using knife sterilized with 0.025% NaOCl. The potato flakes were placed in a 600 mL-glass beaker (90 mm-diameter), thoroughly mixed with sterile water (30 g potato for 40 mL of water (Taylor et al. 2007)) and kept in growth chamber ([25 ± 1 °C, 50–60% RH and photoperiod of 14:10 (L:D]). The beakers were covered with clean, sterile cloths secured with elastic bands to prevent the escape of insects. Subsequent cohorts of *B. impatiens* were generated from equal-aged pupae harvested from the beakers by Pasteur pipette and placing them in new beakers (50 pupae per beaker) with the same medium as described above. Four hardwood bark chip pieces were placed on the surface of the medium to provide dry landing sites

for adult insect once they emerged. The bark chips prevented the insects from being trapped by condensation on the beakers' walls (Taylor et al. 2007). For our experiments, only adults of 2 days old and second-instar larvae were used.

2.2 Pathogenic Spores

The spores of *F. oxysporum* and *P. aphanidermatum* used in the study were provided by the laboratories of Dr. J.A. Sullivan and Dr. J.C. Sutton, Departments of Plant Agriculture and Environmental Biology, University of Guelph, Ontario, Canada, respectively. The spores were either in form of a powder or fluid suspension (10^7 spores per g or per L of the product).

2.3 Biological Control Agent

A formulation of *C. rosea*, containing a nominal 2×10^7 viable CFU (colony forming units) of *C. rosea* per gram of product was used as the suppressing agent for *F. oxysporum* and *P. aphanidermatum.*

The actual viability of *C. rosea* was evaluated before use. Three 0.01 g amounts of the formulated *C. rosea* were suspended each in a 100 ml of distilled water and 0.1% Triton-100 in a 250 mL-flask. The flasks were agitated on a rotary shaker at 125 rpm for 2 h. Then 100 µL of the conidial suspension was spread on Sabouraud dextrose agar (SDA) + penicillin G sodium salt and streptomycin sulphate salt, and stored in dark at 25 °C for 5 days (Toledo et al. 2006). Dissecting microscope was then used to count the CFU.

2.4 Plant Tissues

Fully developed leaves of organically grown strawberries (*Fragaria* X *ananassa* Duch. [Rosaceae]) were used as a substrate for the development of the fungal spores. The harvested leaves were washed with sterile water (autoclaved water at 121 °C in 20 min) then air-dried at room temperature under a sterile fume hood to prevent contamination from saprophyte fungi in the general environment of the laboratory.

2.5 Experimental Design and Procedures

The strawberry leaves were cut in discs of 2.5 cm diameter each with six discs placed in a Petri dish of 9 cm diameter. Some discs were artificially infested with *F. oxysporum* or *P. aphanidermatum* spores suspended into water, while the remaining discs were spread with the powder of *C. rosea* according to the different treatments as describe below. Leaf discs were incubated for 5 days at room temperature (22 ± 2 °C) to promote sporulation of pathogenic fungi. At day 6, sterile laboratory maintained adult insects (2 days old) or second-instar larvae were starved for a day before their release into the Petri dishes with the leaf discs as treated with the three microbes. 6 h later, the Petri dishes were placed in the freezer at −18° (for 10 min) to kill all individual insects. Then, a sterile thin metallic pin (gauge 0 insect pin) was used to remove each individual insect for further processing. Our experiment was made in three phases with six replicates.

In the first phase, we assessed the capacities of larval and adult *B. impatiens* to vector the spores of each of the three kinds of study microbes externally on their bodies and internally by ingestion. Four treatments were evaluated and compared: T1 (*Fusarium* + insect), T2 (*Pythium* + insect), T3 (*Clonostachys* + insect) and the control (water + insect). For each treatment, six leaf discs of strawberry were infested with particular microbial spores submerged in sterile water (10^6 spores in 1 L of water) and stored at room temperature (22 ± 2 °C) for 5 days. At day 6, insects (2 larvae or 2 adults) were then released into Petri dishes with treated strawberry leaf discs (above) that were immediately covered with lids prevent the insects' escape. 6 h later, the Petri dishes were placed in the freezer (for 10 min) to kill all insects and individuals were aseptically assorted for processing as described above.

Two fungus gnats (adult or larva) were released per Petri dish. One insect from each Petri dish was body washed to determine the number of spores that insects carried their bodies. Each was submerged in 1 mL of water (sterile water + Tween 80: Fisher Scientific, Fair Lawn, NJ, USA) contained in 1.5 mL of micro-tube and vortexed for 2 min at 125 rpm. Aliquots (100 μL) were plated on PDA and stored for 5 days in the growth chamber (25 °C and 80% RH) to determine the number of CFU corresponding to spores that each individual insect carried on its body. The other insect from each Petri dish was surface-sterilized and homogenized using porcelain mortar and pestle. The homogenate paste was submerged in 1 mL of water using the same protocol as for the body washes to determine the number of CFU corresponding to spores that each individual insect had ingested.

In the second phase of our experiment to determine the capacity of fungus gnats to vector simultaneously two or three microbes and to test the capacity of *Chlonostachys* to suppress the growth of the pathogens, we had three exposure treatments. T3 (*Fusarium* + *Pythium* + insect), T2 (*Fusarium* + *Pythium* + *Clonost achys* + insect), and T1 that is set as the control (heat inactivated *Fusarium* + heat inactivated *Pythium* + heat inactivated *Clonostachys* + insect). Among six leaf discs, two were infested with 100 mL of *Fusarium* spores suspension as prepared into sterile water (10^6 spores/L) and other two with spores of *Pythium* (same amount and

concentration as for *Fusarium*). The leaf discs were placed into Petri dishes that were stored at room temperature for 5 days. At day 6, the remaining two healthy strawberry leaf discs were either spread with 0.01 grams of powder of the *C. rosea* or sterile water according to the treatment. Three fungus gnats (larvae or adults) were immediately released into each Petri dish that was immediately covered with lid to stop insect from escaping. 6 h later, the dishes were placed in the freezer (for 10 min) to kill all insects. Then, a sterile thin metallic pin was used to remove each individual insect for further processing.

In the third phase, three fungus gnats (adults or larvae) were released per Petri dish. Two of the insects from each Petri dish were processed as described above to determine the number of spores carried externally or ingested. The remaining insect was also surface-sterilized and ground as described above, but the homogenate was spread with a sterile needle onto sterilized wet filter paper in Petri dishes. The Petri dishes were then stored in a growth chamber (25 °C and 80% RH.) for 7 days. After storage, each insect's homogenate preparation was submerged in micro-tube containing water + Tween 20 and processed as described above. The eventuating aliquots (100 µL) were plated on PDA and stored in the growth chamber (25 °C and 80% RH.) for 14 days to assess the spore-vectoring capacity of the insects for each microbe and to simulate the suppression of *Fusarium* and *Pythium* by *Clonostachys*. The suppression of diseases was assessed using a dissecting microscope by estimating the relative areas of the colonized surface areas of PDA covered by conidiophores of *Clonostachys* versus the areas colonized by *Fusarium* or *Pythium*.

2.6 Statistical Analysis

To compare the number of CFU of each microbe on and inside the insects at different stages (larvae and adults) and to assess the capacity of *C. rosea* to suppress *Fusarium* and *Pythium*, the CFU counts of each microbe growing on PDA were log transformed before analysis. The mean numbers of CFU of *Clonostachys, Fusarium* and *Pythium* from each insect and treatment were then compared using the *F*-test by two-way ANOVA (SAS Institute 2001). The type-I error rate was set at $p < 0.05$. CFU data were back-transformed to their original scales for presentation in Tables 1 and 2.

