Environmental Applications of Genetically Modified Viruses Reveals Challenges

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The release of novel genetically modified (GM) virus applications into the environment for agricultural, veterinary, and nature-conservation purposes poses a number of significant challenges for risk assessors and regulatory authorities. Continuous efforts to scan the horizon for emerging applications are needed to gain an overview of new GM virus applications. In addition, appropriate approaches for risk assessment and management have to be developed and implemented. These approaches need to address pertinent challenges, in particular with regard to the environmental release of GM virus applications with a high probability for transmission and spreading, including transboundary movements and a high potential to result in adverse environmental effects. However, the current preparedness at the EU and international level to assess such GM virus application is limited.

Keywords: genetically modified ; virus ; bacteriophage ; environmental risk assessment ; horizon scan ; agriculture ; nature conservation ; veterinary vaccines

1. Introduction

Viruses have traditionally played a significant role in biotechnology from its early days to the recent developments of new applications of genetically modified (GM) viruses. Thus, the spectrum of possible use scenarios for GM viruses is very broad. Initially, viruses served as a source of genetic elements, such as the 35S promoter element from the cauliflower mosaic virus, which is used for the construction of recombinant expression cassettes for the development of GM organisms (GMOs) [1]. More recently, highly complex applications based on GM viruses are being developed in the field of synthetic biology (SynBio) [2].

Plant and animal viruses have been used for quite a long time as tools to introduce recombinant DNA constructs into target cells [3] to either create GM cells or to transiently express desired gene constructs in target cells. Examples include the production of transgenic proteins (VAGE—virus-aided gene expression), the silencing of the expression of endogenous genes (VIGS—virus-induced gene silencing), or the modification of the epigenetic regulation of plant gene expression, e.g., via RNA-dependent DNA methylation (RdDM) [4].

More recently, viral vectors have been used to express the molecular components required for genome editing, such as sequence-specific nucleases and guide RNAs, or to introduce repair templates for targeted genome editing, also called virus-induced genome editing (VIGE) [2][5][6]. Such viral vectors can be used to introduce targeted genome modifications in planta, thus avoiding the transformation of isolated single cells or plant tissue in vitro [7][8]. Some VIGE approaches involve no in vitro steps at all, but are based on viral expression constructs that spread systemically in the inoculated plants. Such approaches can also to generate plants with heritable genome modifications [9][10]. Such approaches, which facilitate the genetic modification of whole plants growing in the environment, are significantly different from the traditional methods used to create GM plants. These are based on the regeneration of modified plants from single transformed cells or plant tissue cultures under contained conditions in the laboratory and the selection of particular transformants for subsequent release and propagation in the field. If GM vectors based on viruses, which can be spread horizontally among plants, e.g., by animal vectors like herbivorous or parasitic insects, are used for these applications, whole plant populations could be modified in the environment. Applications of this kind are referred to as “horizontal environmental genetic alteration agents” (HEGAAs) [11]. A proposal by the US Defense Advanced Research Projects Agency to develop HEGAA applications has sparked a debate whether such approaches, which may be associated with a substantial potential for unintended environmental effects and/or misuse, should be pursued [12].

Other environmental applications of GM plant viruses concern the use of plant virus constructs developed to express transgenes in infected plants to protect them against certain plant pathogens, such as bacterial pathogens that are causing devastating diseases in orange groves in the USA [13] or in some European olive-producing areas [14].

GM viruses may also be used for animal biocontrol purposes, building on existing experience with the use of non-modified entomopathogenic viruses for the biological control of agricultural insect pests [15] or insect pests, which are vectors for human pathogens [16]. GM viruses have also been explored for the population control of certain animals, such as foxes, rabbits, stoats, mice, etc., by means of immunocontraception, i.e., applications to induce infertility in pest animals [17][18].

