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Going Viral: Virus-Based Biological Control Agents for Plant Protection

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Abstract

The most economically important biotic stresses in crop production are caused by fungi, oomycetes, insects, viruses, and bacteria. Often chemical control is still the most commonly used method to manage them. However, the development of resistance in the different pathogens/pests, the putative damage on the natural ecosystem, the toxic residues in the field, and, thus, the contamination of the environment have stimulated the search for safer

alternatives such as the use of biological control agents (BCAs). Among BCAs, viruses, a major driver for controlling host populations and evolution, are somewhat underused, mostly because of regulatory hurdles that make the cost of registration of such host-specific BCAs not affordable in comparison with the limited potential market. Here, we provide a comprehensive overview of the state of the art of virus-based BCAs against fungi, bacteria, viruses, and insects, with a specific focus on new approaches that rely on not only the direct biocidal virus component but also the complex ecological interactions between viruses and their hosts that do not necessarily result in direct damage to the host.

1. INTRODUCTION

Viruses are the most abundant entities in the world and are believed to play a crucial ecological role in keeping organism populations in balance. Biocontrol is the control of pest and pathogen populations through direct or indirect biocidal potential to limit their impact on production as much as possible. Viruses can infect cellular organisms from all three domains of life, with the noticeable exception of some endocellular bacteria, such as phytoplasmas. Although the majority of viruses do not have measurable harmful effects on their hosts, viruses are often considered the ultimate parasites and are thus attractive candidates as potential biological control agents (BCAs). The predominant biotic stresses in crop production are caused by pathogens (fungi, oomycetes, viruses, and bacteria) and insects (123). Examples of viruses interfering with the damage caused by these biotic stresses exist for each class of plant disease–causing agents (**Figure 1**). In fact, implementing viruses as BCAs for plant biotic stress is not new: The use of bacteriophages as BCAs in bacteria was first hypothesized a century ago (76). In fungi, the first example of a virus as a successful BCA was the use of the mycovirus Cryphonectria hypovirus 1 (CHV1), which was discovered to cause hypovirulence in the ascomycetous fungus *Cryphonectria parasitica* and has since been deployed to interfere with chestnut blight disease in chestnut orchards and forests (8). In insects, the first examples of virus biocontrol were the uses of baculoviruses to control populations of lepidopteran insects (87). More surprisingly, viruses can also be used to control diseases caused by plant viruses: In this case, viral biocontrol relies on the use of attenuated strains of plant viruses to prime plants against an infection of more severe viral strains of the same virus species. This concept is known as cross-protection or preimmunization (95).

Here, we compare approaches that use virus-based BCAs, summarize the possibilities offered by viruses to control plant pathogens and pests, propose new approaches that could increase their implementation as BCAs able to overcome technical limitations, and discuss the safety and risk assessments of their use.

2. PHAGE BIOCONTROL AGAINST PLANT-PATHOGENIC BACTERIA

One of the greatest global emergencies in the upcoming years is the insurgence of more and more cases of multidrug-resistant (MDR) bacteria. Resistance to antibiotics has certain environmental aspects (113, 138) that suggest the necessity of forbidding their use in agriculture against phytopathogenic bacteria (127). This has revived alternative approaches in both human medicine, e.g., phage therapy (97), and various agricultural fields (129), e.g., phage biocontrol against bacterial diseases of plants.