Table 1 Mean (± SE) numbers of spores that individual fungus gnat carried on bodies or ingested in 6 h when the insect was separately exposed to a particular growing spores of fungi (*Clonostachys rosea*, *Fusarium oxysporum* and *Pythium aphanidermatum*) on leaf disc of strawberry plant

Pest stage		*Clonostachys*	*Fusarium*	*Pythium*	Average
CFU on body	Larva	459 ± 6	1959 ± 26	993 ± 31	1137 ± 440A
	Adult	1922 ± 60	3535 ± 52	1156 ± 36	22,056 ± 702A
CFU ingested	Larva	301 ± 6	1065 ± 20	742 ± 9	703 ± 222 a
	Adult	18 ± 1	43 ± 1	37 ± 3	33 ± 8 b

Averages of number of CFU recovered on/in insect stages followed by different letters are significantly different between them at $P < 0.005$ using *F*-test

Table 2 Mean (± SE) numbers of spores that individual fungus gnat carried externally or ingested in 6 h when the insect was exposed simultaneously to all fungi. For spores carried internally, the insects were first surface-sterilized before processing

Pest stage		*Clonostachys*	*Fusarium*	*Pythium*	Average
CFU on body	Larva	832 ± 6	1218 ± 11	693 ± 11	914 ± 157 A
	Adult	661 ± 4	1420 ± 17	686 ± 13	922 ± 249 A
CFU ingested	Larva	507 ± 11	570 ± 7	601 ± 10	559 ± 28 a
	Adult	47 ± 2	48 ± 4	72 ± 4	55 ± 8 b

Averages of number of CFU recovered on/in insect stages followed by different letters are significantly different between them at $P < 0.005$ using *F*-test

3 Results

3.1 The Capacity of Bradysia impatiens Gnat to Vector Single Fungus

The fungus gnats at each tested stage (adult and larva) could vector the study fungi externally and internally. Adult gnats carried externally twice as many as the larvae ($F_{1,10} = 1.66$, $P = 0.2665$) (Table 1), but larvae ingested and carried about 21 times more spores than did the adults ($F_{1,10} = 9.15$, $P = 0.0390$) (Table 1).

3.2 The Capacity of Bradysia impatiens to Vector Simultaneously Three Fungi

The pest showed the capacity to carry and ingest each of fungi when they were together exposed to it. No significant difference was found between adult and larva in the amount of spores carried on their bodies ($F_{1,10} = 0.00$, $P = 0.9792$) (Table 2). In contrast, larvae ingested about 10 times more spores compared to adults ($F_{1,10} = 308.3$, $P < 0.0001$) (Table 2).

3.3 Simulation of Suppressive Effect of Clonostachys rosea on Fusarium oxysporum and Pythium aphanidermatum

The antagonistic fungus, *C. rosea* has shown the capacity to suppress the spores of disease causal agents, *F. oxysporum* and *P. aphanidermatum*. The area covered by sporulation of fungal disease in the presence of *C. rosea* was small compared to the area where the fungal pathogen sporulated in the absence of the suppressive agent, *C. rosea*. Therefore, the *C. rosea* reduced the sporulation of fungal spores ($F_{1,10} = 70.6$, $P < 0.0001$) (Table 3).

Table 3 Mean (± SE) percentage of sporulation of disease from spores that were ingested by each stage of fungus gnat. Insect was surface-sterilized before grinding and the paste was stored for 7 days following with their spread on PDA plates. Plates were then kept in growth chamber for 14 days prior to disease assessment using dissecting microscope

Insect stage	*Fusarium* and *Pythium* sporulation in the control plates	*Fusarium* and *Pythium* sporulation in the presence of *Clonostachys*
Adult	85 ± 3 a	78 ± 11 a
Larva	85 ± 3 b	36 ± 5 a

Averages of percent of area with disease in a row followed by different letters are significantly different at $P < 0.005$ using *F*-test

4 Discussion

The objective of the study was to determine the capacity and potential of the pest *B. impatiens* to vector a plant-pathogen antagonistic fungus *C. rosea*, while at the same time vectoring the spores of pathogenic fungi, *F. oxysporum* and *P. aphanidermatum*. *Bradysia impatiens* demonstrated its capacity to vector individually and collectively the three fungi with the possibility of reducing sporulation and the spread of the two pathogenic agents.

In our studies, adult and larval stages carried internally and externally the spores (Tables 1 and 2) with a maximum of 1090 active spores ingested per larva and only 45 per adult. That fact that adult stages do not often feed likely contributes to fewer spores found within them when compared to larvae that need to feed actively for larval development. The count of spores on *B. impatiens* showed about 3590 infectious spores on the adult and 1985 on the larva. This can be explained by the fact alate adults are frequently in motion compared to the larva and therefore exposed to contract more spores.

The fungus gnat carried beneficial spores of *C. rosea*, while vectoring the infectious spores of *Fusarium* and *Pythium*. The adult carried 14 times more spores externally on the body compared to ingested spores. The larva carried approximately 1.6 times more spores carried on the body than ingested, but ingested many more spores than the adult stage. Results given in Table 2 demonstrate that the fungus gnats carried the beneficial agent as well as the pathogenic spores of *Fusarium* and *Pythium*. The usefulness of fungus gnat as means to vector the biological control agent *Clonostachys* for the suppression of *Fusarium* and *Pythium*, can be confirmed by the fact that sporulation of fungal spores on PDA plates after 14 days in the presence of *Clonostachys* decreased when compared with the control without *Clonostachys*. The *Clonostachys* reduced the sporulation only in plates where spores were ingested by the larvae (Table 3). The cause of this difference in disease suppression between two stages of fungus gnat would probably be attributed to the fact that adults are generally non-feeding (Kennedy 1976). Hence, we found fewer spores inside of this stage of the insect after it was surface-sterilized compared to the larval stages (Table 2). The reduced number of suppressive spores might reduce the effectiveness of *Clonostachys* to suppress and inhibit the sporulation of disease caused by *Fusarium* and *Pythium*.

From the simulation trial, the fungus gnat constituted a tool for vectoring a beneficial fungus straight to the point of disease infection. If it carried the pathogen, it can also be used to vector the beneficial agent that will control the disease agents. This confirms the hypothesis of fungal disease being controlled by spores that were carried by the same vectoring insect.

In conclusion, the fungus gnat can be converted into a beneficial insect although it is usually associated the spread of the pathogenic spores. The ingestion of the spores of *Chlonostachys* did not change the germination power of the spores and the beneficial fungus did not lose its antagonistic effect. However, the beneficial fungus *C. rosea* does need to be applied to areas where the larval fungus gnats can have access to it either by feeding or picking it up. Because this was a laboratory trial, the next steps are controlled field trials. We urge any curious scientist to extend the present initiative to large scale production so that the technique can be profitable to growers who face crop injuries from the pest, the fungus gnat.

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Regulatory Processes Surrounding the Risk Assessment of Microbial Pesticides for Pollinators

Emily A. McVey and Jacoba Wassenberg

1 The ICPPR Working Group on Microbials and Bees

With the rising concerns over the health of pollinators in recent years, efforts have been made to refine the approach to risk assessment for bees and other pollinators for conventional pesticides. While biopesticides generally have a lower risk profile, honey bee toxicity/pathogenicity data are nonetheless required for most microbial pesticide registration applications. Many stakeholders have recognized the need for improvements in the guidance available for testing with honey bees, particularly given the increasing interest in development and registration of microbial pesticides (e.g. Pathak et al. 2017; Scheepmaker and Butt 2010). The EU passed a package of legislative measures in 2009 based around IPM, including the Framework Directive on the Sustainable Use of Pesticides (EU DG Environment). The government of the Netherlands instituted the "Green Deal" to stimulate, among other initiatives, the quick evaluation and registration of microbial and "green" plant protection products in the Netherlands (https://www.greendeals.nl/sites/default/files/uploads/2015/06/GD083-Nationale-Federatie-Stadsgerichte-landbouw.pdf). Although the Green Deal ended in 2017, the minister of Agriculture recently announced a multi-year plan to modernize agriculture in the Netherlands, with a strong focus on sustainability and "green" agriculture (Ministerie van Landbouw, Natuur en Voedselkwaliteit 2018).

With these thoughts in mind, a working group on testing the side-effects of microbials on bees was formed within the International Commission on Plant-Pollinator Relationships (ICP-PR) Bee Protection Group. The ICP-PR Bee Protection Group was founded in 1980 and is a non-profit organization of volunteer

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researchers in a broad range of disciplines from within and outside Europe who share an interest in improving tools for assessing and understanding bee protection within the context of modern, sustainable agriculture. The tasks of the Bee Protection Group consist of developing methods to inform regulatory guidance and guidelines for assessing and managing potential risks to bees and other pollinators from pesticides. The group proposes and discusses current and emerging test methods and organizes ring-testing of promising test methods. The group aims to provide a platform for the exchange of knowledge on the state-of-the-science and leverage the relevant experience of the scientists involved. Within the ICP-PR Bee Protection Group, working groups are formed to study different topics, including the working group on microbials and bees. Its formation is the result of efforts by the pesticide regulatory authorities in the United States (U.S. Environmental Protection Agency, US-EPA) and the Netherlands (the Ctgb) to communicate about common issues related to bee hazard testing and risk assessment of microbials and to seek expert input. The current aim of the group is to foster discussion around the suitability of current bee testing guidance and how it may be improved to provide more reliable and useful results for microbial pesticides. The group held its inaugural meeting at the ICP-PR Bee Protection Group 13th International Symposium on Hazards of Pesticides to Bees in October 2017. The group is co-chaired by risk assessors from the competent authorities of the Netherlands and the US, the Ctgb and the US-EPA, and members include representatives of other government agencies tasked with assessing pesticide risks to bees, industry, and academia.