In the context of veterinary medicine, GM viruses are used successfully as vaccine agents or as vector backbones for veterinary GM vaccines [19]. Some of these viral vaccines are designed to be replication-competent and transmissible between animals [20]. Such a transmissible vaccine based on a GM virus was developed to protect European wild rabbit populations against myxomatosis and rabbit hemorrhagic disease. However, the development was discontinued after initial field testing in 2000 [21].

In the EU, GM virus applications are subject to the existing GMO regulations, such as Dir. 2001/18/EC, which requires a mandatory risk assessment to be conducted prior to authorization being granted for environmental releases for either field or clinical testing or for placing GM viruses or products developed from them on the market. However, the multitude of possible applications of GM viruses and the prospects for more GM virus applications being used in the environment is likely to present challenges for the competent authorities and institutions involved in the environmental risk assessment (ERA) of such applications at the national and EU level. Both at the EU level [22] and internationally [2], efforts have been made to identify emerging GM virus applications. The study of van der Vlugt [22], which surveyed developments relevant for the EU, focused on the agricultural applications of SynBio microorganisms with relevance for food production and thus did not provide a comprehensive overview of GM virus applications, specifically with regard to environmental applications.

2. Considerations for the Assessment of Environmental Effects of GM Virus Applications

Applications of GM viruses are subject to different regulations. For example, in the EU, there is the current legislation for the use of GMOs in contained use (Dir. 2009/41/EC) and for deliberate release and placing on the market (Dir. 2001/18/EC) as well as the regulations for veterinary medical products (Reg. (EU) 2019/6). These regulations mandate requirements for the risk assessment of GM products before their use may be authorized. Dir. 2001/18/EC provides the general framework for the environmental risk assessment (ERA) of GMOs that are notified for environmental release for experimental trials or for placing on the market of GM products. For veterinary medical products that are GMOs or contain GMOs, Reg. (EU) 2019/6 requires an ERA according to the requirements stipulated in Dir. 2001/18/EC. This ERA comprises a comprehensive case-specific assessment of the potential adverse environmental effects that could result from the characteristics of the specific GM viruses, the exposure of the receiving environments, and from the interactions with target or non-target organisms in these exposed environments. The ERA required by the EMA is considered to provide an in-depth and complex assessment [23].

Several examples for applications are discussed in the following chapters to illustrate the specific scenarios of use and exposures and to identify the potential effects that need to be considered during a risk assessment. The examples for such use cases were based on the different types of applications that were identified by the horizon scanning and additional (regulatory) information on newly developed applications of GM viruses. In particular, the researchers selected applications that could potentially affect biodiversity or nature conservation and could result in negative effects on human and animal health. Earlier considerations regarding the ERA of GM virus applications, e.g., GM virus vectors for vaccine development [23][24][25], were taken into account for the analysis of the following examples:

- A GM virus vector vaccine against communicable facial cancer in Tasmanian devils;
- A GM citrus tristeza virus expressing defensins to protect orange trees from a bacterial disease (citrus greening disease);
- GM bacteriophages for the biocontrol of the plant pathogen *Xylella fastidiosa*.

These examples were chosen with a view to cover the recent developments in several fields of application, such as agricultural applications, including the control of pathogens affecting agriculturally relevant plants (e.g., orange trees and olive trees) as well as veterinary applications targeting threatened wild animals of conservation concern, such as Tasmanian devils. Two of the chosen examples are currently undergoing field testing based on authorizations for deliberate release (GM citrus tristeza virus and GM virus vector vaccine). The third example (GM bacteriophages for biocontrol) represents a proposal for a future use scenario that would involve the spread of the released GM phages into the environment and could lead to a wider and less predictable pattern of environmental exposure.

2.1. GM Virus Vector Vaccine against Facial Cancer in Tasmanian Devils

2.1.1. Description of the Application

The GM vaccine used in this application (Wild Immunity Vector Adenovirus 20-WIVA20) consists of a replication-defective human adenovirus serotype 5 (HAdV-5) vector, which was modified to express tumor antigens to protect Tasmanian devils against devil facial tumor disease (DFTD).