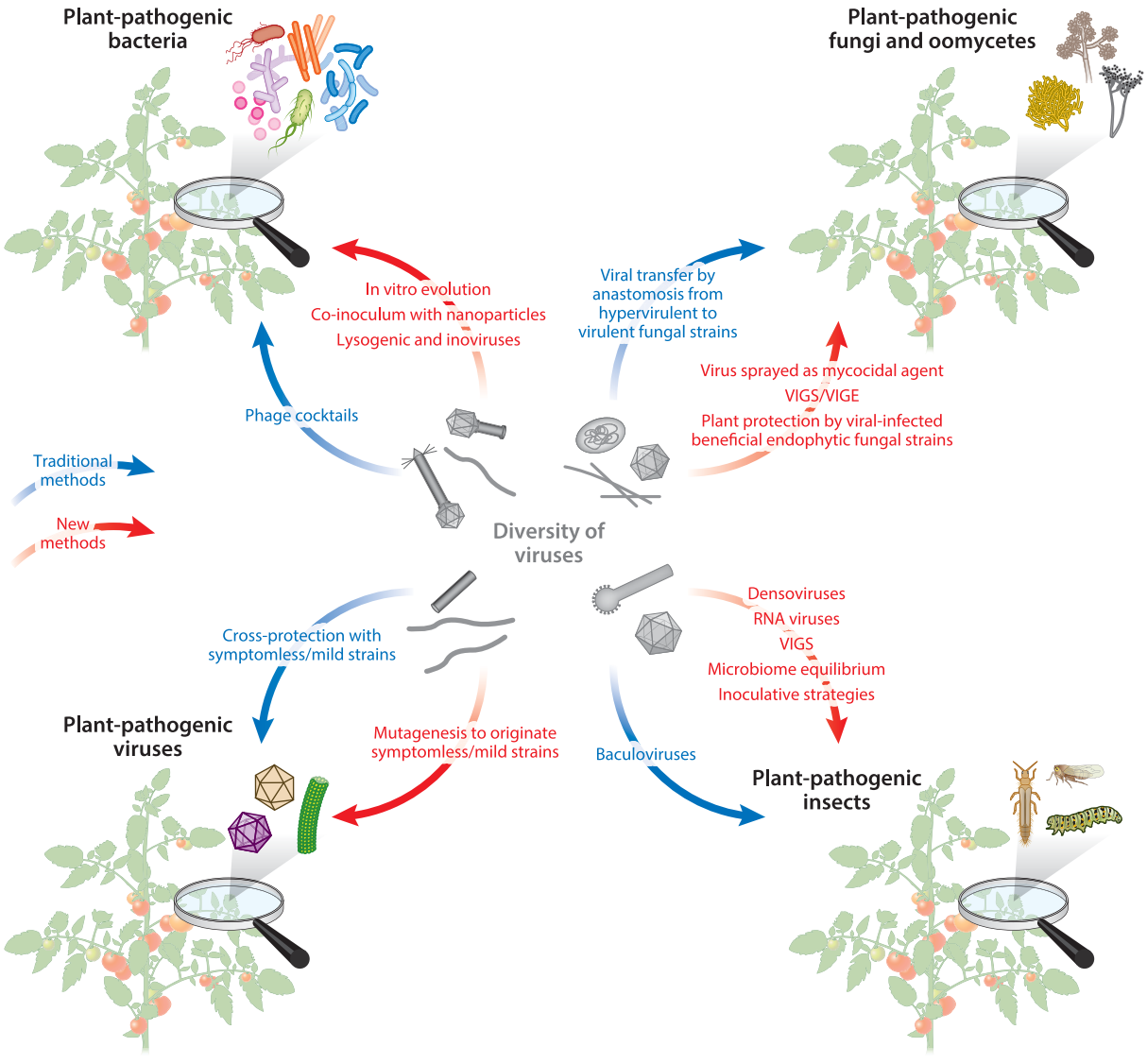

Figure 1

Use of virus biodiversity for traditional and new approaches to control pests and pathogens. Viruses can be used to limit phytopathogenic bacteria (phage biological control agents), fungi/oomycetes (mycovirus), and insect pests and vectors (entomovirus). Viruses can also be used to limit plant viral disease through cross-protection. Viruses not drawn to scale. Abbreviations: VIGE, virus-induced gene editing; VIGS, virus-induced gene silencing.

2.1. A Limited Number of Phage-Based Biobactericides Is Available on the Market, Especially in Europe

Among the low impact measures against phytopathogenic bacteria is the use of bacterial viruses or bacteriophages as biocontrol tools. Promising results and technical limitations have been reviewed elsewhere (21, 56, 121). Even though there is clearly great potential for the implementation of phage biocontrol in crop production, there is currently only a limited number of commercially

available phage cocktails on the crop protection market, especially in Europe, likely because, in the European Union, biologicals need to pass a regulatory process similar to that of synthetic pesticides. This leads to rather lengthy and costly procedures, although regulatory agencies could issue waivers for phage products, considering the inherent biological safety of these natural substances for farmers and the general public (44).

Since 2005, the US Environmental Protection Agency (EPA) has authorized four different phage cocktails as active substances for bactericides, all from Omnilytics (part of Phagelux Agrihealth). The first cocktail, called AgriPhage, contains bacteriophages against *Xanthomonas campestris* pv. *vesicatoria* and *Pseudomonas syringae* pv. *tomato* and protects tomato and pepper plants from bacterial spot and speck in both the greenhouse and open field. Similarly, Omnilytics also developed cocktails against bacterial canker disease on tomatoes and citrus caused by *Clavibacter michiganensis* subsp. *michiganensis* and *Xanthomonas citri* subsp. *citri*, respectively, and against *Erwinia amylovora* fire blight on apple and pear trees. Unfortunately, no concrete details on the phage contents and expected symptom development reductions of the current cocktails are described in the literature, although early formulations of the *X. campestris* pv. *vesicatoria* phage cocktail reduced bacterial spot disease severity on tomato plants by an average of 17% (15, 39). A cocktail against *X. citri* subsp. *citri* containing at least some of the Omnilytics phages was able to reduce citrus canker disease severity by 59% on average in a set of five greenhouse experiments (14). In 2021, the EPA approved another phage product, XylPhi-PD, from the Otsuka Pharmaceutical subsidiary A&P Inphatec that protects grapevines in California from Pierce's disease, which is caused by *Xylella fastidiosa*. The company reported that consistent XylPhi-PD injection in the xylem reduced disease incidence by 57% in a four-site California field study.

In Europe, the European Commission is the agency that approves active substances for plant protection products (PPPs). To date, no phages have been registered as a pesticides' active substance in Europe, which is in sharp contrast to other viral BCAs (e.g., baculoviruses). However, at the national level, a member state can give provisional authorization under specific circumstances, even if the active substance has not yet been approved (44). Under this umbrella, since 2018 the Hungarian government has authorized Enviroinvest to locally sell the phage cocktail Erwiphage Plus as a preventive bactericide against *E. amylovora*. This product is adapted yearly to reduce resistance development and can be applied only under strict conditions during the blossoming period, and this emergency authorization is approved yearly for only 120 days from mid-March to mid-July (79). The Erwiphage Plus cocktail contains two specific phage species, PhiEaH1 (79) and PhiEaH2 (31), which were isolated locally and successfully reduced the occurrence of fire blight cases in field experiments when no artificial infection was applied (**https://www.erwiphage.com**). Although not registered as a PPP, APS Biocontrol sells a phage product within Europe: Biolyse-PB reduces *Pectobacterium* soft rot of potato tubers packed for human consumption.

2.2. Recent Advances to Further Improve the Efficacy of Phage Biocontrol

Based on several phage biocontrol studies and the few available commercial products against different bacterial pathogens, it is clear that an in-depth knowledge of the pathosystem is crucial to select an efficacious phage application method (56). Moreover, a careful phage cocktail selection, which increases the success rate of the treatment, is another point of attention. Indeed, although biologicals such as phages have important advantages over synthetic pesticides, their efficacy generally seems to be lower because of their bacterial host hyperspecificity. Therefore, developed phage cocktails may not work simply because they do not contain phages that recognize the specific pathogen present in a certain greenhouse or field. Therefore, phage application requires monitoring the genetic features of bacterial populations even after the product is being applied.

Thus, new upcoming strains that cannot be efficiently killed by the current cocktails can be used to create host range mutant phages, e.g., by in vitro evolution (20).

Another way to partly overcome the host specificity problem is to deliver precision phage biocontrol matching a phage cocktail to the particular strain of interest. Precision biocontrol could rely on algorithms to match the strain genome to the appropriate phage cocktail. Machine learning can link the genome sequence of pathogenic bacteria to candidate bacteriophages (19, 73, 77), and provide the ability to monitor a larger number of available phages via the prediction of the relationship between phage and pathogen structures (97). Although precision therapy is applicable to human phage therapy, precision biocontrol in crop production may only be profitable in, e.g., high-value cash crops, ornamental plants, or postharvest control measures.