2 Biopesticides

Biopesticides are pesticides derived from such natural materials as animals, plants, bacteria, and certain minerals. They can be considered to fall into four broad but separate categories:

- *Microbials,* including bacteria, fungi, viruses and protozoa;
- *Natural Products,* such as plant extracts or minerals exhibiting insecticidal or fungicidal properties;
- *Semiochemicals,* which are synthetic analogues of insect pheromones that can be used to lure insects into traps or repel them from crops; and,
- *Macrobials,* which are live insects used to control pest populations.

In this chapter, we will focus on the unique problems of registering microbial pesticides and determining their potential risks to pollinators, however, many of the points mentioned could be true for the other “biopesticides” as well.

3 Microbial Pesticides

Microbial pesticides include bacteria, fungi, viruses and protozoa which are used for the control of insects, plant pathogens and weeds. The most well-known microbial pesticide is probably *Bacillus thuringiensis* (Bt), which produces a protein crystal (the Bt δ-endotoxin) during bacterial spore formation and is toxic to susceptible insects. However, there are many other types of microbial pesticides already in use, including entomopathogenic baculoviruses (Moscardi 1999) and fungi (Faria and Wraight 2007).

4 Regulatory Frameworks

Registration requirements for microbial pesticides vary among different countries, and in some cases may differ for microbial pesticides compared to conventional pesticides. The need for differentiation in registration requirements has been recognized by the WHO/FAO in their recently published "Guidelines for the registration of microbial, botanical and semiochemical pest control agents for plant protection and public health uses" (WHO/FAO 2017).

In the United States, pesticides must be registered by the US-EPA, and meet the legal standards set forth in the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) and the Federal Food, Drug, and Cosmetic Act (FFDCA). Under FIFRA, a pesticide must not "cause unreasonable adverse effects on human and environmental health, when used in accordance with widespread and commonly recognized practice". The EPA includes microbial pesticides in the category of "biopesticides", which are pesticidal substances derived from natural materials. While biopesticides must meet the legal standards of FIFRA and FFDCA, the EPA recognizes that biopesticides may have certain benefits, such as increased specificity and a lower risk profile (Leahy et al. 2014). As a result, the EPA has developed a regulatory approach that encourages the development of biopesticides and streamlines their registration via shorter deadlines (*i.e.*, 18 months for registration of new biopesticide active ingredients used on food, compared to 24 months for a similar action for conventional pesticides) and lower registration fees. Due to the generally lower toxicity of biopesticides, data requirements are reduced and can be more easily waived.

In the EU, microbials are registered under European Commission Regulation (EC) No. 1107/2009. Under this Regulation, a microorganism is defined as any microbiological entity, including lower fungi and viruses, cellular or non-cellular, capable of replication or of transferring genetic material. The approval of microbial active substances is at the strain/isolate level. An exception to this is *Baculoviruses,* which have been approved on the species level.

Although it is acknowledged that due to the ability of microorganisms to proliferate there is a clear difference between chemical active substances and microbial active substances, and that potential hazards arising from microbial active substances

are not necessarily of the same nature as chemicals, EU Guidance on risk assessment of microbial active substances is lacking. Nonetheless, there is general agreement amongst EU regulators that these differences should be taken into account in the regulatory risk assessment. Similar to the situation in the United States, data requirements for microbials in the EU are less extensive than for chemical substances and are more often waived based on background knowledge about the microorganism. Although there are no harmonized guidance documents specific to the evaluation of microbials in the EU, the OECD Guidance on the environmental safety evaluation of microbial biocontrol agents (OECD Series on Pesticides No. 67) is generally followed. The European Food Safety Authority (EFSA) literature review on microbial organisms used in plant protection products (EFSA 2013) includes a broad summary of the public literature on microbials used in agriculture and may be referred to as a source of information on characteristics of specific microorganisms.

European Commission Regulation 1107/2009 itself does not contain specific procedural considerations for microorganisms or indeed any biopesticides; however, it contains the category 'Low risk substance/product', which contains several of the micro-organisms assessed so far under the Regulation. For substances that fall within this category, reduced timelines for evaluation and an increased length of authorization apply. Fees are often based on recalculation after completion of the dossier, which, due to the reduced time needed for evaluation, means that the evaluation procedure is also generally less costly.

5 Protection of Pollinators

As a part of the regulatory risk assessment of plant protection products, most countries require a risk assessment for pollinators. Often, these requirements focus specifically on honey bees, or on managed pollinators, rather than wild pollinator species. For the purposes of this chapter, much of the focus is on honey bees, as they are the representative species for the pollinator protection goals in the European Union (EFSA 2013; Croft et al. 2018) and United States (USEPA 2012), however, it is acknowledged that other pollinators are nonetheless vital to the overall pollination of plants worldwide (Kremen et al. 2002; Greenleaf and Kremen 2006; EFSA 2013; Garibaldi et al. 2013).

Other than pollination services provided by honey and bumble bees, they have also been used as vectors for microbial plant protection products (Butt et al. 1998; Al-Mazra'Awi et al. 2007; Kapongo et al. 2008). This area has been identified as a gap in knowledge (see "knowledge gaps", below) for the risk assessment of microbial plant protection products, and will not be directly addressed in the following section on the current regulatory risk assessment for honey bees, mainly because the risk assessments do not currently directly take this into account.

6 Regulatory Risk Assessment for Pollinators

The FAO Guideline states that data waivers may be accepted based on whether the MPCA is indigenous, if the amount released is similar to or below, or above the levels that commonly occur in the natural environment, and if there are relevant secondary metabolites. Information from good quality literature or studies of the pathogenicity of the organism for bees should definitely be provided for insect pathogenic MPCAs.

In the United States, FIFRA's implementing regulations set forth the data requirements for pesticide registration within Title 40 (Protection of the Environment) of the Code of Federal Regulations at Part 158 (Data Requirements for Pesticides; abbreviated as 40 CFR Part 158). However, data routinely required under Part 158 may not always be sufficient to assess whether there are unreasonable adverse effects on the environment. Therefore, the US-EPA has flexibility under 40 CFR Part 158.30(b) and 40 CFR Part 158.75 to require additional data when needed to fully characterize the effects of a pesticide, and to modify data requirements on a case-by-case basis. Compared to the conventional pesticides, fewer environmental fate and effects studies are required for microbial pesticides, and ecological risk assessments for microbials are generally hazard based and often more qualitative. Because environmental exposure cannot be accurately predicted for microbial pesticides, risk assessment and data requirements focus primarily on non-target organism effects (which include effects on pollinators). As with conventional pesticides, specific data requirements are influenced by use pattern, but are also graduated according to a tiered process based on testing outcomes, which also determine the scope of analyses for risk assessment. Microbial pesticide non-target data requirements are described in 40 CFR 158.2150, and consist of four tiers. Tier I describes toxicity and pathogenicity testing that is performed on the same set of taxa as with conventional pesticides (with additional requirements for testing with insects other than bees). Tier II describes environmental expression (persistence) data required in terrestrial and aquatic environments. Tier III consists of host range and chronic testing, and Tier IV consists of semi-field and field testing. Under this scheme, Tier I testing is performed first, and testing is done using the maximum hazard dose (MHD, usually at 10× – 100× estimated environmental concentrations). Since true exposure to living microbes cannot be reliably predicted, the MHD is meant to test whether a microbial pesticide causes toxic and/or pathogenic effects at levels that are reasonably higher than any exposure that may be encountered with microbial growth following application. If no adverse effects are observed in these studies, and unless other information (*e.g.*, literature reports; incident reports) indicate other concerns, testing is complete and the risk to non-targets is determined to be minimal. If adverse effects are observed at Tier I, testing advances to Tier II, which provides data on environmental concentration and persistence after exposure. If Tier II testing indicates environmental presence at levels similar to levels consistent with adverse effects observed in Tier I toxicity testing, then further testing at Tier III and/or Tier IV would be triggered. Typically, testing beyond Tier I is infrequent for

microbial pesticides, and many data requirements (*e.g.*, saltwater fish and invertebrate testing) are routinely waived.

