Arthropod-Plant Interactions (2011) 5:81–95
DOI 10.1007/s11829-011-9123-x

REVIEW ARTICLE

Entomovectoring in plant protection

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Received: 19 July 2010 / Accepted: 12 January 2011 / Published online: 26 January 2011

Abstract This paper gives an overview on the unique concept of the entomovector technology to employ pollinating insects, including honey bees and bumble bees in the context of biological control of insect pests and diseases. After a brief introductory description, the multifaceted aspects of this intriguing technology are highlighted by describing the most significant results and achievements of research groups around the world concerning: (1) the importance of vector selection, as this determines the transport efficacy of biocontrol agents into the crop and is influenced by the vector–plant interactions, (2) the different potential biocontrol agents used so far, (3) the significance of the diluent and formulation for an increased vector loading and transport, (4) the different dispenser types developed over the past 20 years, and (5) the safety of this technology to the environment and humans. For all these interactions, we identify in a critical manner the limitations and the successes obtained so far. The needs for further research are also discussed to increase the potential of the entomovector technology in practical use.

Handling Editors: Sam Cook and Heikki Hokkanen.

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Keywords Pollinator · *Apis mellifera* · *Bombus terrestris* · Vector · Crop protection · Dispenser · Biological control agent · Microbial control · Formulation

Introduction

In agriculture and horticulture pollination plays a key role in the establishment of successful fruit setting, which is of high economic importance with an annual worldwide value estimated to be 153 billion euro (Velthuis and van Doorn 2006; Gallai et al. 2009). Since the dawn of their existence insects and plants have co-evolved to benefit from each other, but also plant pathogens have co-adapted for optimal dispersion. Short and long distance dispersion of diseases is mediated by abiotic factors, but also insect vectors such as pollinators can contribute to this process. Indeed they are known for their capacity to transmit viruses and fungal and bacterial spores together with pollen (Card et al. 2007). Based on this, the concept originated of using pollinators as vectors in a new, environmentally friendly control strategy to disseminate control agents against plant pathogens and insect pests into crop flowers (Peng et al. 1992). In this context pollinators are not only important for their pollination services, but they fulfil an important dual role.

The term "entomovector technology" was first used by Hokkanen and Menzler-Hokkanen (2007), and the approach incorporates different ecological components such as pollinators, biocontrol agents and plant pathogens/insect pests (Kevan et al. 2008). However, its success is 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 the human health (Fig. 1).

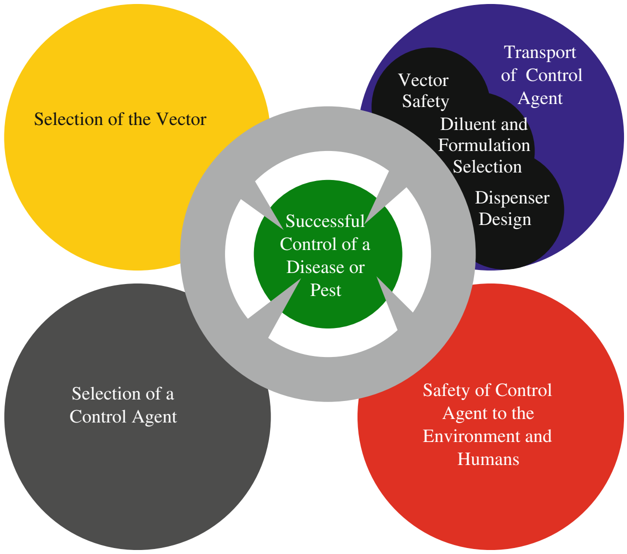

Fig. 1 Schematic view of the multifaceted interactions within the entomovector technology. Adapted from Kevan et al. (2008)

Since the first report by Peng et al. (1992) almost 20 years ago, three vector species have been used in entomovectoring studies, namely honey bees (*Apis mellifera* Linnaeus (Hymenoptera: Apidae)), bumble bees (*Bombus impatiens* Cresson (Hymenoptera: Apidae) and *Bombus terrestris* Linnaeus (Hymenoptera: Apidae)) and in some particular cases the mason bee (*Osmia cornuta* Latreille (Hymenoptera: Megachilidae)). The limited number of vector species can be explained by their commercial availability. Bumble bees and honey bees are available the year around, allowing pollination to be synchronized with the blooming period of crops, whereas *Osmia* management remains a challenge. Several laboratories, however, are making progress to develop standardized protocols to control the time of emergence of *Osmia* (Pitts-Singer 2008; Sgolastra et al. 2010).

The primary reasons to introduce the entomovector technology as biocontrol strategy were to reduce the application of synthetic pesticides because of concerns of their impact on human health and the environment, and because of the development of resistance by pests against commonly used chemical pesticides. Also the poor control results achieved by spraying biocontrol agents, contributed to the employment of the entomovector technology. The assumption was that the entomovector technology performs better than spraying of a biocontrol agent because of the ability to deliver the agents directly onto the target location, i.e. the flowers. Moreover, flowers of crops play an important role in the life cycle of several important plant pathogens (reviewed by Ngugi and Scherm 2006) and after successful infection result in economic losses. Similarly, pests inhabiting flowers are also known to affect yields (Jones 2004; Dedryver et al. 2010). In this context, we discuss in this review the role of microbial control agents (MCAs) including fungi, bacteria and viruses, and the usefulness of vectoring by pollinating insects in the control of economically important diseases and pests in agriculture and horticulture.

For transport of the MCAs by the entomovector to the target, the formulation of the MCA is of crucial importance. As many commercial, powdery MCA formulations are developed for a water-spray application, these can be improved for an optimal transport by vectors. However, the acquisition of the product on the body of the vector is not only affected by the formulation, as also the dispenser needs to be appropriate. Over the past 20 years, multiple authors have reported on the development of different dispenser systems to allow the loading of vectors with a biocontrol agent. To date, eight dispenser types and a few modified versions have been designed for *A. mellifera* (Fig. 2). In parallel, six dispensers were developed for bumble bees, whereof two and a modified version for *B. impatiens* and three for *B. terrestris* (Fig. 3), and one type for the solitary orchard pollinator *O. cornuta* (Fig. 4). However, so far only few have been used in practice, and thus the efficacy and limitations of each system will be discussed on a more general level.