Another limit to the implementation of bacteriophages as BCAs is the risk of bacterial resistance development. Therefore, identifying evolution-proof strategies to prevent this is also crucial to increasing the efficacy of phage biocontrol. Evolution-proof applications could encompass combinations of phages and antagonistic bacteria (140) or phages in combination with other pest management strategies. As resistance to one of these stressors causes increased sensitivity to the other, pathogens facing multiple natural enemies do not easily evolve resistance, thereby increasing the efficacy of the combination treatment. In this regard, Ibrahim et al. (57) tested an *X. citri* subsp. *citri* phage cocktail in combination with systemic acquired resistance (SAR)-inducing compound acibenzolar-S-methyl (ASM), which significantly reduced Asiatic citrus canker disease incidence on lime leaves by 18.3–75.2% under greenhouse conditions. This was a comparable reduction of 12.8% for a copper hydroxide–treated control group. Similarly, Lang et al. (68) tested a phage cocktail targeting *Xanthomonas axonopodis* pv. *allii* on onion, the combination of the phage cocktail with ManKocide (containing the bactericidal copper hydroxide and the fungicidal mancozeb), and the phage cocktail with ASM for two subsequent years at different geographical locations. During these trials, it showed that phages, both with and without additives, perform as well as a traditional copper hydroxide treatment.

Nanotechnology can help develop phage-based BCAs. Nanoparticles with an extremely small size and a high surface area can stand as potential candidates for future bacteriophage formulations to help improve the efficacy and facilitate the applicability of bacteriophages. Nanoparticles are being investigated for their ability to control antibiotic-resistant pathogenic bacteria (69). They have been shown to reduce the exopolysaccharide production and biofilm formation of bacteria, thereby interfering with quorum-sensing signaling. This in turn prevents bacteria from expressing several density-dependent traits, including antibiotic production, biofilm formation, virulence, swarming, and evolution of resistance (118). From an environmental perspective, silicon nanoparticles may be particularly relevant, as they can be produced by plant materials and can have beneficial effects on plant health (99). The synergies between phages and nanoparticles are currently still under investigation.

A last approach to drastically reducing resistance development is to use multiple phages for each pathogenic strain (140) that together put pressure on a pathogen until it evolves a resistant phenotype weakened in other essential traits, resulting in "evolutionary suicide."

Researchers are also exploring the use of phages other than the conventionally used strictly lytic phages that immediately propagate inside and lyse their bacterial host as biocontrol tools. Temperate phages, which are able to undergo a lysogenic infection cycle during which they reside in the host genome, were traditionally excluded from biocontrol approaches, but a recent review outlines a theoretical framework that includes them among feasible tools to control bacterial infections (86). One other approach besides the use of lytic phages is the use of filamentous phages (these types of phages chronically infect their host, thereby continuously releasing viral progeny without lysing the host cell) (5). In this regard, Ahmad and colleagues (3) characterized

filamentous phages influencing the virulence of their host *X. axonopodis* subsp. *citri*. Similarly, Akremi et al. (4) showed that the use of filamentous phages against *E. amylovora* could be an option to treat fire blight in pears.

2.3. Some Risk Assessment Considerations

In contrast to chemical PPPs, phages consist of proteins and nucleic acids, both of which are biodegradable and widely abundant in the environment. The formation of toxic metabolites or bound residues, which must be considered for chemical PPPs, is not expected for these phage BCAs. Phages and viruses, however, can replicate and thereby increase their abundance above the amounts that have actually been released during their application.

For their environmental safety, and in line with the environmental risk assessment of chemical pesticides (54) and genetically modified organisms (10), the impact of phages and viruses should consider potential effects on nontarget organisms (NTOs). These effects depend on the properties of both the phage/virus product and the receiving environments. Host specificity defines the range at which NTOs may be affected. The mode of phage replication may strongly affect the exposure time and dissemination into neighboring ecosystems. Other host-related properties include UV resistance, desiccation tolerance, and affinity for adsorption onto clay minerals. For the receiving environment, one major factor is the presence of hosts that allow phage or virus infection and replication. Generally, the host range of a phage is expected to be narrow and specific for a target organism, but the lack of knowledge on the presence of potential hosts within soil or other environmental microbiomes may increase the predictive uncertainty. Also, some phages are less specific and can infect hosts from different genera (124).

Indirect effects of phages on NTOs must also be considered. These may potentially be more drastic than just a transient elimination of a host. For aquatic ecosystems, it has been shown that phage–bacteria interactions can strongly influence global biogeochemical cycles (141). Comparable data are not available for soil, and the mobility of phage and viral particles is limited by adsorption to soil particle surfaces (48). However, soil viromes are not fully inactive and thus may have effects on soil microbiomes and their activities (101).

Most emerging soil microbial activities result from complex interactions. Will a bacteriophage host removed from a community be simply replaced by another taxon without affecting the interaction networks? Or will it trigger cascade reactions that ultimately result in completely different communities? A way forward to addressing these questions and predicting indirect effects is network analysis, which reveals the interaction potentials, e.g., whether an affected taxon is a keystone species or hub or just a poorly connected community member. When developing a test system for the construction of microbial networks, it is advisable to analyze distinct individual soil aggregates, as the focus on such small spatial entities increases the probability of detecting interacting partners instead of only incidental correlations (130).

Given the tremendous potential of phages and viruses as PPPs, risk assessment agencies should ask themselves whether current procedures for assessing their environmental risks are fit-for-purpose or whether adjustments in response to phage-specific and virus-specific properties and new knowledge on environmental microbiomes should be applied to enhance safe applications of phage and virus PPP products.

3. MYCOVIRUSES AS PUTATIVE BIOCONTROL AGENTS OF PLANT-PATHOGENIC FUNGI

Mycoviruses are obligate parasites prevalent in most economically important plant-pathogenic fungi. Mycovirus–fungus interactions are frequently associated with latent infections; however,

mycovirus infection can also be beneficial or detrimental to the fungal host. Mycoviruses can have many types of genomes: mainly positive- and negative-sense single-stranded RNA (ssRNA+/−) or double-stranded RNA (dsRNA) genomes and, much less frequently, single-stranded DNA (ssDNA) genomes, with the noticeable exclusion of dsDNA viruses (27, 110, 146). Their genomes are mono- or multi-segmented, naked, or encapsidated (135). The historical success of using mycovirus-infected hypovirulent *Cryphonectria parasitica* for the control of chestnut blight has stimulated additional studies on mycoviruses and their potential for biocontrol of plant-pathogenic fungi in other pathosystems (90, 94, 143).