In the United States, current microbial pesticide data requirements specific to pollinators are described in 40 CFR Part 158 Subpart V, and include Tier I honey bee testing (OCSPP 885.4380) and Tier IV simulated or actual field testing with insect pollinators (OCSPP 850.3040). Tier I testing with honey bees is intended to examine the potential for both toxic and pathogenic effects, and is required for all aquatic and terrestrial food/feed and non-food uses, forestry uses, and outdoor residential uses. Tier IV testing is conditionally required depending on effects observed in testing at lower tiers.

In the European Union, the data requirements for microorganisms in Commission Regulations (EU) No 283/2013 and 284/2013 specify information on toxicity, infectiveness and pathogenicity to non-target organisms. The choice of the appropriate test organism should be based on the identity of the microorganism (including the host-specificity, mode of action and ecology of the organism).

As mentioned in the previous section, there are no specific regulations or guidance documents related to the assessment of microbial actives or products in Europe. However, the Ctgb in the Netherlands has developed an evaluation manual (https://www.ctgb.nl/gewasbeschermingsmiddelen/toetsingskader/biopesticides-evaluation-manual) that outlines its general approach to risk assessment for microbial actives in the context of Regulation 1107/2009. Generally speaking, an active microorganism may give rise to risks because of its potential to infect and multiply in host systems, or due to its ability to produce relevant toxic metabolites during the production of the microbial pest control agent (MPCA) and/or in contact with the (non-)target organism. Therefore, the risk for non-target organisms should be assessed, unless it can be demonstrated that non-target organisms will not be exposed.

For the environmental risk assessment, information obtained by the characterization and identification of a microorganism forms the starting point. The proposed manner of use defines the nature and extent of potential exposure. In short, the risk assessment should take into consideration the following information:

- Mode of action and other biological properties;
- Survival and dispersal of the active microorganism in the environment;
- The ecological niche of the microorganism;
- The natural background level of the active microorganism, where it is indigenous;
- Where relevant, other authorized uses of the plant protection product in the area of envisaged use, containing the same active substance or which give rise to the same residues; and,
- Studies on toxicity, pathogenicity and infectivity.

During EU member state expert meetings on general issues on the risk assessment for microorganisms in 2007 and 2009 (the ‘List 4 meeting’ and PRAPeR M2, respectively, organized by the European Commission and European Food Safety Authority, respectively) agreements were reached on how to calculate initial off-crop

exposure densities in soil and water but not on the risk assessment for bees. For any given environmental compartment, the risk characterization should, when possible, contain a comparison of the predicted exposure with the available toxicity values from effect studies with the microorganism. However, when such a comparison is made, regulatory thresholds must be available with which to decide whether the risk is acceptable or not. The regulatory thresholds (assessment factors) used for chemical substances are not validated for microorganisms, and are only used for relevant metabolites/toxins, according to the decision criteria in Regulation (EU) No 546/2011.

Therefore, in most cases the risk assessment for microorganisms performed in Europe consists of a qualitative or semi-quantitative evaluation of the likelihood that an adverse effect will occur under the expected conditions of exposure. Based on this evaluation, it is determined whether the risk is acceptable or not.

The data requirements for pollinators are essentially the same as the data requirements for chemical active substances, with the caveat that some data can be waived considering the scope of use, expected exposure, type of microorganism and natural background levels. OECD No. 67, section 4.3.2 is often referred to in order to determine a testing scheme; however, regarding the current data requirements under Commission Regulations (EU) No 283/2013 and 284/2013, this Guidance is somewhat outdated. The available test guidelines referred to are USEPA Guidelines 885.4380 (the aforementioned Tier 1 testing), OECD 213/214 (the adult bee acute oral and contact toxicity studies, also utilized in chemical active substance risk assessment), the methodology for bee brood testing, as outlined in Aupinel et al. (2005), and the EPPO 170 guidelines (2010), which provide guidance for colony-level (semi-)field testing. All except the first of these tests was developed with chemical substances in mind and are routinely used for risk assessment of chemical active substances in Europe and the US/Canada. In addition, chronic adult honey bee (OECD 245) and 10 day repeated dose larval honey bee (OECD 239) toxicity study guidelines have recently become available, and are regularly recommended and included in recent microbial dossiers.

Unlike in the case of chemical active substances, as mentioned above, for microbials there is no quantitative assessment performed according to a hazard quotient (HQ), as is performed for the risk assessment of bees from exposure to conventional active substances. The OECD 67 recommends a similar, more qualitative risk assessment similar to the methodology used in the United States, mentioned above, with special consideration for bumble bees (*Bombus* species) due to lower hive temperatures which may be more conducive to microbial growth. Waivers are recommended in cases of negligible or minimal exposure, or non-entomopathogenic agents, if data are available to support that claim.

7 Knowledge Gaps and Areas of Uncertainty

Among the first tasks for the ICPPR working group on microbials and bees is to produce a white paper presenting issues and concerns with current guidance and providing options for improvement to help streamline and focus future efforts. Specifically, the paper will identify knowledge gaps that should be addressed, provide clarity as to areas that may already be addressed with current testing approaches or other information, consider common problems encountered in risk assessment and registration processes in the Americas and Europe, and provide insight into how best to characterize uncertainty and relay risk estimates to risk managers.

Knowledge gaps related to bee toxicity testing with microbial pesticides have been considered at an international level within the Organization for Economic Cooperation and Development (OECD) Expert Group on Biopesticides (EGBP, formerly the Biopesticide Steering Group). In a 2012 survey, the OECD EGBP identified several needs for bee testing, including further guidance or updated guidelines, including testing with brood, more guidance on the applicability of existing OECD test guidelines for ecotoxicological effects, and specifically the need to update the much-used US EPA OCSPP 855.4380 guideline, with specific consideration given to appropriate study duration (30 days, as currently required, is too long).

These knowledge gaps specifically refer to testing needs, however, there are also knowledge gaps related to broader questions about the context of the test within the risk assessment, including the life stage which is most appropriate for testing, the exposure/dose level and route, specific considerations for specific types of microbes (see example, below), the appropriate bee species to be tested (or whether multiple species should be tested), how to account for infection by-products and secondary metabolites, what place might behavioral effects and effects on gut microbes and hive microbial community have in the risk assessment, and are there cases where testing is actually not necessary to perform an adequate risk assessment.

As mentioned above, ideally, harmonized "considerations" could be available to determine optimal testing conditions depending upon the species, or even strain, being tested. For example, currently, when testing a fungi, it is unclear whether testing be performed at the optimal temperature and humidity for the bees (i.e. according to the Guideline) would represent an adequate worst-case situation. A case might be made for choosing a medium level between the optimal range for the bees and for the fungi, but it remains unclear whether the actual "worst case" situation has been tested, increasing the uncertainty in the risk assessment. Furthermore, different risk assessors may be of a different opinion of what would be the best way to test in order to achieve the most certainty in the risk assessment.

Another gap, as alluded to above, is the lack of accounting for the use of bees as vectors for certain microbial plant protection products in the risk assessment. Although work has been done on the safety of bees in the development of microbial plant protection products for use in combination with bees as distribution vectors (Butt et al. 1998; Al-Mazra'Awi et al. 2007; Kapongo et al. 2008), this information is not taken into account in the risk assessment for pollinators in any formal way,

nor is there a specific mechanism to do so. Furthermore, it is not clear whether a risk assessment should be performed for bees to be used as vectors – are they accounted for under the relevant protection goals? Will market pressures adequately ensure that the commercially obtained bees used as vectors for plant protection product application will at least be healthy enough to complete their tasks as vectors, anyway?

8 Conclusion

As shown in this brief chapter, much work remains to be done in the area of risk assessment for pollinators from microbial pesticides. Risk assessors and risk managers must be reasonably sure that no adverse effects on pollinators will occur, but existing test guidelines cannot address the unique potential risks from microbial products. In-depth knowledge of the micro-organism is required to decide whether testing is necessary at all, and, if so, to design a test that adequately represents a realistic worst case for use in a risk assessment. As a result, risk assessment, and likely risk management, of micro-organisms may require significant background knowledge and a more flexible mindset than is currently required for conventional plant protection products. Nevertheless, progress is being made toward addressing the most urgent gaps in knowledge for risk assessment. The aforementioned white paper should be completed within the next 2 years, and is envisaged to provide scoping for focusing of resources to address the most critical areas where knowledge is lacking and to improve harmonization of risk assessments world-wide. The greater aim of this effort is to provide the appropriate level of support for the development of pollinator-safe plant protection products for the Future of Agriculture.