A final aspect to which attention must be drawn is the environmental and human safety of the entomovector strategy. The MCAs used so far have been isolated from the environment and thus are present in the target ecosystem. However, considering their niche, which mainly is the soil or the foliage, vectors rarely come into contact with them under natural conditions, and thus their safety towards the vector must be evaluated. So far vectoring studies only observed mortality when disseminating insecticidal MCAs, but care is needed as sublethal effects are not always obvious.

The aim of this review is to demonstrate the capacity of the entomovector technology for the dissemination of MCAs in the context of the biological control of plant diseases and insect pests, as explored since the early 1990s.

Selection of the vector

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.

As listed in Table 1, honey bees and solitary mason bees are used to vector MCAs onto crops under field conditions. However, it is known that honey bee foraging activity is sensitive to environmental conditions. A good example is that honey bee workers do not fly, or fly only poorly during

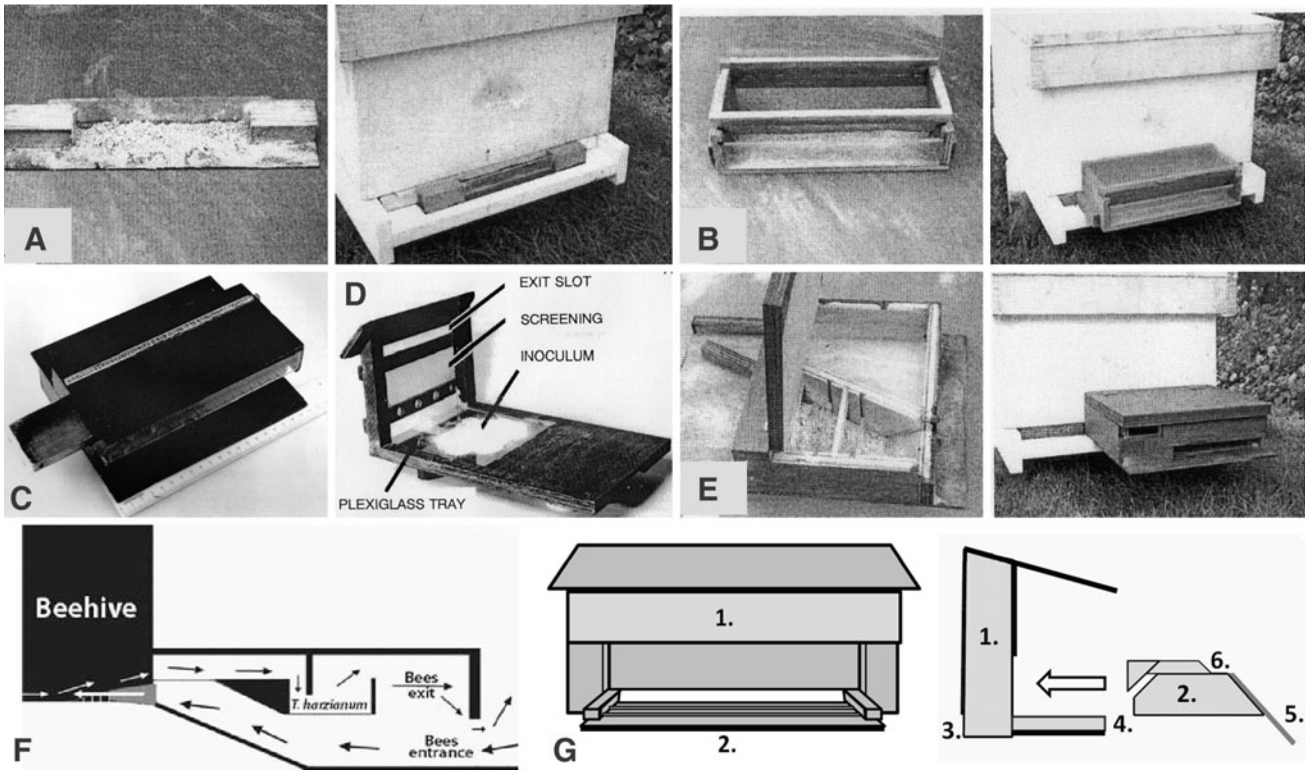

Fig. 2 Overview of the different hive-mounted dispensers developed over the past 20 years for honeybees. One-way type dispensers: **a** Tub and **b** Hardwood, and two-way type dispensers: **c** Gross et al. (1994), **d** Peng and **e** Triwaks, **f** Houle and **g** BeeTreat. Adapted from Peng et al. (1992), Gross et al. (1994), Bilu et al. (2004), Albano et al. (2009) and Hokkanen et al. (2011)

rainy days, and as a consequence, unvisited flowers are less protected and are likely to become infected. Indeed Vanneste (1996) and Maccagnani et al. (1999) postulated that weather conditions affect the capacity of the bees to transmit biocontrol agents. However, future studies should investigate the impact of weather, specifically dry conditions, on the potency of the MCA and/or the vectoring activity. For honey bees, it is also well known that they are less appropriate to be used for pollination in greenhouse long-blooming crops (Cribb and Hand 1993; Guerra-Sanz 2008). For *O. cornuta*, it should be mentioned that studies have been performed in orchard crops only so far (see for review Bosch and Kemp 2002). In contrast, for bumble bees, most entomovector studies have been conducted under greenhouse conditions (Table 1) as they are economically widely used to pollinate greenhouse crops because of their tolerance to the high temperature fluctuations typical to the greenhouse (Guerra-Sanz 2008). However, bumble bees are also suitable pollinators in open fields, and Goulson (2010) defined them as bad weather foragers compared to honey bees. Another important vector-species dependent factor is the range of foraging. Compared to honey bees, *O. cornuta* and bumble bees like *B. terrestris* stay in the close proximity of their nests, ranging between 100 and 200 m (Vicens and Bosch 2000) and 800–1,500 m (Wolf and Moritz 2008; Osborne et al. 2008), whereas effective foraging distances up to 3 km have been reported for honey bees.