The GM adenovirus construct was prepared using a commercially available platform that allows customized DNA fragments derived from the HAdV-5 genome to be modified and assembled into a linear double-stranded DNA construct [26]. Adenoviruses can infect a wide variety of vertebrates; human adenoviruses have a host range limited to only mammals [27].

The following modifications were introduced into the GM HAdV-5 vector virus:

- The HAdV-5 early transcribed E1 region was deleted. The E1 genes are essential for viral gene expression and replication, and the deletion of these genes prevents the multiplication and expression of late-transcribed genes, thus limiting the production of infectious virus particles [28];
- The early-transcribed E3 region was deleted as well. This region contains genes that are necessary for wild-type adenoviruses to evade host immune responses. The deletion of the E3 region results in an increased immunogenic response in host animals to the GM virus;
- An expression cassette was inserted into the early transcribed region to express two antigen constructs designed to induce immune responses against DFTD tumor cells. One antigen was designed to elicit a response against DFT1 tumor cells; the second antigen was a construct that contained 18 short epitopes specific to DFT1 and DFT2 tumor cells (i.e., a polypeptide neoantigen) and a fluorescent marker gene.

The GM WIVA20 virus can express the antigen genes in the target animal [29], but it cannot replicate or cause a disease [27]. The expression of the GM antigens is transient, as the GM vaccine would be cleared by the host immune system within days or weeks, except in case of integration of the viral DNA into the host genome, which is considered to be a very rare event [30].

The obtained GM vector virus is similar in structure to other previously designed HAdV-5-based vaccines, for example, vaccines that are used in humans (e.g., the Vaxzevria COVID-19 vaccine produced by AstraZeneca), vaccines used for veterinary purposes (e.g., the ONRAB® rabies vaccine authorized in North America, which is based on a replication-competent GM HAdV-5 vector), and vaccines used in cancer immunotherapy [27].

2.1.2. Exposure Pathways and ERA Considerations

For the inoculation of the test animals, the GM WIVA20 vaccine was produced in the form of infectious viral particles in helper cells and was administered via intramuscular or intratumoral injection or by direct instillation into the oral cavity to test different methods of inoculation [27]. After inoculation, the GM viral particles may be distributed in the body of the vaccinated animals and can be excreted or shed. Such shedding is considered the main route of environmental exposure in the case of replication-incompetent adenovirus-based vaccine agents [27]. The amount of shed virus particles, however, would be limited by the dose of the viral vaccine that was used for the vaccination. Accidental exposure of human staff to the vaccine agent itself or to the vaccinated animals needs to be considered, as does the exposure of non-target animals to vaccinated Tasmanian devils and to virus particles that are shed by them, e.g., via saliva, feces, or, to a lesser degree, urine.

The following risk issues were considered for the risk assessment of the time-limited, partly contained test trial [27]:

- Infection of human staff who are accidentally exposed to infectious material during immunization or the handling of immunized animals. Due to the characteristics of the vaccine, infections would not be sustained over longer periods, and then only limited health effects, if any, were to be expected [27];
- Infection of non-target animals after contact with vaccinated Tasmanian devils and with saliva or feces from vaccinated animals that contain virus particles. While contact with other mammals was restricted in the test facilities, contact of birds with vaccinated animals or shed virus particles would be plausible. However, the effects were considered to be minimal, as HAdV-5 is different to adenoviruses occurring in birds and exposure is not believed to lead to adverse effects according to the OGTR [27];

- Generation of replication-competent GM viruses could happen via recombination or complementation in animals or humans infected with the GM virus and other AdV variants. However, these scenarios were considered highly unlikely by the OGTR due to the properties of the AdV and the multiple modification events necessary. Also, the likelihood that co-infection and complementation events would occur in the cells of the vaccinated Tasmanian devils or other exposed animals or humans would be very small under the conditions of the trial. Similar conclusions were drawn for recombination events that could also lead to different combinations of GM or wild-type viruses after multiple recombination events, including to GM viruses with an altered host range [27].