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Flying Doctors for a Better Quality in Fruit Production

Maria I. Pozo, Julien Vendeville, Veerle Mommaerts, and Felix Wackers

1 Introduction

Flying insects can cover long distances making them potentially effective pollen vectors. In insect-pollinated plants, pollen movement, rather than movement of seeds, is generally the main component of gene flow (Pasquet et al. 2008). The transport of plant gametophytes via pollinators is therefore essential for plants to have access to the benefits of cross-pollination (Jordano et al. 2003). In the particular case of cultivated plants, current agricultural practices feature large-scale monocultures, requiring high peak pollination services to achieve commercial yield. At the same time, large fields and stringent weed control do not necessarily stimulate the presence of wild pollinators, given the low diversity in floral resources and extremely uneven distribution of floral resources in time (Bukovinszky et al. 2017). This agricultural intensification and loss of botanical diversity is a main driver of the global decline of wild pollinators, together with the use of pesticides, and in some cases incidence of pathogens (Goulson et al. 2015). Domesticated pollinators, such as honeybee and bumblebee colonies, are thus frequently employed in a range of horticultural crops to ensure sufficient fruit set (Corbet et al. 1991; Abrol 2012).

In addition to their role in crop pollination, these honeybee or bumblebee colonies can also be used for targeted application of (biological) crop protection products or other agricultural inputs, through so-called entomovectoring (Hokkanen and Menzler-Hokkanen 2007; Mommaerts and Smagghe 2011). Specific dispensers have been developed that load the outgoing bees with a product when the exit the hive. As these bees are flower foragers, the product is primarily vectored towards the

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inflorescences, making this application especially effective against flower-borne diseases and flower dwelling pests. Having functioning dispenser systems available, these can also be used to load (bumble)bees with crop pollen as a strategy to improve pollen transfer and hence pollination (Smagghe et al. 2012).

Honeybee colonies tend to be large (between 20.000–80.000 workers) and individuals may cover foraging bouts of up to 10 km (Beekman and Ratnieks 2000). However, their use for entomovectoring can be compromised by weather-dependent flight activity and due to the ability of honeybees to communicate the presence of prolific resources to colony members. This means that the presence of attractive flowering alternatives in the wider environment may distract the entire colony away from the target crop (Sapir et al. 2017). Moreover, honeybees show little attraction towards a range of crops that offer limited rewards in terms of pollen and or nectar quantity or quality (Delaplane et al. 2000). In a number of crops, growers facing this issue refer to mechanical pollen application as an alternative means to achieve sufficient pollination. In this technology, commercially available, hand-collected pollen is blown onto the flowering crop using hand-held or tractor-mounted blowers. The pollen application is typically performed once or twice a week. This mechanical pollen dusting is an extremely costly pollination method, given that the hand collected pollen often costs €1000–€3000/kilo. As most product is waisted during the blanket blowing of the crop, amounts of 500 g–1000 g are often needed per application. Add to this the substantial labour cost and the fact that not all flowers will receive pollen during their brief receptive phase and it becomes clear why there is a keen demand from growers for more (cost-) effective alternatives.

Domesticated bumblebees, on the contrary, consist of relatively small colonies that last up to 8 weeks in the field. While foraging distances may vary among species, they rarely exceed 3–4 km (Walther-Hellwig and Frankl 2000). As bumblebees are more limited in their communication capabilities regarding feeding resources, their foraging decisions are typically based on individual experience, which is advantageous for the pollination of lower-rewarding crops (Dornhaus and Chittka 2001 Mena-Granero et al. 2005). Their ability to creat their own heat, means that Bumblebees can fly at cool and overcast conditions. Bumblebees also have a large, hairy body that ensures a good loading for entomovectoring purposes. Finally, bumblebees show so-called “buzz pollination”, meaning that they grip the flower tightly and subsequently loosen the pollen by quick wing vibrations (audible as a short buzzing). This buzzing also effectively releases products that the bumblebees might be carrying, making them particularly effective in vectoring products to flowers (De Luca and Vallejo-Marin 2013). The commercial use of bumblebees for entomovectoring has become possible since the launch of the “Flying Doctors dispenser” (patented in 2013). The dispenser has a double chamber system, with hinged doors to ensure one-way traffic. The bumblebees exit through the top chamber with the replaceable pollen tray. Re-entry is through a second chamber underneath, allowing direct entrance into the hive (Fig. 1). The “one-way traffic” also prevents that bumblebees may collect the pollen from the tray directly, to bring it to the colony. The entrance is transparent to ensure maximum light incidence, encouraging bumblebees to leave the nest and forage. Product loading is optimized through the length of

Fig. 1 Flying Doctor hive. When bumblebees leave their nest, they pass through the dispenser tray. Pollen sticks to their bodies and legs and during flower visits pollen is delivered directly to the pistil (Biobest group)

the dispenser (Mommaerts 2010). To load the bees with microbial control agents, normally a carrier is used to increase the adherence of the product to the bee body. Pollen, on the contrary, has a very fine structure and low weight, so it typically adheres without the need to add carriers. The product to be dispersed may adhere to different parts of the bumblebee body with the bulk attached to the thorax and abdomen, while lower levels are adhering to legs (Mommaerts and Smagghe 2011). Since the launch of the "Flying Doctors" concept, we have tested the feasibility of pollen vectoring in a number of outdoor crops. Results from two case studies will be described here. The first involves open field studies in kiwi vines (France), while the second concerns pear orchards in Belgium.

2 Outdoor Use of Flying Doctors for Kiwi Pollination[1]

Kiwi (*Actinidia deliciosa*) is functionally a dioecious vine. The pistillate flowers contain non-viable pollen. Male plants have staminate flowers with a rudimentary pistil and viable pollen. Effective pollination is crucial to kiwi fruit production, determining fruit formation, weight and size (Abrol 2012). More than 90% of pistillate flowers can develop into fruit when properly fertilized (Hopping 1990). Each female pistil needs to receive about 3000 pollen grains to develop the required minimum of 700 seeds per fruit. Fruit quality variables, such as size or weight, are positively correlated with the number of seeds, as it has been consistently found in

[1] Content adapted from Pozo, M.I., Vendeville, J., Kay, C. and Wackers, F. (2018). Entomovectoring technology in kiwifruit pollination. Acta Hortic. 1218, 381–390.

other crops (McKay 1978; Pyke and Alspach 1986; Testolin 1991; Vaissiere et al. 1991).

Kiwi flowers show adaptations to wind pollination, as well as insect pollination, but the sole action of wind often results in an uneven pollen donation that does not yield marketable fruits (Pyke and Alspach 1986; Corbet et al. 1991). An optimal pollination, considering the current agricultural practices, requires the combined action of wind and insect pollination (Costa et al. 1993). When male plants are grown in the orchard, growers typically rely on naturally occurring pollinators (e.g. solitary bees, syrphid flies) or on honeybee hives to ensure pollination. A yield increase between 29–300% has been reported due to insect pollination in commercial kiwi orchards (Abrol 2012). However, kiwi flowers are nectarless, limiting their attractiveness to most naturally present pollinators. Honeybees show little attraction towards kiwi flowers, so the transfer of pollen is quite uneven throughout the orchard and blooming period (Bomben et al. 1999). Moreover, nowadays kiwi growers tend to produce their kiwis under hail nets or plastic covers, which hampers honeybee orientation, and limits their use even further. Hence, it is challenging to realize sufficient pollination in kiwis and to achieve optimal fruit set.

Artificial pollination has been widely recommended to secure a good crop of large-size fruits (Sano 1987; Holcroft 1989; Hopping 1990; Costa et al. 1993). Nowadays, manual applications of male pollen, either by dusting or by spraying a wet pollen suspension, are the most widely used supplemented pollination methods. However, both techniques require substantial labor, which has been estimated to often exceed 50 h/ ha (Hii 2004). The scarcity of adequate commercially available male pollen means that kiwi pollen prices are high (3500 $ per kilo in 2016). As a result of these cost factors, growers are applying male pollen twice during the blooming season. However, kiwi pistillate flowers are most receptive during 4 days after anthesis, which means that a great proportion of flowers will not be dusted with male pollen at the right time (González and Coque-Fuertes 1996).