Major factors determining the crop visitation rate by the vector are the vector–plant interactions. Kovach et al. (2000) reported on the impact of the attractiveness of strawberry crops for honey bees in open field trials. Similarly in a vectoring study, Peng et al. (1992) sprayed a bee-attractant (Bee-Scent®) to increase attractiveness, whereas other vectoring studies assured honey bee visits by increasing the colony size from 5,000 to 9,000 bees/hive (Shafir et al. 2006) and even up to 50,000 bees/hive (Escande et al. 2002). In addition, in open fields the population levels of other bees in the vicinity might affect the frequency of bee visits to the crop (Vanneste 1996). Here the decision of a bee to visit a flower is driven by floral-related cues such as size (Spaethe et al. 2001; Lunau et al. 2009), colour (Forrest and Thomson 2009), odour (Farina et al. 2007; Molet et al. 2009), temperature (Rands and Whitney 2008; Whitney et al. 2008) and by the floral reward (Stout and Goulson 2002; Raine and Chittka 2007; Gil 2010). Wolf et al. (1999) and Roldàn-Serrano et al. (2005) showed that varying nectar sugar concentration among plant cultivars affected the bumble bee visitation rate. Therefore many vectoring studies used up to four plant cultivars per

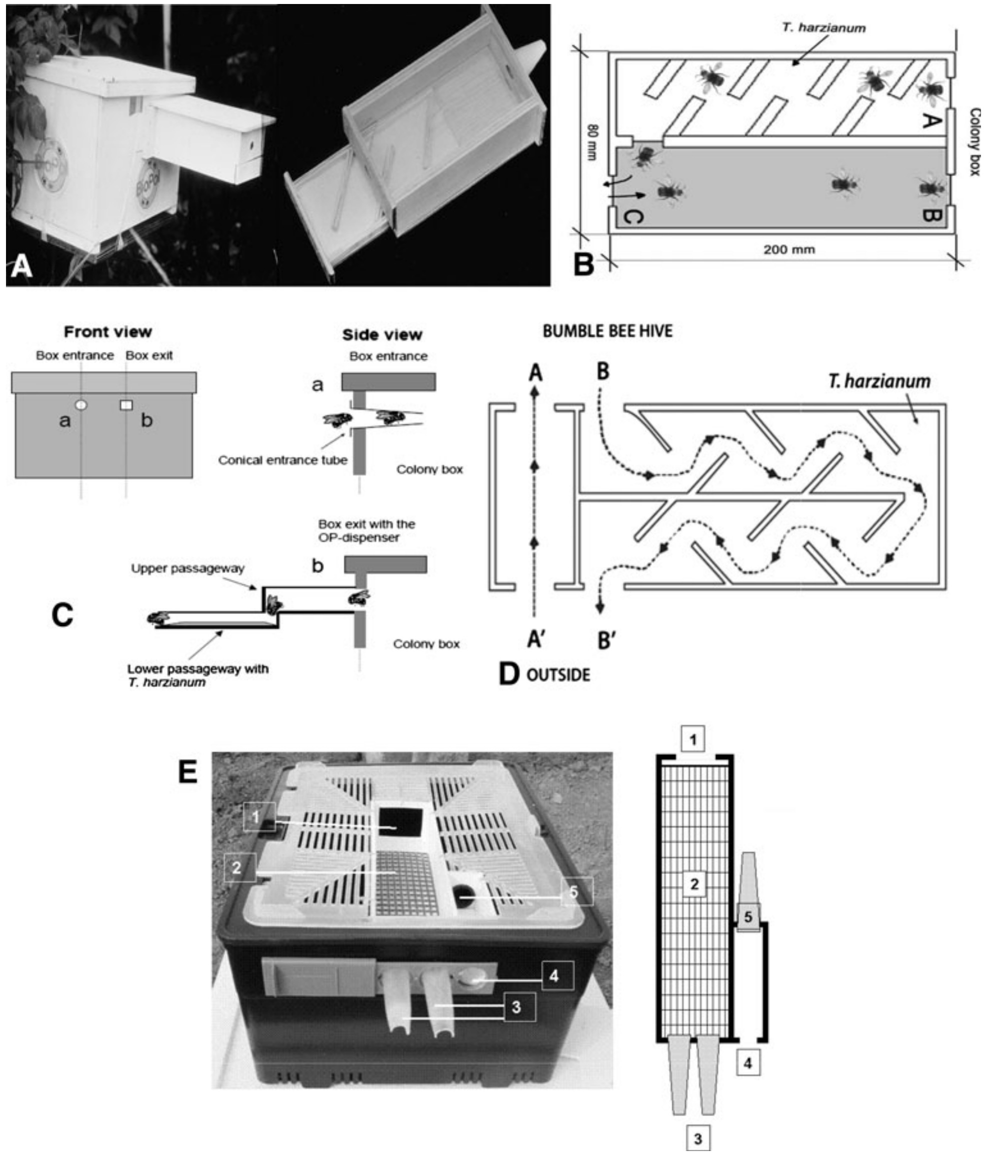

Fig. 3 Overview of dispensers developed for the bumblebee *B. impatiens* and *B. terrestris*. One way type dispensers: **a** Yu and Sutton (1997), and **b** SSP and two-way type dispensers: **c** OP; **d** Houle and **e** the dispenser as developed by Mommaerts et al. (2010b). Adapted from Yu and Sutton (1997), Maccagnani et al. (2005), Albano et al. (2009), and Mommaerts et al. (2010b)

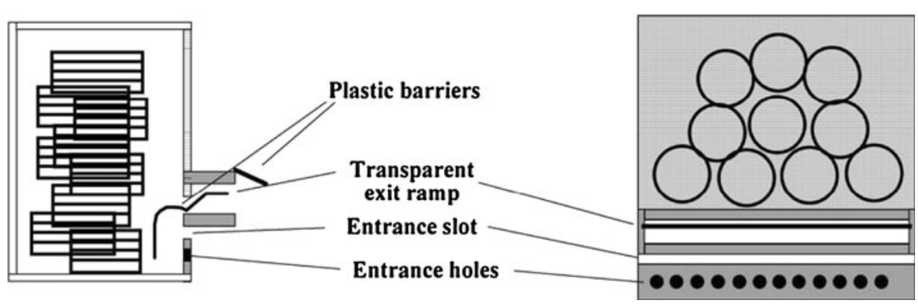

Fig. 4 The two-way dispenser developed for the mason bee, *O. cornuta*. Adapted from Maccagnani et al. (2006)

field plot to increase bee attraction (Yu and Sutton 1997; Kovach et al. 2000; Escande et al. 2002). Since the rapid decline of the nectar sugar concentration during anthesis and the correlation between flower age and host susceptibility (Ngugi et al. 2002), vectoring should start before most of the flowers are open.