3.1. Hypovirulent Strains for the Biocontrol of Chestnut Blight

A successful case example of biocontrol of a fungal disease with a mycovirus is the use of Cryphonectria hypovirus 1 (CHV1) against its fungal host *Cryphonectria parasitica*, the causal agent of chestnut blight, a severe disease of chestnut with characteristic canker symptoms (necrosis of the bark and cambium) that can girdle the trunk and lead to death of the canopy (90).

Comprehensive reviews have been written on the history of controlling this invasive species with hypoviruses and the molecular aspects of the hypovirus-fungus interaction (55, 83, 90, 133). Here, we mainly focus on the practical aspects of this hypovirus-based biocontrol approach, the reasons behind some failed attempts at controlling this disease with mycoviruses in the United States, and some possible new approaches to overcome such failures.

The successful story of CHV1-caused hypovirulence in Europe. Hypovirulence was first described by Antonio Biraghi in Italy in the early 1950s followed by approaches to develop the hypovirus-containing strain as BCA (106, 133). The demonstration of its cytoplasmic localization, the presence of a dsRNA molecule, and, finally, its viral nature (133) were the three discoveries that led to implementation of an active biocontrol strategy in both Europe and the United States. However, no mycovirus-based PPP is registered for commercial use.

In Europe, hypovirulent isolates of *C. parasitica* have been used to treat chestnut blight disease. These attempts began in France with the efforts and protocol from Grente in the 1970s (106). Several steps were identified for the introduction of hypovirulence: (*a*) the vegetative compatibility (VC) groups present in a specific location must be identified; (*b*) a virulent, local representative of each VC group needs to be transfected with CHV1; and (*c*) isolates, or a mix of isolates from a virulent, local representative of each VC group are used to treat actively growing cankers. The BCAs are inoculated by placing mycelia in holes made with a cork borer at the canker margin. Molecular markers for both the fungus and the virus are used to monitor the efficacy of the treatment on single cankers and the establishment of the BCAs. The goal is to maintain a limited disease pressure without continuous reintroduction of hypovirulent isolates (102). Although the therapeutic effect on the treated cankers was mostly successful, these natural biocontrol tools did not persist in the field and the role of the specific virus isolate seems crucial: Aggressive isolates are likely better at treating single cankers, but their reduced fitness (slower growth and reduced conidia) can hamper their long-term effects. This was shown for some French CHV1 isolates similar to CHV1-EP713, the isolates used as BCA in most biocontrol experiments. However, Italian isolates, which are milder in relation to hypovirulence, have better fitness (judged by growth and conidia production) and often establish in the long-term in areas treated for biocontrol purposes (108).

Deployment of virus-caused hypovirulence in the United States. Introducing stable natural biocontrol in the United States has been unsuccessful (83). The major reasons seem to be the higher susceptibility of American chestnut to *C. parasitica* and the greater diversity of VC groups that are locally present, which makes natural spread of the hypovirus more difficult (33).

Successful biocontrol relies on horizontal transfer of a hypovirus through anastomosis. When isolates belonging to different VC groups attempt hyphal fusion, cell death occurs. Theoretically, up to 64 VC groups are possible, controlled by six loci with two alleles at each locus.

The American regulatory landscape is more open to biotechnological manipulation of BCAs. In the mid-nineties, a system to initiate CHV1 infection from a *trans*-gene copy of the viral cDNA was established (24). The release of transgenic fungal strains in chestnut orchards and coppices is expected to increase the spread of hypovirulence because (*a*) all the conidia will be infected by the virus (as opposed to cytoplasmic CHV1, which is transmitted only to a variable and limited extent to conidial progeny); (*b*) ascospores originated from outcrosses using transgenic conidia as spermatia will segregate for the presence of CHV1 (whereas no ascospore is infected from cytoplasmic CHV1); and (*c*) mating will increase the variability of CHV1-infected VC groups, resulting from recombination at the VC loci. Two studies evaluated the introduction of transgenic *C. parasitica* carrying the CHV1 virus and both reported that several ascospores carried the virus (although in a lower number compared to the expected segregation ratio) but without changing the likeliness of hypovirulence naturally spreading in the orchard/coppice (33, 109).

The possibility to use genetically manipulated organisms in the United States also suggested a further approach to overcome the limitations to natural hypovirulence spread due to the high diversity of VC groups: In this case, a superdonor strain of *C. parasitica* was obtained by knocking out four of the five vegetative incompatibility (vic) loci that restrict virus transmission (one locus is not involved in restricting CHV1 transmission). This strain is therefore able to establish anastomosis with a great number of the 64 VC groups and thus can be used to efficiently spread hypovirulence in the field, without VC restrictions (148). Indeed, when two such superdonor strains were used to treat cankers, in a natural and *vic*-genotypically diverse *C. parasitica* population, they were successful in transmitting the virus to most of the local *vic* genotypes (120).

3.2. Mycoviruses as Biofungicides

As shown above, the use of mycoviruses as BCAs has relied on the infected hypovirulent fungal strain to transmit the mycovirus to the virulent fungal strain by hyphal anastomosis. However, the formulation of mycoviruses in such ways that they could be used as external fungicides, similar to the commercial products based on other microorganisms such as phages, fungi, and bacteria, would be more promising for market success. In this way, field transmission barriers due to the fungal host vegetative incompatibility could be avoided.

In this regard, there are a few examples of extracellular infections of fungi using mycoviral particles. In 2010, Yu and coworkers discovered the first mycovirus with an ssDNA circular genome associated with the fungus *Sclerotinia sclerotiorum* [Sclerotinia sclerotiorum hypovirulence-associated DNA virus 1 (SsHADV-1)] (146). The treatment of *Arabidopsis* leaves and *Brassica napus* plants with SsHADV-1 virions as a virus-based fungicide was protective against *S. sclerotiorum* infection; the results were comparable with the application of carbendazim, a chemical fungicide frequently used for fungal control (147). Moreover, the isolation of ssDNA-infected strains from the treated leaves indicated that the inhibition of the infection was due to the conversion from virulent to hypovirulent variants by mycoviral infection. Furthermore, in rapeseed field trials, the application of SsHADV-1 particles also prevented secondary infections, reduced both the incidence and severity of *Sclerotinia* stem rot, and significantly improved seed yield (147).