Kiwi production is concentrated in a limited number of countries and the market is dominated by New Zealand and Italy. In France kiwis have been grown increasingly, and the global production (approximately 80,000 t) makes it the second European producer. In the last years, netting is being increasingly used to avoid the devastating effects of hail. As a result, insect pollination by honeybees or wild bees is very limited because their activity is greatly hampered under the net. Commercial bumblebees could be an alternative to honeybees. Here we conduct comparison tests in France between different pollination methods, namely wind, mechanical pollen dusting, wild pollinators plus honeybees, and Flying doctors technology by using commercial bumblebee hives. Pollination success was measured by fruit set, fruit quality at harvest, and number of seeds per fruit. In the particular case of the use of commercial bumblebee hives, we also evaluated flight activity of the hives and transfer of pollen by bumblebees.

In order to compare the four different pollination treatments, the total surface was divided in 4 plots of 150 m^2 each at two different experimental sites (commercial plantations of Hayward kiwi vines, named as M and D hereafter). Mesh was use to prevent the entrance of other visitors excepting the “open” compartment in which

wild pollinators and honeybees were present. In order to provide an overview on pollen use, dusting was applied twice during the blooming season, at a dose of 250 g pollen/ha/application. Bumblebees hives with Flying Doctors® dispenser were filled with 6 grams of male kiwifruit pollen and the pollen was refreshed daily during the experiment.

Our results show that bumblebee hive activity was significantly higher in M site, where both hives had a better flight activity throughout the blooming season ($\chi^2 = 156.50$, $p = 0.018$). The amount of pollen transported per hive was positively correlated with overall activity (Rs = 32.68%, $p = 0.03$). However, there were no differences among the two experimental sites in pollen transport ($\chi^2 = 64.00$, $p = 0.6649$). There was variation among dates, and we could see a trend towards a slight decrease over time ($\chi^2 = 10.69$, $df = 5$, $p = 0.0579$).

Due to the large amount of flowers that are produced in mature kiwi vines, the amount of fruits produced per tree was estimated for each plot (N = 4 treatments∗2 plots/treatment), which led to a low statistical power. Nevertheless, we saw that treatment had a significant effect on the number of fruits produced ($\chi^2 = 10.21$, $df = 3$, $p = 0.0168$). Such a difference was mainly driven by the difference between wild pollinators + honeybees and wind alone (Tukey HSD test, $p = 0.0460$). For both sites, wind resulted in the smallest amount of fruits produced per plot (234 and 980 in D and M sites, respectively), compared to more than 2200 fruits per plot that were consistently counted for the honeybees and wild pollinators plots at both sites. More limited fruit production in wind only vs. insect pollination is consistent with the findings by Costa et al. (1993). According to this research, wind only led to fruit set of around 81%, while fruit set above 98% could be achieved by the introduction of honeybee hives. However, the advantage of using honeybees strongly depends on the specific conditions of the trial, as their foraging activity is substantially affected by weather conditions, the orchard layout and the location of other plant resources. Bumblebees are endothermic pollinators that can fly at low temperatures. In addition, they need a large food supply to fuel their foraging needs, together with pollen to feed their brood (Goulson 2010). When used at the advised introduction rate, bumblebee colonies ensure a good fruit set, while limiting labor in manual thinning.

Fruit quality results are summarized in Fig. 2. The complexity of the techniques is depicted in Fig. 2 by using a darker color from white (wind only) to black (Flying Doctors), with dusting and open pollination as intermediate treatments. Comparing the two dates at which the quality was assessed, we saw that the size of the fruits was smaller at the later date. High temperatures during the late summer period has been shown to slow down the developmental rate of kiwi fruits. Therefore, the fruits of early flowering kiwi vines tend to be larger (Snelgar et al. 2005). For a given date of monitoring, the use of more complex pollination treatments significantly improved the size of the fruit in both experimental sites (Fig. 2a). Wind pollination led to the smallest fruits in all cases, followed by dusting and honeybees + wild pollination, while the largest fruits were obtained in the Flying Doctors treatment. Our results are in agreement with previous studies that showed a limited success of dusting as pollination technique when this is not applied frequently (Razeto et al. 2005).

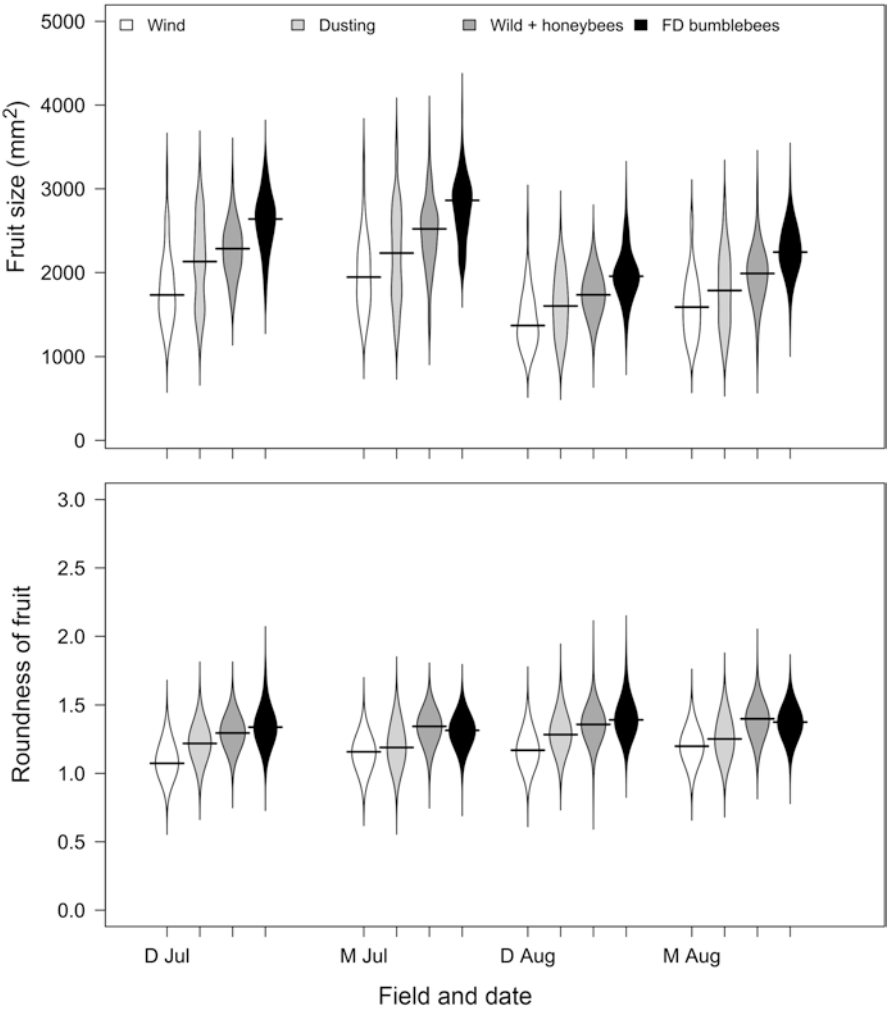

Fig. 2 Fruit size (mm^2, upper panel) and fruit roundness coefficient of individual kiwi fruits that were achieved by using different pollination techniques (wind, dusting, wild pollinators plus honeybees and Flying Doctor bumblebee hives) in two commercial orchards in France. Reprinted from 'Pozo, M.I., Vendeville, J., Kay, C. and Wackers, F. (2018). Entomovectoring technology in kiwifruit pollination. Acta Hortic. 1218, 381–390.' Copyright 2018 by ISHS

However, a higher frequency of dusting is often unpractical and uneconomical in terms of labor cost and pollen use. In the particular case of D site, fruit size did not differ between dusting and honeybees + wild pollination at both dates. This can be explained by a lower abundance of pollinators in open plot at D site, compared to M (results not shown). Consistently, at the M site there were more pollinators present. Here the size of the fruits approached the size of the Flying Doctors treatment. This indicates that a continuous vectoring of pollen, as provided by wild pollination +

honeybees and by the Flying Doctors technology, optimizes pollination by increasing chances of pollen transfer at the optimal flower age.

The fact that the use of the Flying Doctors treatment yielded the biggest fruits was seen in both fields, and this size difference remained significant in the two consecutive harvesting dates (results not shown). This technology produced fruits of similar size in both fields for the first set, while for the second date fruits were larger in the M field. This result is consistent with the higher flight activity that we registered at this site.