Concerning unrestricted release scenarios, the following aspects would need to be considered:

The GM virus could be present in inoculated animals (or tumors) for several weeks or even months. There is some uncertainty about the possible duration and extent of shedding and the likelihood of a possible infection of other vertebrate hosts (including people, horses, cattle, pigs, sheep, goats and domestic fowl, wild birds, bats, and reptiles). In addition, AdVs can remain infectious for long periods in the environment, for weeks in tap water, sewage effluent, and sea water [31], and for from 7 days to 3 months on dry surfaces [32].

There is some uncertainty concerning the stability of the GM virus. Some mutations were detected in the GM virus that was produced for use as the vaccine. The number of these mutations were in line with the mutation frequencies that can be expected for the replication of HAdV-5 viruses [33]. According to an analysis by the applicant, the identified mutations did not impact functions of the GM WIVA20 vaccines [27]. Another relevant issue involves possible recombination events upon the co-infection of humans and animals with GM WIVA20 as well as other AdVs. Such viruses, including non-human AdVs, would likely be present in the environment. However, the environmental spread of recombinant viruses expressing GM immunogenic peptides will be limited if the expression of the GM insert has a negative impact on viral replication, i.e., by eliciting an immune response. GM inserts in recombinant vector vaccines that are not beneficial for the propagation of the GM virus will likely get lost during sequential viral replication/hybridization events [34]. The interplay of the factors influencing transgene stability, such as the characteristics of the modified vector genome and the inserted transgenic sequences, the expression level of the transgene(s), the host cell environment, and the virus abundance, is complex, poorly understood, and challenging to predict [24][35]. The ERA requirements, according to Dir. 2001/18/EC, do not mandate the provision of (WGS) data to address this issue appropriately [24].

During the trial, the risks resulting from the co-infection of Tasmanian devils or other host species with multiple adenovirus strains and the recombination of naturally occurring adenoviruses with the GM WIVA20 virus were considered to be negligible by the OGTR due to the conditions imposed by the regulator. These conditions mandate that freshly vaccinated animals are kept in confinement for the time when shedding occurs and are only relocated when tests indicate that shedding has stopped.

There are uncertainties concerning potential effects for non-target organisms infected with GM WIVA20 expressing polypeptide neoantigens. The used promoter could drive the expression of recombinant proteins in a wide range of mammalian cells. A potential cross-reactivity with healthy tissues of humans, birds, or other animals could occur with a low risk of autoimmunity in humans and animal species, as concluded by OGTR [27].

2.2. GM Citrus Tristeza Virus to Protect Citrus Trees against a Bacterial Disease

2.2.1. Description of the Application

Citrus greening disease, or Huanglongbing (HLB), is a bacterial disease (caused by *Liberibacter asiaticus*) affecting citrus and orange trees [36]. The disease and its vector species, psyllids (e.g., *Diaphorina citri*), were introduced into the Americas in the 2000s. Since 2005, the disease has caused significant damage to commercial orange and citrus fruit production in the USA, e.g., in Florida [37]. The pathogenic bacterium colonizes the phloem of infected trees and inhibits the flow of essential nutrients in the vascular system. This leads to damage in the root system as well as subsequent damage to leaves and fruits. The pathogen is spread efficiently by its highly mobile and fast-reproducing vector, *Diaphorina citri* [38], which shows increased fitness and fecundity upon infection with *Liberibacter asiaticus* [39].