Deposition of the MCA on the target is necessary for the success of the entomovector technology. However to guarantee deposition on the flower organs the vector needs to be an efficient pollinator of the crop. Indeed, a good control of *Erwinia amylovora* (Burrill) Winslow (Enterobacteriales: Enterobacteriaceae) was achieved in studies

Table 1 Overview of the pollinator-and-vector studies so far conducted with honey bees, bumble bees and a mason bee species

Vector	Active ingredient (CFU/g)	Crop	Dispenser	Bee load direct (CFU/bee)	Flower load (CFU/flower)	Target	Control	Reference
A. mellifera	*Gliocladium roseum* (5×10^8)	Strawberry	Peng			*Botrytis cinerea*		Peng et al. (1992)
		F*		6.3×10^4	$1.6–27 \times 10^3$		S	
		F		5.7×10^5	$2.0–114 \times 10^2$		NS	
A. mellifera	*Pseudomonas fluorescens A506* (1.7×10^9)	Apple and pear	Antles	$5.0–8.0 \times 10^3$	$0–1 \times 10^3$	NC	NC	Thomson et al. (1992)
	Erwinia herbicola Eh318 (3.1×10^8)	F		2.5×10^3	$0–5.4 \times 10^3$			
A. mellifera	*Gliocladium roseum* (1.2×10^9)	Raspberry	Peng	H:$1.3–81 \times 10^4$	H: 0–8000	*Botrytis cinerea*	S*	Yu and Sutton (1997)
		F*	Yu & Sutton					
		F*		B: $0.3–128 \times 10^4$	B:0–20666			
B. impatiens		G			B:945 ± 1255			
A. mellifera	*Trichoderma harzianum* ($1.0–4.9 \times 10^8$)	Strawberry	NI		$0.4 \pm 0.3 \times 10^5$	*Botrytis cinerea*	NS	Maccagnani et al. (1999)
		F*		$2.7–45 \times 10^5$	$4.1 \pm 0.5 \times 10^5$			
A. mellifera	*Trichoderma harzianum* 1295-22 ($1 \times 10^8–10^{10}$)	Strawberry	H: Tray	H: 1.0×10^5	NC	*Botrytis cinerea*	S	Kovach et al. (2000)
B. impatiens		F	B: Tube	B: NC				
A. mellifera	*Trichoderma harzianum* T39 (1.0×10^9)	Strawberry	Triwaks			*Botrytis cinerea*	S*	Shafir et al. (2006)
		F		1.5×10^5	$2.2 \pm 0.5 \times 10^4$			
A. mellifera	*Bacillus subtilis* QRD132	Blueberry	Gross			*Monilinia vaccinii-corymbosi*	S	Dedej et al. (2004)
		F*		$5.1–6.4 \times 10^5$	5.1×10^3			
A. mellifera	*Trichoderma koningii*, *Trichoderma aureoviride*, *Trichoderma longibrachiatum*, *Trichoderma harzianum* sp. (13×10^9)	Sunflower	NI	NC		*Sclerotinia sclerotiorum*	S	Escande et al. (2002)
		F*			$1 \times 10^7–10^9$		S*	
		F			NC			
A. mellifera	*Bacillus subtilis* BS-F4^{rif} ($1 \times 10^{10}–10^{11}$)	Pear				NC	NC	Maccagnani et al. (2006)
O. cornuta		F*	A: NI	A: 1×10^4	A: $1–2 \times 10^4$ (= 1st–3rd flower)			
		F*	O: Maccagnani	O:$1 \times 10^4–10^7$	O: $4–14 \times 10^4$ (= 1st–3rd flower)			
		F			O: $1 \times 10^2–10^6$			

Table 1 continued

Vector	Active ingredient (CFU/g)	Crop	Dispenser	Bee load direct (CFU/bee)	Flower load (CFU/flower)	Target	Control	Reference
B. impatiens	*Clonostachys rosea* + *Beauveria bassiana* GHA ($1.4 \times 10^7 + 6.3 \times 10^{10}$)	(1)Tomato	NI	(1)2.6×10^4	(1)$4.3 \pm 0.3 \times 10^3$	*Botrytis cinerea*	S	Kapongo et al. (2008a)
		(2)Sweet pepper		(2)5.5×10^5	(2)1.6×10^4	*Trialeurodes vaporariorum*	M	
		G		(1)5.0×10^4	(1)$4.8 \pm 0.2 \times 10^3$	*Lygus lineolaris*		
				(2)8.0×10^5	(2)1.0×10^4			
B. impatiens	*Beauveria bassiana* GHA (9×10^9–6.2×10^{10}–2×10^{11})	(1)Tomato	Modified Yu & Sutton	(1)1.7×10^3–3.1×10^5	(1)0.4–3.2×10^3	*Trialeurodes vaporariorum*	M	Kapongo et al. (2008b)
		(2)Sweet pepper		(2)1.1–7.4×10^5	(2)0.3–2.4×10^3	*Lygus lineolaris*		
		G				*Myzus persicae*		
A. mellifera	*Trichoderma harzianum* T22 (9.8×10^6)	Strawberry	Houle			NC	NC	Albano et al. (2009)
		H: F		H: $3.9 \pm 1.8 \times 10^3$	H: 26 ± 90			
B. impatiens		B: G		B: $7.2 \pm 2.2 \times 10^4$	B: $1.3 \pm 0.9 \times 10^3$			
B. terrestris	*Trichoderma harzianum* + *Gliocladium virens* (1×10^8)	Tomato	SSP	$1.3 \pm 4.0 \times 10^2$	$0.7 \pm 0.3 \times 10^2$	NC	NC	Maccagnani et al. (2005)
	Trichoderma harzianum (13/3 RBDPH1,15/2RBD5 and 4/18RBD1) (3.5×10^9)	G	OP	$4.3 \pm 4.2 \times 10^4$	$1.3 \pm 1.1 \times 10^2$			
B. terrestris	*Trichoderma atroviride* + *Hypocrea parapilulifera* (7.0×10^7)	NC	Mommaerts	$4.3 \pm 0.2 \times 10^5$	NC	NC	NC	Mommaerts et al. (2010b)
A. mellifera	*Heliothis* nuclear polyhedrosis virus (106×10^{12} IU/g)	Crimson clover	Gross	NC	NC	*Helicoverpa zea* larvae	M	Gross et al. (1994)
		F						
A. mellifera	*Metarhizium anisopliae*	Oil seed rape	Peng	NC	NC	*Meligethes aeneus*	M	Butt et al. (1998)
		F*						
A. mellifera	*Bacillus thuringiensis var kurstaki*	Sunflower	Modified Gross	NC	NC	*Cochylis hospes* larvae	M	Jyoti and Brewer (1999)
		F						
A. mellifera	*Beauveria bassiana* GHA (1×10^9)	Canola	NI	NC	NC	*Lygus lineolaris*	M	Al-mazra'awi et al. (2006a)
		G						