3.3. New Approaches for Mycovirus-Based Biocontrol Strategies

The deployment of fungal isolates with virus-mediated hypovirulence had the advantage of limiting the environmental spread of the mycovirus, raising very little concern for off-target effects;

nevertheless, such approaches that rely on the horizontal spread of the virus among pathogen populations with delayed efficacy are not suitable for annual crops but remain particularly useful for forest diseases in natural settings. Recent new approaches seem to be better adapted to protect annual crops, using mycovirus-infected strains, but have the advantage of not relying on horizontal transfer of the virus for effective biocontrol.

Mycovirus-caused change of fungal lifestyle. A more recent mycovirus-based biocontrol approach relies on the discovery of the complex relationship of a mycovirus-infected hypovirulent strain with the plants that need protection. Three groundbreaking works have been published in the past year sharing the discovery that a mycovirus can switch the lifestyle of a fungal host from a pathogen to an endophyte and that the presence of the endophytic fungus in the plant hosts has positive effects on the growth of the plant and protection from the virus-free pathogen. Zhang and coauthors (149) started from the observation that SsHADV-1 DNA could be found in rapeseed pods long after treatment with the hypovirulent strain, suggesting that mycovirus-infected *S. sclerotiorum* could colonize *B. napus*; they showed that mycovirus-infected *S. sclerotiorum* isolates formed compound appressoria and could grow on the surface and under the leaf epidermis without killing the cells (as normally done by virus-free isogenic isolates). Looking at mycovirus-caused differential expression patterns, the authors showed that the mycovirus suppressed the production of pathogenicity/virulence factors and provided the fungus with the ability to regulate plant defense and hormone function, stimulating plant growth and resistance to pathogen infection, including *Botrytis cinerea* (149).

Zhou and coauthors (151) looked at a different host–pathogen system: *Pestalotiopsis theae*, a pathogen of tea. *Pestalotiopsis* spp. are commonly found as nonpathogenic endophytes, but sequence and phylogenetic analysis have failed to distinguish closely related pathogenic and endophytic *Pestalotiopsis*. These authors associated the endophytic lifestyle with the presence of a chrysovirus and assessed the potential for a BCA based on such mycovirus-infected endophytic strain (151).

In a surprising development of the SsHADV-1-caused endophytic switch in its fungal host, Tian and coauthors (131) discovered that *S. sclerotiorum* can establish a protective endophytic relationship also with gramineous hosts (which were formerly thought to be nonhosts and used in rotation with dicotyledonous *S. sclerotiorum* hosts), treating the seed and priming the plant to protect itself from several different fungal pathogens such as *Fusarium graminearum*, *Magnaporthe oryzae*, and *Puccinia* spp. (131). In these approaches, the product that is sprayed on the crop is indeed a fungal culture that carries the mycovirus intracellularly; therefore, there is no open release of the virus in the environment; nevertheless, it acts as a PPP without the necessity to convert the pathogenic recipient strain through anastomosis.

Virus-induced gene silencing and synthetic biology. Most mycoviruses that are found through metatranscriptomic data are not causing hypovirulence, because they coevolved with their host, which tamed their pathogenic potential. Nevertheless, there are biotechnological approaches that can make those viruses pathogenic to their fungal host, interfering with the fungal life cycle or specifically interfering with their virulence potential for causing disease on plants. Virus-induced gene silencing (VIGS) is an approach in which a fragment of a target host gene is inserted into a viral genome (into a viral infectious clone). From CHV1 infectious clones, an expression vector was derived (128) and the same site of insertion can be used to insert a fragment of a fungal host gene that would be downregulated during infection.

Furthermore, the mycoviruses that can be used for biocontrol strategies can include those that result from the amplification and assembly of infectious clones from metasamples analyzed in silico, as recently demonstrated for an ssDNA virus amplified from a plant metagenome (38). Such

infectious clones can be adapted to become virus-induced gene editing (VIGE) tools, increasing the potential for application (29, 91).

3.4. Biosafety and Risk Assessment

Before the commercial use of new BCAs, their safety, taking into account their dispersal and persistence in the environment, needs to be assessed. Known mycoviruses with RNA genomes are strictly intracellular, and as BCAs they need to be carried to the field inside the mycelia or spores of their host fungus. This also means introducing new inocula of the host pathogen into the environment. For this reason, only environments already heavily infested with the pathogen can be considered, and the introduced strain of the pathogen should be clearly hypovirulent. Moreover, unless the introduced host strain has the same genotype as the resident population, the genetic diversity of the host may increase, which could potentially lead to the generation of new, more virulent host genotypes. In general, mycoviruses are expected to persist in the environment for a long time after application because of their intracellular nature. This is beneficial in terms of biocontrol efficacy, as has been seen in the case of *C. parasitica* hypoviruses that are naturally spreading in the forest, but it also means that any harmful effects would be difficult to mitigate.

Another risk to be assessed is the potential virus transmission to NTOs. In general, mycoviruses are highly species-specific and no major interspecies spread is expected. However, mycoviruses have been shown to be occasionally transmitted between different species of the same fungal genus (72, 80, 112, 134) or possibly even more distantly related fungal species in the same environment (9, 136). This is not expected to lead to problems if a common mycovirus pool is naturally shared between multiple host species, in which case each species would be equally adapted to the virus effects. However, the outcome is more unpredictable if non-native viruses are used. Because of the lack of coevolution and adaptation, viral host jumps are expected to be harmful to the fungus, as has been demonstrated in laboratory experiments (23, 61, 63). If the introduced viruses infect co-occurring ecologically important fungal species such as mutualistic endophytes or mycorrhizal species, concomitant negative outcomes may extend to the plant community and thereby the whole ecosystem.

An even more problematic scenario would be posed by virus transmission into other organisms that are closely connected with fungi, such as plants and arthropods. Recent high-throughput sequencing studies have suggested the occasional occurrence of such transmissions in nature (27, 114) or experimentally (18). Ultimately, human exposure needs to be taken into account during risk assessment. The only currently known mycoviruses with DNA genomes are gemycircularviruses in the family *Genomoviridae*, which have been shown to be capable of extracellular transmission and are vectored by insects (71). Vectoring by insects is highly beneficial considering practical virocontrol applications, as it could eliminate the necessity of co-distributing the fungal host pathogen into the environment. However, some caution should be taken, as gemycircularviruses have been widely detected in various environmental samples (65, 116) as well as immunosuppressed humans (75).