To protect orange trees, an approach was developed based on a GM plant virus vector derived from modified CTV isolates to express a variety of antimicrobial proteins (defensins) in citrus trees. CTV is an RNA plant virus (from the genus *closterovirus*) that can infect various citrus plants. The virus is associated with the phloem of the host plant and multiplies in the cytoplasm of phloem parenchyma cells [40]. The GM CTV vector is derived from wild-type CTV strains (T30 and T36), which occur naturally in Florida and do not lead to severe disease symptoms (stunting, slow or quick decline, stem pitting) in the orange varieties grown in Florida. The genes expressing the defensins (SoD2, SoD7, SoD8,

SoD9, SoD11, SoD12, and/or SoD13) were derived from spinach. Previous studies, e.g., to create HLB-resistant GM orange trees, indicated that these defensins can protect orange trees against infection with *L. asiaticus* and thus against HLB disease [41][42].

2.2.2. Exposure Pathways and ERA Considerations

For the treatment, scions (i.e., stem, leaf, or bark pieces) infected in the laboratory with CTV-SoD virus were grafted onto healthy orange trees in orange groves and tree nurseries or orange trees that were already infected with Liberibacter asiaticus. Upon inoculation, the GM CTV-SoD virus spread systemically in the vascular tissue of the infected citrus trees and expressed the defensins in the very plant tissues that were colonized and affected by *Liberibacter asiaticus*. CTV-SoD is expected to persist for longer periods (of up to several years) in infected citrus plants. It is not expected to introduce genetic changes into the genome of treated orange plants.

No other forms of release of the GM CTV-SoD virus, e.g., spreading by infected vector insects, are foreseen, and such exposure pathways were consequently not addressed by the USDA in its assessment [41]. However, the possible spread of CTV-SoD by vector insects, starting from inoculated trees, would lead to a similar result and needs to be taken into account when analyzing additional exposure pathways and impact areas.

In case the exposed areas are indeed limited to orange trees deliberately inoculated by humans, the range of exposed organisms would include the target pathogen and non-target organisms that come into contact with GM CTV-SoD-infected orange trees, including non-target bacteria and fungi as well as non-target animals such as psyllids, aphids, mites, and nematodes. If the further spread of CTV-SoD by (insect) vectors, either accidentally or via the unauthorized movement of infected scions, were to occur, the potentially exposed areas would comprise further areas and possibly also other (citrus) host plants and a wider range of associated non-target species.

The relevant issues to be considered for the risk assessment of GM CTV-SoD are as follows:

- Adverse effects may occur through changes in the infectivity and pathogenicity of the GM CTV virus as well as through changes in its host range. Evolutionary changes due to the higher mutation rates in RNA plant viruses like GM CTV-SoD as well as through recombination/complementation can be expected. Indications of such changes can be provided by a thorough molecular characterization of the GM virus [43]. Based on a comparison of CTV-SoD with the CTV strains endemic in Florida (in particular, the CTV strains T30 and T36), the USDA concluded that CTV-SoD does not show novel biological characteristics [41]. However, for an application of GM CTV-SoD in the EU, the CTV strains that occur in Europe would need to be considered [40];

- Additional environmental hazards could arise due to the spreading and establishment of the GM virus in other host plants outside of the areas of intended use. For this scenario, GM CTV-SoD would have to spread from the inoculated trees into the surrounding ecosystems via parts of the GM-virus-infected plants or by transmission through animal vectors. Uncertainties exist whether this is possible. In Florida, transmission by the CTV vector insects was not detected [41]. However, the complementation of poorly transmissible CTV strains (T36) with other naturally occurring CTV strains in the laboratory did increase the ability of CTV-SoD to be transmitted by aphids, such as the main US vector of CTV, the brown citrus aphid (*Toxoptera citricida*) [44]. In case the insects known to vector CTV in Europe [40] could also transmit GM CTV-SoD, the possible spread to other citrus plants relevant in the Mediterranean region of Europe would need to be investigated. This would concern plants such as lemon, lime, sweet and sour orange, mandarin, tangerine, grapefruit, trifoliate orange (*Poncirus*), and dwarf orange (kumquats);