Table 1 continued

Vector	Active ingredient (CFU/g)	Crop	Dispenser	Bee load direct (CFU/bee)	Flower load (CFU/flower)	Target	Control	Reference
		F*		4.5–6.0 × 10^5	2.7–3.7 × 10^4			
		F		NC	NC			
B. impatiens	*Beauveria bassiana* GHA (1 × 10^9)	Sweet pepper	NI	4.5 ± 0.5 × 10^5	1.8 ± 0.5 × 10^4	*Lygus lineolaris*	M	Al-mazra'awi et al. (2006b)
		G		3.8 ± 0.8 × 10^5	3.2 ± 1.0 × 10^4	*Frankliniella occidentalis*		
A. mellifera	*Metarhizium anisopliae*	Canola				*Meligethes aeneus*	M	Carreck et al. (2007)
						Ceutorhynchys assimilis		

F field; *F** covered field; *G* greenhouse; *M* significant mortality of the pest; *NC* not considered; *NI* no information; *NS* no suppression; *S* suppression; *S** suppression at low and medium disease level but not at high level

when the MCA was directly applied on the stigma of flowers (Scherm et al. 2004), or when it was established before the presence of the plant pathogen (Wilson et al. 1992; Johnson et al. 1993a, b; Wilson and Lindow 1993; Alexandrova et al. 2002).

Selection of the biocontrol agent

Criteria for suitable biocontrol agents

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

What has already been done with the entomovector technology?

Contribution of pollinators to the control of plant pathogens

In the past, honey bees have been used with success for the dissemination of MCAs against economically important plant pathogens of orchard fruits (apple and pear), strawberry, raspberry, blueberry and sunflowers (Table 1). Under open field conditions, the success of suppression of plant diseases was shown to depend on the frequency of the visits by the vector to the crop. Indeed as long as a high frequency of bee visits to the crop was maintained, the vectoring of *Clonostachys rosea* (Link.: Fr.) Schroers, Samuels, Seifert & Gams (formerly *Gliocladium roseum* Bainier) (Hypocreales: Bionectriaceae) reduced the incidence of *Botrytis cinerea* Pers.:Fr. (Helotiales: Sclerotiniaceae) on strawberry flower organs from 53 to 35% in stamens and in the petals from 18 to 15% (Peng et al. 1992). Similarly, a high density of honey bees vectoring a combination of *Trichoderma* species protected sunflower crops until physiological maturity of the flower (Escande et al. 2002). A significant suppression of plant pathogens was also obtained when experiments were performed in covered areas such as cages placed in open field and in greenhouses. Good examples are: *Monilinia vaccinii-corymbosi* (JM Reade) Honey (Helotiales: Sclerotiniaceae) incidence was reduced from 21–67% to 7–44% in blueberries by vectoring *Bacillus subtilis* (Ehrenberg) Cohn (Bacillales: Bacilaceae) QRD132 (Dedej et al. 2004), *B. cinerea* was suppressed from 90 to 68%, and from 64 to 48% in raspberries and strawberries, respectively, when *G. roseum* was vectored (Peng et al. 1992; Yu and Sutton 1997), and sunflower *Sclerotinia sclerotiorum* (Lib.) de Bary (Helotiales: Sclerotiniaceae) infection was suppressed by vectoring *Trichoderma* species up to 31 days after application of the pathogen (Escande et al. 2002). Therefore it can be concluded that when a satisfactory level of the MCA into flowers is realized, a good control level can be achieved. To date, all studies performed with honey bees as vector reported a mean MCA deposition between 10^3 and 10^4 CFU/flower. Interestingly, these numbers of CFU per flower agree with Elad and Freeman (2002) who reported that this amount is sufficient to suppress the plant pathogen *B. cinerea*. However, several authors reported a high variability in numbers of CFU per flower when vectored by honey bees, ranging between 0 and 10^4 CFU (Thomson et al. 1992; Kovach et al. 2000; Shafir et al. 2006; Albano et al. 2009). As a consequence, the efficacy of suppression was also variable, and even in some cases there was a failure of control when the disease pressure was high (Shafir et al. 2006). In addition, it is to be remarked that the vectoring was favourable for the viability of the MCA. Yu and Sutton (1997) and Kovach et al. (2000) reported higher numbers of CFU per flower after delivery via honey bees, and also a higher viability, compared to spray application. Typically, <10 CFU were scored in the flower within 2 days after a spray application.

For bumble bees, the few studies conducted under field conditions (i.e. open fields and/or greenhouses) demonstrated the potency of these vectors in raspberry, strawberry, tomato and sweet pepper. Yu and Sutton (1997), Kapongo et al. (2008a) and Albano et al. (2009) reported a good dissemination of the MCA by *B. impatiens* into flowers of several different crops. For instance, 1,000–5,000 CFU were deposited per flower by *B. impatiens,* resulting in a suppression by 57–59% of *B. cinerea* in tomato and sweet pepper (Kapongo et al. 2008a). Similarly, *B. cinerea* could be suppressed in raspberry and strawberry in a satisfactory manner (Yu and Sutton 1997; Kovach et al. 2000). Based on these results it can be concluded that vectoring by *B. impatiens* having a colony size that is only 1/60 of that of the honey bees, results in an equal suppression of the plant pathogen compared to honey bees. In addition, studies with *B. terrestris* workers documented a lower amount of MCA deposited into the flowers: 70 ± 31 CFU/flower and 135 ± 106 CFU/flower, and a high variability in the numbers of flowers containing MCA (67.5 ± 33.8%) (Maccagnani et al. 2005). However, according to Mommaerts et al. (2010b), the low transport efficacy can be explained by the use of inefficient dispensers, as these authors demonstrated a ten times higher acquisition on the bumble bee workers with a newly developed dispenser, compared to the one-way side-by-side passageway (SSP) dispenser. Moreover, drawing conclusions about the vector efficiency for both *B. impatiens* and *B. terrestris* is difficult due to difference in size of the experimental plots used: cages and small greenhouses of 6, 10 or 80 m^2 (Yu and Sutton 1997; Kapongo et al. 2008a; Albano et al. 2009), whereas the vector capacity of *B. terrestris* was evaluated under more realistic conditions, namely in a greenhouse of 480 and 800 m^2 (Maccagnani et al. 2005; Mommaerts et al. 2010b).