4. VIRUSES AND INSECT BIOCONTROL: SOMETHING OLD, SOMETHING NEW

Insects form the largest living animal group on Earth, representing a major part of its biodiversity. Insects have conflicting relationships with humans: They are beneficial for a plethora of biological processes and ecological services, but they are also competing with humans for food and resources. As such, they constitute a major economic burden on food crop production by either direct feeding (pre- and postharvest) or transmitting pathogens to plants.

Viruses are integral components of ecosystems and as such they cause epizootics in insects (35). The first field trials using a virus occurred in the 1950s, using a baculovirus to control alfalfa caterpillars (100). Since then, the use of entomopathogenic viruses to control agricultural pests and disease vectors represents one of the most promising approaches in sustainable pest control strategies (125). The success of baculoviruses as BCAs has been undeniable, but they have a limited target that excludes most of the insect vectors of viruses and phytoplasmas; furthermore, their intensive use has inevitably selected for resistance (11, 12), leaving us with few alternative solutions. Today, there is an urgent need to diversify the arsenal of viral pesticides as BCAs. This section describes the baculovirus success stories and the potential new virus resources and virus-based strategies for insect control.

4.1. Entomopathogenic Viruses: The Baculovirus Success Stories

Entomopathogenic viruses belonging to at least 20 taxonomic groups (119) have been associated with epizootic outbreaks in insects from natural ecosystems or rearing facilities, among which we find baculoviruses, cypoviruses, densoviruses, entomopoxviruses, and insect-infecting picornaviruses. However, not all these viruses have the potential to become BCAs. Indeed, the criteria required for a "good" viral BCA in agriculture include specificity, oral infectivity and pathogenicity, safe and easy production, and stability in the environment. Although more viruses could meet these criteria, application has so far concentrated on baculoviruses.

Baculoviruses belong to two genetically related virus families, *Baculoviridae* and *Nudiviridae*. These viruses have been characterized in arthropods only, mainly in insects of the Lepidoptera, Diptera, and Coleoptera orders. Surprisingly, the recent characterization of insect viromes did not broaden the baculovirus host range to other relevant agricultural pest and vectors (26, 89, 92).

Approximately thirty-five baculovirus products are commercialized at present against various pests, mostly Lepidoptera (100, 126). These are mainly specific baculoviruses, yet few relatively broad-spectrum baculovirus products have also been developed. Despite the multiple products in use, so far host resistance to baculoviruses has rarely been reported, primarily in populations of the codling moth *Cydia pomonella* treated with the baculovirus Cydia pomonella granulovirus (CpGV) (11, 12). Genetic resistances occurred independently in different populations. Insect resistance was overcome by introducing naturally occurring CpGV genetic diversity from field sampling (37).

Molecular biology and genetics studies have also opened the possibility of genetic modification, in particular for improving the baculovirus speed-to-kill (122). Research on genetically modified baculoviruses remains limited mainly because of the general societal reluctance to embrace genetically modified organisms, but research is ongoing in this field.

4.2. Densoviruses: Possible Biological Control Agents with High Potential

Densoviruses were discovered in the 1960s following mortality in mass rearing of the lepidoptera *Galleria melonella* (81). They were next associated with natural epizootics in various lepidopteran populations, including agricultural and forest pests (82, 107).

Densoviruses are the smallest known animal viruses. A nonenveloped icosahedric capsid (19–25 nm) protects a linear ssDNA genome (4–6 kb) ended by inverted terminal repeats. Densovirus genomes encode 3–7 proteins. Arthropod-infecting densoviruses can be found in two subfamilies of the family *Parvoviridae*: *Densovirinae* and *Hamaparvovirinae* (30). The diversity in sequence, genomic organization, and structure identified eight genera in *Densovirinae* and five genera in *Hamaparvovirinae*. Although only 65 assembled genomes have been described, metagenomic analyses and data mining in transcriptomic and genomic public databases have identified partial genomes and densovirus-related sequences. Diversity and prevalence of these

viruses in all ecosystems (42, 43, 132) suggest that densoviruses can be considered as potential BCAs against several insect families, including Lepidoptera, Diptera, Orthoptera, Hemiptera, Blattoidea, Thysanoptera, etc. Densovirus transmission is thought to be mostly horizontal, although vertical transmission (from adults to progeny) has been often described (6, 96).

A few successful field trials have been reported in South America and Africa to control lepidopteran pests of palm and coconut trees (47), but research remained low profile and densoviruses were never developed further against agricultural pests despite interesting results for a few densovirus candidates (85). Viroden, the first densovirus-based product, was commercialized in Ukraine in the 1980s to control mosquitoes (59), and a densovirus pathogenic for the smoky brown cockroach *Periplaneta fuliginosa* has been commercialized in China (126).

Densoviruses are also easy to manipulate and their entire genome can be placed into an infectious plasmid, which can be transfected in insects or in cell culture to recover a viral population (60). Mosquito densovirus vectors have been used to deliver short hairpin RNA to mediate gene silencing and interfere with the transmission of arthropod-borne viruses (50, 70).

The small size of their DNA genome makes densoviruses easy to engineer and produce, and densovirus capsids can be exploited as specific nanovehicles for gene or product transduction, opening the design of precision products.

4.3. Diversifying Virus-Based Biocontrol Strategies Against Insects

Pest control strategies using viruses have been modeled on those of chemicals and viral pesticides evaluated with the same criteria, i.e., specificity, efficacy, persistence, and biosafety. Most virus-based strategies designed so far have relied on the periodical release of viruses against targeted pests. However, due to their close relationships with their hosts, viral BCAs can be more versatile than conventional pesticides. The knowledge and experience accumulated with baculoviruses over the years encouraged diversifying the strategies of use (137).

Viral ecology–based strategies. Viruses have been associated with natural population cyclic dynamics by directly reducing the host population peak but also by having sublethal effects that indirectly influence population decline. But not all insect populations undergo epizootic fluctuation episodes (88). Field tests of the impact of baculoviruses have shown that for host populations that undergo natural epizootics, early infection may be beneficial for biocontrol (93). The diversity of responses that insects may have to virus infection likely relies on intrinsic (host genetics, symbionts) and extrinsic (plant, environment) factors. Taking the complexity of the biological system into account is important in evaluating chances of success for a viral BCA.