There are several references in the literature about the relative contribution of wind and insects in kiwi pollination, and how this relates to fruit quality. Clinch (1984) found that honeybees were the most numerous visitors in the kiwi orchard, as wild bees were spotted too infrequently to have impact on pollination success. We found that honeybees increased fruit set, but this improvement did not translate into a substantial improvement of fruit quality. This outcome is consistent with Costa et al. (1993), who found that honeybee pollinated kiwi fruits had an average weight of just 70 g. Such an outcome might be explained by the foraging behavior of honeybees compared to other bees. Honeybees have a complex communication system that allows the colony to focus on specific pollen and nectar sources. Therefore, honeybee hives tend to focus either on female or male kiwi vines, and they would not contribute to pollen flow from male vines to female flowers (Craig and Stewart 1988). Overall, it seems that honeybees show floral sex constancy and a preference towards female kiwi flowers (Goodwin and Steven 1993). Despite the fact that bumblebees are social as well, they show more limited communication abilities and workers of the same colony make individual foraging choices, based on their own experience (Abrol 2012). Donovan and Wier (1978) found that bumblebees were the most effective pollinators for kiwifruit due to their foraging activity under adverse climatic conditions and the large contact between their body parts and stigmas. Consistently, adding more commercial bumblebees to a kiwi orchard helped to increase fruit size according to Wearing (1986).

When comparing standard bumblebee colonies to Flying Doctors colonies equipped with the pollen tray, the latter proved to further improve pollination efficacy. When male and female plants bloom synchronously, the sole use of insect vectors that fly to both sexes would ensure a good fruit production. However, the blooming season of males and female kiwi vines is strongly affected by weather conditions, and both sexes may bloom with a difference of up to 10 days. In those cases, having male trees in the orchard does not improve pollination, as they are not releasing any pollen during the most receptive period of pistillate vine blooming. Hence, the use of Flying Doctors hives, that ensure continuous “dusting” of the pistillate vines, together with pollen donation from male trees would be optimal to guarantee pollination under all conditions.

The analyses of shape are indicative of an adequate natural pollination, as the application of plant growth hormones leads to misshaped fruits (Crane 1969; Martin et al. 1970; Ogata et al. 1989). For each date and field, we saw that shape analyses matched with the trend we saw for fruit size: the more “complex” the pollination system, the more rounded the fruits. In this case, the use of Flying Doctors and wild

pollination + honeybees did not differ in terms of roundness of the fruits (Fig. 2b). These results are consistent with the pollination success that we inferred from fruit size measures in Fig. 2a. Besides indicating the quality of the pollination process, fruit shape is one of the most important quality parameters for commercial evaluation of kiwi fruits.

Based on the differences that were obtained in fruit quality by using different pollination techniques, we decided to do a more detailed trial with Gold3 variety in France in which we compare the best treatment (Flying Doctors) against a positive control in which we aim to have a perfect pollination by manually pollinating flowers at the right floral stage. This technique has been consistently used as a reference pollination system that ensures a well pollinated fruit of more than 100 g (Costa et al. 1993). As male trees tend to be used in orchards, and considering the promising results we got for wild pollinators and honeybees, we decided to use regular bumblebee hives as well, to assess pollen donation from male trees present in the orchard.

Firstly, we checked the flight activity of both types of hives, and hives with the Flying Doctors dispenser had an overall activity of 0.60 ± 0.11 bees per minute, while regular hives had an activity of 0.40 ± 0.10 per minute. This activity was rather low, which is likely explained by the poor weather conditions during the trial. The differences in flight activity between treatments translated into a slightly higher presence of bumblebees on kiwi flowers in the Flying Doctors plot, compared to the one in which regular hives were used (0.45 ± 1.25 and 0.40 ± 1.12 bumblebees recorded in 200 open kiwi flowers, respectively).

Fruit quality in bumblebee-pollinated vines was equally good as in our positive reference treatment, with an average fruit weight of more than 130 grams (Fig. 3). Previous attempts to assess bumblebee efficiency as kiwifruit pollinator did not find differences in seed content, as indicator of the success of the pollination process (Wearing 1986). In our trial, bumblebee and Flying Doctors treatments (bumblebee hives with and without pollen dispenser) had a higher number of seeds per fruit than the positive control, hand-pollinated fruits ($Z = 8.40$ and $Z = 5.25$, respectively, $p < 0.0001$, Fig. 3).

Our results show that bumblebees can achieve similar to even better results than hand-pollination, with a substantial reduction in pollen and labor. At our sampling site and date, weather resulted in a large overlap in the blooming period of female and male plants. This synchrony allowed a very good donation of pollen from male trees by using regular bumblebee hives. However, each blooming season is subject to great environmental stochasticity. In addition, kiwi plantations strongly differ in the ratio of male trees, and sometimes male trees are totally absent. The use of the Flying Doctors dispenser, pre-filled with kiwi male pollen, offers an optimal solution in such circumstances and thus provides growers assurance of pollination success.

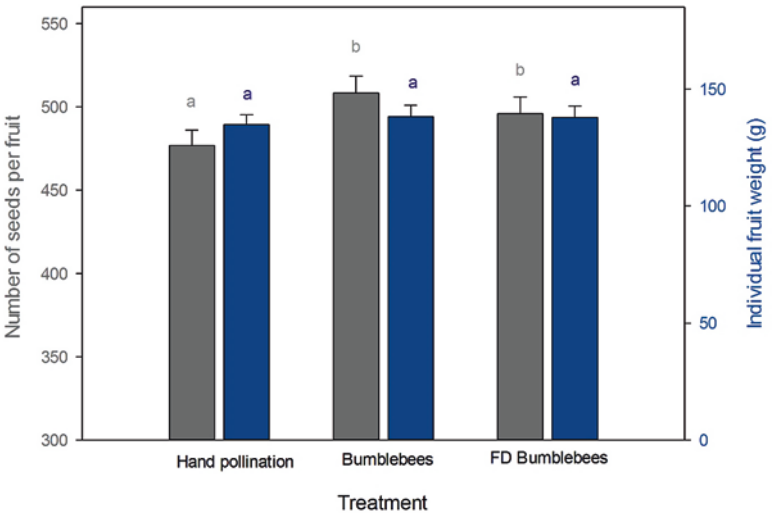

Fig. 3 Number of seeds per fruit (left Y axis, indicative of the quality of the pollination) and individual fruit weight (right Y axis, indicative of fruit quality) per treatment. Pollination treatments are shown along the x axis. Different letters denote means that are statistically significant at $p < 0.05$. Reprinted from 'Pozo, M.I., Vendeville, J., Kay, C. and Wackers, F. (2018). Entomovectoring technology in kiwifruit pollination. Acta Hortic. 1218, 381–390.' Copyright 2018 by ISHS

3 Use of Flying Doctor for Pear Pollination

The yields of cultivars of the European pear (*Pyrus communis*) are frequently poor on young trees and inconsistent from season to season on mature trees. Yields on pear trees are influenced by the number and quality of flowers produced, by the efficiency of pollination and fruit set, by the severity of natural or induced abscission of fruitlets and by the degree and rate of cell division and expansion in the persisting fruits (Webster 2000). Blooming occurs in early spring, under sometimes unfavourable weather conditions (e.g. frost, rain and low temperatures), which can limit pollination by insects.

Pears may produce parthenocarpic fruits, i.e. fruits that are formed even without fertilization of ovules. "Conference', the economic most important pear cultivar in Belgium, is a self-fertile cultivar (Nyéki et al. 1993). However, when cross-pollination takes place, fruit quality -in terms of fruit size and symmetry- significantly improves (Sedgley and Griffin 1989; Bellini 1993; Delaplane et al. 2000). In Belgium, the variety Doyenne do Comice is commonly planted in the same orchard as pollinizer given its compatibility and bloom synchrony (Free and Spencer-Booth 1964). In practice, plant growth hormones such as gibberellins are also commonly used to improve fruit set artificially, resulting in fruits that are entirely or partially seedless (Richard et al. 2001; Tromp and Wertheim 2005). In Zhang et al. (2008), GA3, GA4 and GA7 treatments ensured an increase in fruit set percentage of unpollinated *Pyrus communis* flowers. Still, when using this technique, yields are

compromised due to the high percentages of small and misshapen fruits (Yamada et al. 1991).