It is of interest that besides its presence in the flowers, MCA was also recovered on the leaves as result of load loss during flight. Kapongo et al. (2008a) showed that 90% of the sampled tomato leaves and 76% of the sampled sweet pepper leaves contained *C. rosea,* vectored by *B. impatiens*. Therefore, it is likely that the entomovector technology can be of help in protecting also other plant structures against *B. cinerea*, a fungus known to grow on every plant part (Mertley et al. 2002; Williamson et al. 2007), and to control foliar diseases such as powdery mildews.

So far one study has been conducted using the mason bee *Osmia cornuta* (Latreille) (Hymenoptera: Megachilidae) as vector (Maccagnani et al. 2006). In general, the study indicated that *O. cornuta* was better suited than honey bees to disseminate powdery MCA formulations into pear flowers, as higher CFU per flower (10^4 vs. 10^4–10^7) were obtained, and a higher deposition of CFU/flower up to the 6th consecutively visited flower. However, firm conclusions can only be drawn in future studies when biocontrol activity will be considered.

Contribution of pollinators to insect pest control

Multiple studies have reported on the success of both honey bees and bumble bees to vector different entomopathogenic control agents into flowers to control pest insects which feed on, or inhabit, the flowers. An overview is given in Table 1.

As for the control of plant pathogens, successful control of several pest insects was achieved when honey bees were placed in caged field plots. For field crops such as for crimson clover, mortality of *Helicoverpa zea* Boddy (Lepidoptera: Noctuidae) larvae was 74–87% when *Heliothis* nuclear polyhedrosis virus (HNPV) was vectored (Gross et al. 1994). Similarly, the vectoring of *M. anisopliae* on oil seed rape and canola resulted in high mortality of insect pests, including larvae/adults of *Meligethes* *aeneus* Fabricius (Coleoptera: Nitidulidae) and *Ceuthorhynchus assimilis* Dejean (Coleoptera: Curculionidae) (Butt et al. 1998; Carreck et al. 2007). Jyoti and Brewer (1999) demonstrated that larvae of *Cochylis hospes* in sunflowers could be controlled by vectoring of *B. thuringiensis var kurstaki* (*Bt*), and that control levels were comparable or even higher than achieved by a spray application of *Bt*. Also vectoring of *B. bassiana* GHA in canola killed 22–56% of *Lygus lineolaris* (Palisot de Beauvois) (Hemiptera: Miridae) (Al-mazra'awi et al. 2006a). Overall, studies with honey bees reported high mortality rates of the target pest, indicating that sufficient amount of the MCA had been transported to the target; however, this was not investigated. Besides, MCAs such as *B. bassiana* and *M. anisopliae* have been indicated in the past as inefficient when applied as spray application, but this was due to the difficulty of suspending the conidia in water because of their hydrophobic cell walls (Noma and Strickler 2000), and the adverse effects on the viability of the conidia (Nilsson and Gripwall 1999). Consequently, this first success of insect pest control on field crops via the entomovector technology stimulated research on insect pest control in greenhouses.

To date the bumble bee *B. impatiens* is the only vector used for insect pest control in greenhouses. Here vectoring of *B. bassiana* GHA against major greenhouse pests such as thrips (*Frankliniella occidentalis* Pergande (Thysanoptera: Thripidae)), tarnished plant bug (*L. lineolaris*), whiteflies (*Trialeurodes vaporariorum* Westwood (Hemiptera: Aleyrodidae) and aphids (*Myzus persicae* Sulzer (Hemiptera: Aphididae) resulted in infection rates of 34-70% of the insect pests in tomato and in sweet pepper (Al-mazra'awi et al. 2006b; Kapongo et al. 2008a, b). The latter greenhouse studies achieved control with 10^3–10^4 CFU/flower. Although these results are promising, validation under more realistic conditions is still needed as the studies performed by Al-mazra'awi et al. (2006b) and Kapongo et al. (2008a) were done in cages of 6–8 m^2 which is only a fraction of the size of a commercial greenhouse or an open field.

In addition, in studies using cages and/or greenhouses the MCA was also recovered at high amounts on the sampled leaves: 92% for tomato, 87–92% for sweet pepper and 70–82% for canola (Al-mazra'awi et al. 2006a; Kapongo et al. 2008b). Therefore, it can be concluded as above for plant pathogens that the entomovector technology is not limited to targeting insect pests of flowers only.

Alternatives to commercial MCAs to enhance control success by using the entomovector technology

To date entomovector studies achieved suppression of plant pathogens and insect pests after vectoring a MCA. However, the sensitivity of these control agents to environmental conditions might reduce their activity (Williamson et al. 2007). A valuable alternative that several authors have suggested is to use mixtures of MCAs, whose components differ in their mode of action, in order to achieve higher control levels. It should be stressed here, however, that good knowledge of the mode of action is crucial so that antagonistic interactions can be avoided. For instance, Robinson-Boyer et al. (2009) reported a lower control after a simultaneous application of Sentinel (based on *Trichoderma atroviride* P. Karsten (Hypocreales: Hypocreaceae) LC52) and Trianum (based on *T. harzianum* T22), whereas sequential applications resulted in a higher biocontrol efficacy. However, successes via enhancing control of *B. cinerea* by co-application has already been reported by multiple authors on cucumber, tomato and strawberry leaves when MCAs were used which either differ in their ecological requirements for survival, development and biocontrol activity (Elad et al. 1998; Guetsky et al. 2001; 2002), or inhabit different niches such as the foliar biocontrol agent *U. atrum* and the mycorrhizal fungus *Glomus mosseae* (T.H. Nicolson & Gerd.) Gerd. & Trappe (Glomerales: Glomeraceae) (Møller et al. 2009). A final promising alternative to enhance control is the use of a combination of a MCA and a control agent which is not influenced by environmental conditions (e.g. synthetic fungicides). Such combined use was already proven successful against post-harvest diseases (Zhou et al. 2002; Errampalli and Brubacher 2006; Sugar and Basile 2008; Nallathambi et al. 2009). For example, isolates of *Trichoderma viride* combined with 50 µg/g of the synthetic fungicide mancozeb were shown to control the plant pathogen *Alternaria alternate* Fr. (Kleissler) (Pleosporales: Pleosporaceae) by >70%. Also in the control of *B. cinerea*, Elad et al. (1993) reported reduced pathogen incidence on cucumber leaves after alternated/simultaneous use of *T. harzianum* and the chemical botryticide iprodione. Here it is evident that the compatibility between the control agents needs to be assessed. For instance, captan decreased the mycelium growth of *C. rosea* by 75% and that of conidia by 65% (Cota et al. 2009). Moreover, before use in practice can be recommended, additional tests on residues, consumer safety and development of resistance are needed for all MCAs. Indeed in the past, resistance of pathogenic strains was reported after repeated fungicidal spray applications (Dianez et al. 2002; Myresiotis et al. 2007; Bardas et al. 2008; Kretschmer et al. 2009). However, in future the entomovector technologies may also face the common problem of resistance evolution. Finally, there is a growing interest in the biocontrol activity of fungal metabolites. A good example is the biscorbicillinoids from *Trichoderma*, which strongly interfere with the biology of aphids (Evidente et al. 2009).