- It is also uncertain whether pre-infection with naturally occurring CTV variants could protect possible EU host plants from additional infection by GM CTV-SoD due to the current limited understanding of cross protection (superinfection exclusion) [45];

- Regarding the effects on the target organisms, it needs to be assessed whether the target organism *Liberibacter asicaticus* can develop resistance to the spinach defensin(s). Genetic instability of the transgene(s) in GM CTV-SoD could also lead to a loss of efficacy to control the target pathogen and has to be considered, as does the evaluation of the efficacy of the GM CTV-SoD application;

- Possible effects on non-target organisms were not assessed comprehensively by the USDA [41]. Their assessment focused on the experience associated with the human consumption of spinach, which indicates no antinutritive effects of the defensins as part of a diet including spinach [46]. However, spinach defensins can have adverse effects on exposed non-target bacteria. The defensins were shown to exert an antimicrobial effect on Gram-negative bacterial

species and a less strong effect on fungi (e.g., *Fusarium*) and Gram-positive bacteria [47]. However, the mode of action of their effects is currently not well understood;
- Other non-target organisms could also be exposed to the spinach defensins upon feeding on orange trees infected with GM CTV-SoD. A report by the National Academies of Science [48] indicated a wide variety of species that could be exposed and possibly affected in the USA, including the citrus leaf miner (Phyllocnistis citrella), several species of scale insects (Unaspis citri, Chrysomphalus aonidium, Lepidosaphes beckii) and whiteflies (Dialeurodes citri, D. citrifollii, Aleurothrixus floccosus, A. woglumi), the citrus mealybug (Planococcus citri), root-associated beetles (Diaprepes abbreviatus, Pachnaeus litus, Pachnaeus opalus), citrus mites (Aculops pelekassi, Phyllocoptruta oleivora, Eutetranychus banksi), and nematodes (Tylenchulus semipenetrans, Radopholus similis, Belonolaimus longicaudatus, Pratylenchus coffeae, P. brachyurus). Related species should be considered as non-target organisms in Europe.

The assessment of the possible environmental effects of the GM CTV-SoD virus is associated with a variety of relevant uncertainties. It is currently unclear whether the risk assessment conclusions relevant for the USA [41] are transferrable to other regions such as Europe, e.g., also considering uncertainties regarding target and non-target organisms as well as the range of vector insects occurring in the EU. The results of the ongoing field trials and the studies supporting the risk assessment by the USDA need to be analyzed regarding whether they provide relevant data for a number of risk hypotheses, such as possible adverse effects of the transgenic defensins on non-target organisms or the possible spread to other CTV host plants. The EFSA noted that the US assessment [41] did not cover all the risk areas that would need to be considered in an environmental risk assessment in the EU under Directive 2001/18/EC [43].

A possible transmission of the GM virus by vectors to other host plants would significantly increase the area impacted by the GM virus and the citrus species, which would need to be monitored. Furthermore, the spread of the GM virus outside areas of commercial cultivation of orange trees would result in the exposure of natural habitats and ecosystems. The exposure of relevant non-target organisms in EU environments has not been conclusively clarified yet and requires further assessment.

2.3. GM Bacteriophages for the Biocontrol of a Plant Pathogen

2.3.1. Description of the Application

The proposal for the “Xylencer” application [49] represents a particularly elaborated approach to using GM bacteriophages to protect or cure crop and horticultural plants, e.g., olive trees, from a bacterial plant pathogen, *Xylella fastidiosa*. The development the application is in the early research stage; several aspects of its application have been developed in laboratory studies.