Viral metagenomics and high-throughput sequencing have provided the tools to explore the insect virome, unraveling the prevalence and diversity of viruses. One scientific challenge is to understand the structure of virus communities in insects and test how these viruses may influence interactions with the host plant. Data-driven approaches, including statistical methods, may help refine our understanding of the key drivers (and the levers to act on) of the damage for the insect pest/vector and the plant.

Inoculative applications for virus-based biocontrol. Inoculative biological control consists of the intentional release of the BCA into the environment with the expectation that it will multiply and control the pest for long periods. Conservative biological control refers to the modification of the environment to protect and enhance the specific agent (34). Inoculative and conservative methods are often used in biological control using predators and parasitoids. However, these methods have hardly been used with microbial control agents. For viral pathogens, a few attempts were

carried out using baculovirus for the control of forest pests such as the gypsy moth (*Lymantria dispar*), European spruce sawfly (*Gilpinia hercyniae*), and European pine sawfly (*Neodiprion sertifer*) (51).

Multiple studies have revealed that covert infections (also known as persistent or silent infections) with DNA and RNA viruses are very common in field insects, and many stress factors have been discovered to activate lethal infections, encouraging further research into their regulation of the host populations and their potential applications in pest control (45, 98). Although viral covert infections are naturally maintained in populations by vertical transmission, they could also be generated by inoculative applications of the viruses into the system. Strategies aimed to exploit natural and inoculated viral infections to reduce pest populations by triggering epizootics of disease in field populations would need to address certain pitfalls to understand the mechanism regulating the covert/overt interaction with the host, the broad-range effects of viral infection on host fitness and their integration into the cropping system.

Metagenomic analyses of insect populations have significantly increased the rate of virus discovery and revealed that covert virus infections are ubiquitous. However, the mechanisms governing the maintenance of the infection, and, more importantly, the transition from covert to overt infections, remain to be elucidated. Insects have developed a complex immune system to fight against infections (16). However, viral strategies for the maintenance of covert infections include circumventing the host defenses by repressing or inhibiting the immune system or infecting specific cell types (62). Studies on host immunity may potentially clarify the mechanisms involved in the maintenance of covert infections and suggest new molecular tools for promoting the transition to the overt stage.

Application of a baculovirus produces obvious lethal symptomatology on the targeted pest. However, covert infections with baculovirus and with most of the newly discovered RNA viruses do not reveal a clear symptomatology, and their long-term effects on insect populations are not well known. So far, clear effects of RNA viruses on insect fitness have been mainly reported in reared beneficial insects such as honeybees and silkworms. For example, Sacbrood disease in honeybee larvae is a fatal disease caused by the Sacbrood iflavirus, and dual infection together with deformed wing virus (DWV) has synergistic effects on the host egg transcriptome. In addition, in the case of DWV infection, besides the direct effect on bee development, long-term covert infection may seriously hamper foraging behavior and colony survival (7, 17). In this context, it is likely that a more detailed study of the broad-range effects of viral infections with newly discovered or poorly studied viruses may reveal long-term effects, perhaps relevant to the detrimental effects on population dynamics. It is also possible that covert infections with these RNA viruses could provide certain advantages to the host. For example, a picorna-like virus in pea aphids suppresses the jasmonic acid response in the plant host, thus facilitating aphid adaptation to different plant hosts (74).

The pest and the viral BCA must be considered as components of a complex system, which also includes the plant or cropping system. Additional components influencing the efficacy of a viral pesticide (115) may include epidemiological parameters (pest abundance, density, spatial distribution) and host intrinsic factors (susceptibility, stage, resistance). Alone and together, each parameter can influence the outcome of an infection. In addition, virus-based strategies in biocontrol should be considered in the context of integrated pest management strategies, including compatibility with other BCA agents. So far, the effect of covert viral infections on the use of nonviral microbial pathogens or the efficacy of the host predators and parasitoids has been the subject of limited studies. Some of the few examples of covert viral infection effects on other BCAs revealed different outcomes depending on the virus–BCA–host combinations and interactions. For instance, Junonia coenia densovirus and Helicoverpa armigera densovirus-1 have been shown to

protect lepidopteran pests from other pathogens, including baculovirus and *Bacillus thuringiensis* (46, 144). In contrast, in the lepidopteran *Spodoptera exigua*, sublethal infections with two iflaviruses increase the potency of baculovirus (22).

Alternative virus-based strategies for biocontrol: molecular manipulation of insect viruses and virus-based vehicles. The approval of transgenic plants targeting the Coleoptera *Diabrotica virgifera* (western corn rootworm) by US and Canadian regulatory agencies has opened the way to RNA interference (RNAi)-based control methods of insect pests. RNAi allows silencing of selected genes through the delivery of sequence-specific dsRNAs inside the target host (58). The technique exploits a cellular mechanism devoted to the endogenous regulation of gene expression, together with the immune reaction against invading viruses and transposons. RNAi may be applied to silence vital insect genes, and owing to its sequence specificity, it has the potential to become an efficient and selective insecticide strategy. Yet several important ecological and technical issues need to be addressed, such as (*a*) the selection of target genes to identify the most efficient ones for each insect species at the most appropriate life stage, (*b*) the specificity of the dsRNA sequence to avoid off-target effects, dosage, and saturation of the RNAi machinery, (*c*) the size of the administered dsRNA molecules, (*d*) delivery strategies, especially to phloem or xylem feeders, and (*e*) possible development of resistance (66). In particular, to protect the dsRNA molecules from degradation during application and from the insect environment upon internalization, their absorption within nanoparticles or their integration within the genome of engineered viruses that can infect the target insect host has been proposed (66).

In the same way as mycovirus-based VIGS, insect virus–based VIGS vectors exploit modified viruses for transient knockdown of target genes through RNAi (32). VIGS is also a powerful tool for investigating insect–plant interactions, in most cases for functional description of plant genes (therefore using plant virus–derived VIGS vectors) to improve plant resistance to herbivore pests (28).