Dilutions and formulations

The products that have been disseminated in entomovector studies are typically powdery MCA formulations of a commercial product, or of a self-prepared mixture of a carrier and a microorganism. The purpose of the carrier is to make transport possible by reducing displacement due to air movement caused by bee wing beats during flight. Therefore, an appropriate carrier needs to fulfil three criteria (Kevan et al. 2008): (a) No effect on the life span of the MCA. A good example is that the germination of *Trichoderma* spp. and *B. bassiana* spores was significantly slower when formulated with talc (Hjeljord et al. 2000); (b) Safe for the vector. In the past, minerals such as talc were shown to adversely affect the honey bee brood (Pettis et al. 2004) and to be irritating, causing honey bees to groom (Israel and Boland 1993), whereas with flours as carrier grooming decreased by 50% (Kevan et al. 2008); (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. So far potential, known carrier substances are corn flour (Al-mazra'awi et al. 2006b), 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. Unfortunately until today—20 years after the first entomovector study—there is still inadequate information on the potential of different carriers and their role in vector acquisition.

Dispenser

An optimal dispenser system needs to fulfil three criteria: (a) loads the vector with a sufficient amount of the powdery MCA product, (b) does not interfere with the foraging behaviour of the vector, and (c) has long refilling intervals (>1 day). Overall, the dispensers so far developed can be classified into two groups, namely the 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 the two-way type 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.

Studies evaluating the efficiency of dispenser types report that one-way type dispensers are less suitable for use in the entomovector technology. Indeed for honey bees the Hardwood-dispenser and Tub-dispenser, which were initially designed to disseminate pollen, loaded the vector poorly, resulted in the vector searching for alternative routes to minimize powder contact, and needed to be daily refilled to obtain a satisfactory vector load of $>10^4$ CFU/bee (Thomson et al. 1992; Johnson et al. 1993a; Dag et al. 2000; Bilu et al. 2004).

Similarly the one-way SSP-dispenser developed for *B. terrestris* is not compatible as it resulted in poor loading: only 13% of the bumble bees had detectable amounts of the control agent. In addition, the bees exhibited strong grooming behaviour, and significantly reduced foraging activity (Maccagnani et al. 2005; Mommaerts et al. 2010b). In contrast, for another bumble bee species *B. impatiens* the over-and-under one-way dispenser developed by Yu and Sutton (1997) and its modified version (Kapongo et al. 2008b) resulted in a high loading of $>10^4$ CFU/bee immediately after leaving the dispenser.

To overcome the negative experiences, two-way type dispensers were developed. For honey bees this has resulted in six dispenser types, which are all based on an over-and-under design (see Fig. 2) and which load the vector with a higher amount. For example for the Tray-, Peng- and Triwaks-dispensers, a load of $>10^5$ CFU per honey bee was realized (Kovach et al. 2000; Bilu et al. 2004), Also for the Gross-dispenser satisfactory levels of pathogen suppression and decreased pest survival were obtained (Gross et al. 1994; Jyoto et al. 1999; Dedej et al. 2004). In contrast for the Houle-dispenser, which is an optimisation of the Gross and Tray-dispensers, the honey bee load after leaving the dispenser was only $4 \pm 2 \times 10^2$ CFU/bee (Albano et al. 2009), but the amounts of CFU per gram of the powdery MCA product used was also 100 times less than that applied in the previous studies of Dedej et al. (2004) and Kovach et al. (2000). Further it is to be remarked that it is difficult to decide which dispenser is optimal due to the different experimental setups, formulations and concentrations used over the various studies. So far, there is only one comparative study (Bilu et al. 2004), reporting that the Triwaks-dispenser performed better than the Peng-dispenser: 1.5×10^5 CFU/honey bee versus 1.1×10^5 CFU/honey bee. In the latter study, it became also clear that the dispenser efficiency depends on the effects on foraging activity, and on the refilling intervals. In conclusion, the different two-way type dispensers developed for honey bees appear satisfactory.

In parallel, for *B. terrestris* Maccagnani et al. (2005) developed the two-way overlapping-passageway (OP)-dispenser, which resulted in a higher bumble bee loading as compared with the SSP-dispenser, but here it should be remarked that the initial CFU concentration was 10 times higher. However, further information on the impact of dispenser types on the foraging activity and on the refilling intervals are lacking. Recently Mommaerts et al. (2010b) designed a new two-way dispenser realizing a high loading of *B. terrestris* workers at >10^4 CFU/bee, with no adverse effects on the foraging activity, and with refilling at 3-day intervals. However, the dispenser developed by Mommaerts et al. (2010b) is not yet commercially available, as at the moment validation experiments are being performed under greenhouse conditions. Also for another greenhouse pollinator, a two-way Houle-dispenser was developed, which loaded the vector with >10^4 CFU/bumble bee (Albano et al. 2009), but no further information is given by the authors.

To date only one dispenser, a two-way dispenser, has been developed for *O. cornuta* (Maccagnani et al. 2006). The authors reported an average of 10^4–10^7 CFU per solitary bee, which is high compared to honey bees and bumble bees, but it should be remarked that here a very high CFU in the powdery MCA formulation (10^{10}–10^{11} CFU/g) was used. Moreover, the strong avoidance behaviour for the powder immediately after filling of the dispenser might be a limiting factor for the system. Overall, it can be concluded that *O. cornuta* is suitable as a vector, and that the dispenser developed appears useful, however, no validation results so far are available from practice in the field.