The approach consists of two different interacting elements:

- A set of lytic bacteriophages, which were shown to target the bacterial pathogen *X. fastidiosa* [50][51]. These phages were genetically modified to express a peptide derived from the flagellin protein (flg22) of *X. fastidiosa*, which triggers defense response mechanisms in infected plants. The GM phages also express a chitin-binding protein (PD1764sh). This protein was fused to the capsid protein of the GM phages and mediates adherence to sap-feeding insects for dissemination;

- A bacterium developed from a strain of *Xanthomonas arboricola* was genetically modified and acts as a phage-delivery bacterium (GM PDB). *X. arboricola* is a recognized phytopathogen (EFSA PLH 2014) and can cause canker and bacterial blight, e.g., in stone fruits, almonds, and walnuts [52]. For the Xylencer approach, a non-pathogenic strain, *X. arboricola* CITA 44, was used to construct the GM PDB. The GM bacterium was modified with a sensing mechanism in order to express the GM bacteriophages selectively in crop plants infected by *X. fastidiosa*. The GM PDB was further genetically modified with a kill-switch, which is triggered in plants that are not infected by the pathogen. This kill-switch removes the GM bacteria from non-infected plants within several days.

2.3.2. Exposure Pathways and ERA Considerations

Dissemination of the GM PDB and thus the GM bacteriophages will be achieved through the injection of the PDB into cultivated crop plants. Due to the GM chitin-binding protein, the GM bacteriophages will be dispersed by insect vectors, including species that spread *X. fastidiosa.* Relevant for the determination of exposure pathways are the insect vectors, which can transmit the PDP and/or the GM phages to other infected or non-infected host plants. Besides the primary vector, the highly polyphagous meadow spittlebug *Philaenus spumarius*, other insects feeding on infected host plants need to be considered as potential vectors of dissemination among trees and between orchards [53]. In the EU, climatic factors determine the establishment of the target pathogen. Suitable areas for a potential establishment differ between

Xylella subspecies [53], but in general, horticultural plantations in southern Europe are the most relevant potential receiving environments. About 174 plant species have been identified as potential hosts for *X. fastidiosa*, including economically important plants like olive, grapevine, stone fruit, and forest trees [14][53].

The intended exposure pathways comprise the following routes:

- Artificial inoculation of horticultural trees (e.g., olive trees) infected with *X. fastidiosa* with the GM PDB and the spreading of the expressed GM phages within the plants' xylem. Due to the kill-switch, the GM PDB is assumed to be present for just a few days in plants that are not infected by *X. fastidiosa*. In addition to the targeted pathogen, other bacteria residing in the xylem of the inoculated plants would be exposed, as would insects feeding on the infected plants;

- Spreading to other infected and non-infected horticultural plants by specific insect vectors, i.e., *Philaenus spumarius*, which is intended to spread the expressed GM phages, due to their ability to adhere to the chitin cuticula of the vector.

In addition, several other (unintended) pathways of exposure need to be considered:

- Spreading by other insect vectors, e.g., cicadas, or other insects, such as xylem-feeding species, to non-target plants. The range of such insects that are able to spread the GM phages is considered to be broad due to the ubiquitous occurrence of chitin in insect cuticles; however, limited data are available to identify all the relevant insect vectors [53] [54];

- The exposure of non-target bacteria in microbiomes associated with infected plants. Dependent on the ability of the GM phages to spread throughout the plants' vascular system and possibly to the phyllosphere and the rhizosphere, non-target bacterial species from these microbiomes could be exposed.

For a risk assessment, the properties of the GM PDB as well as of the GM bacteriophages have to be considered. This poses additional challenges to identifying and characterizing the possible pathways to harm that may be triggered by the application. The following aspects need to be considered:

- The survival, spreading, and persistence of the PDB and the GM bacteriophage need separate consideration. The survivability and spreading of the GM phages, if released within the target plant, and their intended spreading by a vector insect are difficult to predict, as habitats other than those intended may be exposed to the GM phages. The persistence of the GM phages in plants is also not known. In general, phages are able to survive in planta for several weeks even in the absence of a bacterial host [51]. A theoretical model has been developed for predicting the spreading of the GM bacteriophages in the Italian region of Apulia based on data for *X. fastidiosa* [55][56]. The results showed that it might spread successfully across a large (10 × 10 km) area over a period of 40 days;