Honeybee colonies (*Apis mellifera*) are often used in fruit orchards for pollination, but pear flowers are not very attractive to honeybees due to their limited nectar production of low sugar content (Jacquemart et al. 2006). Several other bee species have been proposed as alternative pollinators. One of these species is the mason bee *Osmia cornuta*, a solitary bee that occurs in Northern Africa, Central and Southern Europe. This species has been studied for crop pollination in different European countries. They fly early in the year, are present throughout the whole flowering period and have a greater tolerance to inclement weather (rain, wind etc.). However, their natural abundance in pear orchards tends to be too low (Maccagnani et al. 2003). Landscape management may be used to enhance natural pollinator numbers (Campbell et al. 2017), or managed bumblebee colonies may be employed to enhance pollination.

Here we investigated if pollination by bumblebees in combination with the use of gibberellins could improve fruit set and fruit quality of 'Conference' and 'Doyenne' pears. To test this, bumblebee hives (*Bombus terrestris*) with and without Flying Doctors dispenser were introduced in our test fields in Belgium with a minimum distance between treatments of 100 m. The dispenser trays were filled with circa 3 grams of Doyenne de Comice pollen and renewed every 3 days. A selected number of flowers was pollinated by hand as a reference, to establish that the used pollen was viable and leads to fruit development. We used a density of 3 multi-hives (sets of 3 hives) per ha. In total, for this test we used 90 grams of pear pollen. The addition of Lycopodium pink dye allowed us to see donation of pollen from the Flying Doctors tray to the flowers themselves (Fig. 4).

In this trial we estimated the activity of the bumblebee hives and also measured the amount of pollen that was dispersed by each colony, along with fruit quality and yield for each treatment. The number of developed pits per fruit was also scored.

Flight activity was moderate at the beginning of the blooming period (Table 1), which coincided with adverse climatic conditions and very low numbers of mature

Fig. 4 Detail of a pear flower. At the center is can be spotted the addition of pink-stained pollen from the Flying Doctor tray to the stigmas

Table 1 Number of incoming and outgoing bumblebees per 10 min census for all colonies used in the trial and pollen transport in grams, along the blooming period

Average for all nest	25/Apr	29/Apr	3/May	7/May
In+out in 10 min	1	6	14.5	
Pollen transport (g)		4.6	4.8	4.8

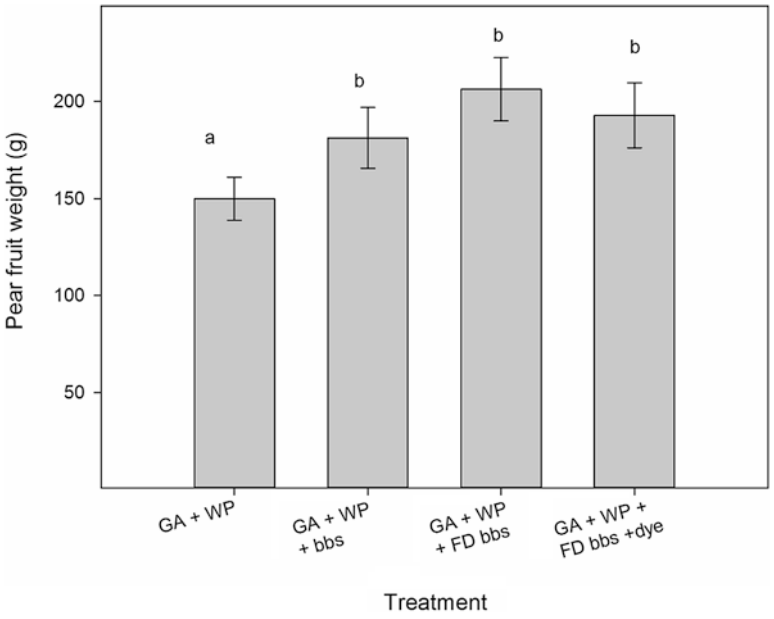

Fig. 5 Average pear fruit weight (+SE) per treatment. All treatments included the use of phytohormones (GA: GA 4/7, 1 L per ha) and wild pollination (WP) as baseline conditions. The use of regular bumblebee colonies (bbs: 3 multihives per ha), bumblebee colonies but with pollen dispenser (FD bbs, with Doyenne pollen), and FD bumblebee colonies in which a dye was added to the pollen to see pollen donation and transport from these hives (FD bbs + dye). Different letters above bars indicate means that were significantly different at $P < 0.05$

flowers (results not shown). At the second week activity increased more than twofold reaching a good flight activity.

Interestingly, fruit quality estimated as average fruit weight, significantly varied across treatments. The application of GA's led to a pear fruit of 150 g on average. The addition of bumblebees increased this fruit weight by 20.7% (Fig. 4). Maximum fruit weight was achieved by the use of the Flying Doctors (206 grams, on average). The use of lycopodium as a dying carrier had a moderately negative effect on fruit weight. Hand-pollinated fruits, contrary to our expectation, did not statistically differ in weight from those in which only GA's were added.

The percentage of fruits with developed pits ranked from less than 14% in the phytohormone plus wild pollination treatment to 100% in hand-pollinated fruits. The addition of bumblebee colonies with or without dispenser led an intermediate 50% number of developed pits in both treatments.

Fruit weight significantly varied across treatments (F_{4324} = 20,43; p-value: <0.0001). Differences were mainly found between the use of phytohormones combined with natural visitation and the remaining 3 treatments (Fig. 5). Such an outcome is in agreement with our previous results for kiwis, where we saw that the use of Flying Doctors, as well as the use of several pollination methods simultaneously-improves yield and fruit quality. Numerous reports have already found that the use of phytohormones alone in parthenocarpic varieties does not yield good fruit production (e.g., Silva et al. 2007 and references therein).

Total yield increased from 51 ton/ha (both for GA's plus natural visitation and for the treatment with the addition of bumblebee colonies without pollen dispenser) to 54 ton/ha when bumblebee colonies with pear pollen were added. This represents a moderate increase that is consistent with previous findings in self-fertile pear cultivars (Zhang et al. 2008). However, the percentage of fruits with 'ideal fruit size' increased from 6% to 55%. Yield per hectare shows that the use of phytohormones plus natural pollinators led to a majority of harvested fruits of low caliber and subsequent lower market value. The use of regular bumblebees improved these figures with fruits of ideal size (65–75 mm) being dominant. When pollen was added via the dispenser, the proportion of fruits of the maximum quality further increased

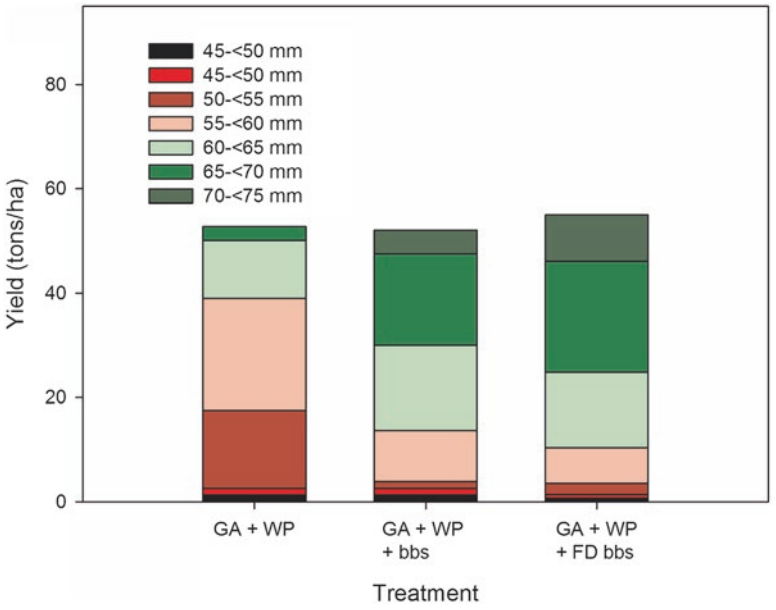

Fig. 6 Yield per ha in conference pears per treatment and fruit category. Ideal fruit size lies between 65 and 75 mm

1.5 fold, relative to bumblebees alone (Fig. 6). This resulted in a added financial yield of around 7000 euro per ha (based on the 2013 price of Conference pears in Belgium).

Our results for the use of commercial bumblebee colonies in pear orchards differ from previous reports. For instance, van den Eijnde (1995) reported that bumblebees showed poor visitation of pear flowers and that the addition of colonies did not have a great impact on fruit set. Here we have proven, using dye plus pollen in a Flying Doctors dispenser, that the commercial bumblebees did visit the pear flowers and transfer the product. The use of bumblebees alone helped to improve fruit quality, but the main added value of bumblebees was seen in the significant increase in fruits of ideal size and top market price. This outcome that was mainly achieved when using colonies equipped with the entomovector technology.

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