Environmental and human safety of the entomovector technology

MCAs used in agriculture and horticulture are registered plant protection products, and thus adverse effects on non-target organisms have been evaluated. However, authorization has been given for spray applications, or for mixing in the soil, with fixed doses, whereas in the entomovector technology the MCAs will be disseminated continuously and potentially also on non-target sites (Brimmer and Boland 2003), where they can as in the case of *Trichoderma* spp. compete with the naturally occurring microflora for nutrients (Vinale et al. 2008).

Besides guaranteeing MCA transport into the flowers, the health of the vector needs to be assessed. For example when considering the use of entomopathogenic control agents, the risks to the vector need to be determined as the agents usually are not host specific. Side effects need to be assessed at three levels: short (acute) and long term (chronic) loss of survival (toxicity), sublethal effects on worker and nest (population) reproduction, and effects on the pollinator foraging behaviour. So far the MCAs used for insect pest control include *Bt*, *B. bassiana* GHA, *M. anisopliae* and HNPV. Based on the results obtained by several authors it can be concluded that entomopathogenic MCAs can adversely affect vector longevity, although the side-effects depend on the pollinator species used, and on the stage (adult vs. larval instar) considered (Vandenbergi 1990; Kangha et al. 2002, 2006; Hokkanen et al. 2004; Kapongo et al. 2008b; Kevan et al. 2008, Mommaerts et al. 2008, 2009, 2010a). *Bt* has been shown to be safe for adult honey bees (Vandenberg and Shimanuki 1986), while dependent on the *Bt* strain Mommaerts et al. (2010a) reported mortality in *B. terrestris* workers. For *B. bassiana* GHA bumble bees were found more susceptible to mycosis by this entomopathogen than honey bees. Indeed, the pathogen was able to grow on the insect body after dermal contact with a suspension of the commercial compound Botanigard resulting in the death of the insect (Mommaerts et al. 2008), but the risks are small as Kapongo et al. (2008b) obtained an acceptable level of vector mortality of 14% and a satisfactory efficacy level of pest control, 40% mortality of the pest by adjusting the initial concentration to 6×10^{10} conidia/g product. Also the use of *M. anisopliae* possesses some risks for bumble bees (Hokkanen et al. 2004) and honeybees. For the latter, however, the control agent has been used for the control of *Varroa* mites (James et al. 2006); so risks are probably minimal. Similarly, HNPV is highly host specific and effective only against certain Lepidopteran pests.

Within the current registration requirements, risk assessment studies with MCAs are to be conducted for the vector *A. mellifera*, and in addition, potential detrimental side-effects of different MCAs have also been evaluated for bumblebees such as *B. terrestris* (Mommaerts et al. 2008, 2009). Although most chemical products and MCAs used for plant disease control can be considered as safe, it is advisable to conduct more risk assessment studies especially when different vectors such as mason bees are considered. In addition, previous studies have demonstrated that simple extrapolation of toxicity values between honey bees and bumble bees is not possible (Thompson and Hunt 1999; Mommaerts et al. 2006).

Besides the reduction in survival, potential sublethal effects should also be evaluated. Although van der Steen et al. (2003) and Mommaerts et al. (2008, 2009) did not report of detrimental sublethal effects on honey bee and bumble bee reproduction, respectively, care is needed as subtle sublethal effects are not always directly obvious. Various pesticides at sublethal concentrations can induce significant changes in the physiology and behaviour of the insect pollinator (e.g. orientation and feeding) (reviewed by Desneux et al. 2007). Behavioural changes affecting the nest performance of *B. terrestris* have been reported after ad libitum uptake of sugar water dosed with sublethal concentrations of *B. bassiana* GHA (Mommaerts et al. 2009). Also other effects such as on orientation performance and learning/olfactory/memory can be assessed. For example in the laboratory, sublethal effects on honeybees have been assessed by a complex maze and the proboscis extension response (PER) (Zhang et al. 1996; Guez et al.

2001; Lambin et al. 2001; Abramson et al. 2004; Decourtye et al. 2003, 2004a, b; El Hassani et al. 2005), whereas in the field various authors have used automatic tracking with a harmonic radar and radio frequency identification devices to follow honey bee behaviour (Reynolds and Riley 2002). Recently, also for bumble bees a test has been developed in the laboratory—the foraging behaviour test—to assess potential risks of sublethal concentrations on the foraging behaviour of free flying bees (Mommaerts et al. 2010c). To date, PER for bumble bees has only been used in the context of studies on associative learning, but not to assess the impact of a pesticide exposure on olfaction and memory (Laloi et al. 1999; Toda et al. 2009).

In addition, the entomovector technology requires the exposure assessment of MCAs as a dry formulation. Behavioural changes have been observed in previous studies upon exposure to dust (Israel and Boland 1993; Vanneste 1996; Pettis et al. 2004). However, the present experimental designs do not allow such assessment and thus the development of an appropriate methodology is needed.

For humans no additional risks are connected with this technology, as the active substances of commercial MCAs are already registered according to the EU Directive 91/414/EEC.

General conclusions and research perspectives

Since the first studies in the early 1990s on the entomovector technology, recent advances have resulted in (i) the development of suitable dispensers for the three vectors that so far have been used; (ii) the efficiency of several pollinators has been evaluated; (iii) potential carriers have been identified; and (iv) several MCAs are now commercially available. At present the entomovector technology is already recommended for practice and is used with success in some countries such as BeeTreat in Finland (Hokkanen and Menzler-Hokkanen 2007, 2009; Hokkanen et al. 2011), but the system will benefit from further improvements. First, the reliability of the system needs to be improved under diverse environmental conditions, and combinations of different MCA strains and/or mixtures of MCAs with low risk chemical pesticides and naturally occurring antimicrobial substances can be investigated in combination with other cultural strategies. Second, to augment MCA deposition in the flowers for a better control capacity under high disease or pest pressure, commercial products need to be further fine-tuned. At present several candidate carries have been identified, but in future their compatibility with the vectors and their effect for a better acquisition on the bee body after flight should be determined. In this context it might also be of interest to investigate the potential of mixtures. Finally, we envisage that further studies on the understanding of the foraging behaviour of pollinators, the vector–plant interactions and the new insights obtained on the mode of action of antagonists under various environmental conditions and during interaction with different plant pathogens will contribute to the increasing success of this control strategy in the future. However it should be remarked that whatever system will be developed and recommended, its practical uptake will be directly dependent on its simplicity and its ease of use, while still providing good control at a low cost.

Acknowledgments This research was funded by the Special Research Fund of VUB (Brussels, Belgium), and a PhD fellowship of the Luxembourg Ministry for Culture, Higher Education and Research.

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