Densoviruses have been explored as VIGS vectors for the prevention of mosquito-borne diseases. A nondefective recombinant Aedes aegypti densovirus microRNA expression system was developed with the aim of clarifying mosquito biology and paratransgenesis parameters for dengue virus control (70). Similarly, Anopheles gambiae densovirus suitability as a VIGS vector was evaluated for malaria control (105). From a plant protection perspective, VIGS is also interesting for its ability to target insect genes, and the construction of an infectious clone of the infectious paralysis dicistrovirus has paved the way to explore other insect viruses as VIGS vectors (64). Construction of insect-specific cDNA libraries together with their insertion into appropriate VIGS vectors to obtain virus-infected plants expressing insect-specific dsRNAs has been proposed as a platform to identify candidate genes with insecticidal and/or repellent activity against *Aphis gossypii* (67). A similar strategy has been explored for other vectors of plant pathogens such as *Diaphorina citri* (52) and *Bactericera cockerelli* (142) psyllids. Phytoplasmas are transmitted by phloem-feeding hemipterans and indirectly controlled by insecticide treatments. The VIGS approach has been applied to the functional genomics analyses of phytoplasma effectors within the host plant (13). In this case, a tobacco rattle virus–based VIGS vector was used to silence the importin α genes (1, 2) of *Nicotiana benthamiana* to confirm their role in the nuclear internalization of the '*Candidatus* Phytoplasma asteris' SAP11 effector. In the case of insect vectors, VIGS can be exploited for direct silencing of vital genes (insecticide activity) but also as a functional genomic tool to screen for insect genes involved in pathogen transmission, especially in the case of phytoplasmas, as they are transmitted by nonmodel insect species.

Exploration of the insect virosphere can also provide the frame for selection of the most appropriate viruses as potential VIGS backbones for phytoplasma vectors, whose control relies

almost exclusively on insecticide treatments. In the case of phloem feeder hemipterans, infection by iflaviruses is quite common (1, 92). Iflaviruses, with their monopartite ssRNA genomes, meet several criteria to be explored for VIGS of phytoplasma vectors (111). Several new positive-strand RNA viruses most closely related to plant sobemovirus, luteovirus, and tombusvirus have been discovered in thrips viromes and could also be easily adapted to VIGS vectors (26).

These approaches involving modified viruses to interfere with pathogen transmission are beyond the scope of biocontrol, but they must be considered as new research avenues in pest management with high innovative potential. This is especially true in the case of vector-borne diseases, as no threshold is acceptable for vector populations to avoid crop infection.

5. VIRUS-BASED BIOCONTROL TOOLS FOR DISEASES CAUSED BY PLANT VIRUSES

It is intriguing that plant virus diseases can be prevented by viruses themselves. The mechanism of virus-based biocontrol tools for virus diseases called cross-protection (152) differs from what we have shown for the other organisms in which viruses are chosen because of their intrinsic parasitic properties and their ability to interfere with host homeostasis in an ecological sense.

To control virus-caused plant diseases with virus-based biocontrol tools, other paradigms better describe the rationale of the approach used, and the most fitting definition, sometimes used in the past, is that of preimmunization with a nonpathogenic (mild) isolate of the virus, as is the case of the live attenuated vaccine against several human viral diseases (84). In fact, virus-based biocontrol tools are mild strains of a virus that, when preinoculated to a host plant, can protect from subsequent infection of a second, severe isolate of the same virus (152).

Cross-protection was first described more than a century ago with mild strains of tobacco mosaic virus protecting tobacco from strains causing a more severe yellow mosaic symptom (78). Since then, cross-protection has been used in open-field conditions to protect against important virus diseases caused by citrus tristeza virus (CTV) on citrus species (40, 49), zucchini yellow mosaic virus (139) on several cucurbit species, and papaya ringspot virus in papaya (145): Detailed analyses of such case studies were provided in a very comprehensive review of this field (152).

Some of the possible molecular mechanisms rely on reprogramming the adaptive immune response of the plant (antiviral silencing pathway), and therefore the analogy with the preimmunization is somewhat congruent with this approach. An appropriate framework for understanding cross-protection is that of the use of satellite viruses, satellite RNAs, and defective interfering RNAs as BCAs that offer some potential for tempering the helper-caused disease severity (103, 117). Nevertheless, the silencing pathway seems not to be involved in some specific cases of cross-protection because it also occurs in silencing deficient mutant plants (153).

Cross-protection was also recently developed and employed on a wide scale in Europe for pepino mosaic virus using several mild strains (2, 53) which were also granted distinct approvals from the European Food Safety Agency (36).

A wealth of literature has dealt with the molecular mechanism of cross-protection. Most likely, different mechanisms are involved in different systems, of which some are protein mediated (40, 41, 150) and others are RNA mediated (104). Different mechanisms of superinfection exclusion at the cellular level seem to be a common theme for the molecular mechanism of cross-protection.

Regarding technological advances in developing mild strains, recent work relies on reverse genetics tools to produce mutants in vitro through site-directed mutagenesis without having to rely on natural mutants (25, 95, 153), a time-consuming and often limiting factor in cross-protection approaches.

6. CONCLUSIONS AND OUTLOOK

Many examples show that viruses are viable as BCAs for limiting the impact phytopathogens have on crops. In any case, care should be taken to select viruses that have the expected properties in terms of applicability and safety. As some examples show, off-target or unpredicted outcomes can occur. Targeted research is thus needed to fully understand the biology of the viruses that are applied. Moreover, an understanding of the effect of BCAs on the natural microbiome (soil and plant microbiome) is needed to further pave the regulatory pathway and ensure biosafety. Finally, social acceptance is an important aspect that should be investigated in more detail as a crucial component of getting the products to the agricultural community.

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AUTHOR CONTRIBUTIONS

M.T. and J.W. wrote the Abstract, Introduction, and Conclusions and Outlook. J.W. assembled and formatted the contributions from all authors. M.T. wrote the Hypovirulent Strains for the Biocontrol of Chestnut Blight section and the Virus-Based Biocontrol Tools for Diseases Caused by Plant Viruses section. J.W. wrote the paragraph on phage products being available on the market and coordinated the contributions from D.H., M. Ravanbakhsh, and M. Rabiey in the section titled Phage Biocontrol Against Plant-Pathogenic Bacteria. C.C.T. wrote on environmental risk assessment in the Phage Biocontrol Against Plant-Pathogenic Bacteria section. M.A.A. wrote on mycovirus-based biocontrol and coordinated the assembly of the contributions related to this topic from M.T. and E.V. M.O. wrote paragraphs in the Viruses and Insect Biocontrol: Something Old, Something New section and coordinated the specific contributions written by S.H. and C.M. related to the same topic.

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