- Horizontal gene transfer (HGT) mediated by bacteriophages has been considered an environmental risk in other contexts (see González-Villalobos and Balcázar [57]). However, virulent (lytic) phages such as those used in the Xylencer application are expected to have a lower capacity for transduction of pathogenic factors [58]. The developers concluded that the transfer of the (inactive) Cas9 (dCas9) protein from the GM phages into the *X. fastidiosa* genome could have negative effects on the efficacy of the Xylencer approach. Due to the functions of the other transgenic elements, the risk for other unintended effects due to HGT is considered low;

- With regard to effects on target organisms, the assessment of the host specificity of the GM phages for the pathogen *X. fastidiosa* is considered an important aspect. The possible development of resistance to the GM phage treatment in the target organism also needs to be assessed. Any evolutionary changes in the virulence and pathogenicity of the GM phages may influence the effects on the target organism(s); however, they are challenging to assess. In addition, it is uncertain whether the predicted genetic changes, such as the loss of transgenic sequences, e.g., of the fusion gene between the phage's capsid protein and the truncated chitin-binding protein, would indeed occur;

- Concerning effects on non-target organisms, the possible range of the host bacteria needs to be considered to determine whether non-target bacteria that occur in exposed plant microbiomes, including beneficial ones, may be affected. As shown by Ahern et al. [50], two of the employed phages are related to a larger group of virulent *Xanthomonas* phages, and the other two are related to phages occurring, e.g., in *Burkholderia* (phage AH2), Enterobacter (phage Enc34), other enteric bacteria (phage Chi), and *Providencia* (phage Redjac). The host range may also be changed by mutations, e.g., those affecting the receptor-binding proteins of the respective phages [59]. A possible reduction in beneficial bacteria may affect the overall performance and therefore the yield of the treated plants

[60]. In addition, potential adverse effects of the transgenic proteins in exposed non-target insects or non-target plants need to be considered;

- The effects on the biogeochemical processes resulting from interactions of the GMO with target and non-target organisms if the bacteria of the soil microbiome are affected need to be considered. Some phages can persist for several weeks in the soil [61];

- Environmental impacts due to changes in the current management need to be considered, as the Xylencer application is meant to replace the current conventional, environmentally harmful management measures to control the target pathogen, i.e., the use of antimicrobial substances or pesticides to control the insect vector(s). Some existing measures may not be compatible with the release of GM phages or PDBs. Therefore, the use of a GM-phage-based approach must be closely coordinated and the efficacy monitored in order to avoid a failure to control the pathogen or subsequent harmful management measures.

Overall, considerable uncertainties exist regarding the long-term stability and interactions of the GM PDB and the GM bacteriophages in natural environments. The long-term efficacy of the approach is uncertain due to the dynamics of the interactions of phages with bacterial hosts, including the development of resistance. Uncertainties are also associated other aspects of the approach, including the loss of capacity for transmission of the GM phages by insects and the efficacy of the kill-switch engineered in the PDB. In this context, approaches have to be developed for the assessment of the long-term robustness and reliability of the novel genetic circuits. Thus, challenges exist for the prediction of evolutionary changes in the GM PDB and the GM phages for potential long-term and large-scale applications in long-lived organisms such as trees.

The confinement of the GM bacteriophages to their target environments is hard to ensure due to uncertainties regarding the spreading dynamics of the target pathogen and the GM bacteriophages as well as their host specificity. Substantial difficulties exist in defining the receiving environments and in assessing the consequences of the spreading of GM bacteriophages to other ecological niches and the resulting interactions in the environment. These uncertainties need to be addressed during the assessment and need to be considered regarding the surveillance and monitoring of the exposure and long-term effects.

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