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Seeing the forest for the trees: Use of phages to treat bacterial tree diseases

Emily R. Grace[1] | Mojgan Rabiey[1] | Ville-Petri Friman[2] | Robert W. Jackson[1]

[1]School of Biosciences and Birmingham Institute of Forest Research, University of Birmingham, Birmingham, UK

[2]Department of Biology, University of York, York, UK

Correspondence
Mojgan Rabiey, School of Biosciences and Birmingham Institute of Forest Research, University of Birmingham, B15 2TT, Birmingham, UK.
Email: m.rabiey@bham.ac.uk

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Abstract

Trees and woody plants can be attacked by many pests and pathogens either individually or as polymicrobial infections. In particular, infections caused by tree-specific bacterial pathogens have become more common during the last decade, causing serious concern for important tree and woody plant species in horticulture, urban environments, and forests. For example, *Xylella* and *Pseudomonas* bacteria are causing significant economic and ecological devastation throughout Europe in olive, cherry, and other stone fruits, mainly because of lack of efficient control methods and the emergence of bacterial resistance to traditional antimicrobial compounds such as copper and antibiotics. Hence, there is an urgent need for innovative approaches to tackle bacterial plant diseases. One way to achieve this could be through the application of biological control, which offers a more environmentally friendly and targeted approach for pathogen management. This review will explore recent advances in use of pathogen-specific viruses, bacteriophages (or phages), for the biocontrol of bacterial tree diseases. Phages are an important component of plant microbiomes and are increasingly studied in plant pathogen control due to their highly specific host ranges and ability to selectively kill only the target pathogenic bacteria. However, their use still poses several challenges and limitations, especially in terms of managing the bacterial diseases of long-lived trees. A particular insight will be given into phage research focusing on controlling *Pseudomonas syringae* pathovars, *Erwinia amylovora*, *Xanthomonas* species, *Ralstonia solanacearum*, and *Agrobacterium tumefaciens*. Recent milestones, current challenges, and future avenues for phage therapy in the management of tree diseases are discussed.

KEYWORDS
bacterial tree diseases, bacteriophage therapy, biological control, integrated pest management, tree health

1 | INTRODUCTION

Trees play an essential role for natural ecosystems, forming a critical source of oxygen and water, contributing to soil carbon balance, providing an ecosystem to countless organisms, and aiding in the restoration of disturbed land (Seth, 2003). Humankind also exploits trees for food and wellbeing (Seth, 2003) and various other resources, including timber, fruit and nuts, biofuel, and medicines (Seth, 2003). The recent rise in the prevalence of bacterial tree diseases is thus concerning (Figure 1). Bacterial pathogens cause many devastating tree diseases worldwide that can have large impacts on economies and societies. For example, in the United Kingdom, the loss of plum and cherry production due to bacterial canker is estimated to cost approximately £5.6 million annually (Roberts & Elphinstone, 2017). Also, citrus huanglongbing, or greening, is the most destructive citrus disease worldwide. The estimated damage of the disease over the past five years amounts to over $1 billion per year in the United States (Li et al., 2020b). Globalization has further exacerbated the spread and economic cost of bacterial tree pathogens, as many are able to enter new countries via import of infected plant material or movement of infected insect vectors. In 2012, potential bacterial diseases that may emerge in Europe were summarized (Janse, 2012). One of the predicted emerging pathogens was *Xylella fastidiosa*, the cause of many leaf scorch diseases, including Pierce's disease of grapevine. As predicted, the disease reached Europe in 2013 and was first detected in Italy and then other parts of southern Europe (DEFRA, 2020). It has devastated olive plantations in Italy and poses a major threat to the crop in other regions. Other bacterial tree diseases on the EU watchlist include 'Candidatus *Liberibacter*' spp., the causative agent of huanglongbing disease and 'Candidatus *Phytoplasma phoenicium*', the causative agent of peach and almond witches' broom disease (Janse, 2012). *Pseudomonas avellanae*, the causative agent of canker and decline of hazelnut and *P. savastanoi* pv. *savastanoi*, the causative agent of olive knot disease, have just been added to the DEFRA control list (https://secure.fera.defra.gov.uk/phiw/riskRegister/).

There is hence an urgent need to develop more efficient control measures to tackle both already established and newly emerging bacterial tree diseases. Chemical control via sprays of copper and antibiotics, such as streptomycin, were originally successful, until their overuse resulted in widespread antimicrobial resistance and toxic bioaccumulation (Bünemann et al., 2006; Sundin & Wang, 2018). Lack of chemical alternatives has thus led to renewed interest in the biological control of plant-pathogenic bacteria. Current biocontrol research is heavily focused on microbe-mediated biocontrol where the pathogen management is achieved via specific interactions with other microbes. These interactions can be driven by antagonism where plant-beneficial bacteria are able to suppress the pathogen due to competition for resources and space, or by direct pathogen inhibition via secretion of antimicrobials (Köhl et al., 2019). Alternatively, it is possible to use microbes that indirectly control the pathogen by inducing changes in plant-induced systemic resistance (ISR), systemic acquired resistance (SAR; Luna et al., 2012; Sundin & Wang, 2018), root exudation patterns, or antimicrobial production (Stringlis et al., 2018). In addition to bacteria, recent work has explored the potential of pathogen-specific phage parasites on plant pathogen biocontrol (Buttimer et al., 2017; Koskella & Taylor, 2018). Phages are viruses that infect and replicate inside bacteria. Discovered independently by Frederick William Twort in 1915 and Felix d'Herelle in 1917, they have since been found to be the most abundant and ubiquitous entities on the planet, present in marine, soil, air, industrial, and eukaryotic microbiomes (Batinovic et al., 2019; Swanson et al., 2009). Their narrow host range makes them suitable for specifically targeting bacterial pathogens in complex microbiomes and phage treatments are currently developed for medical, veterinary, food processing, as well as agricultural applications. This review will explore the recent advances in phage biocontrol research of bacterial tree diseases. A particular insight will be placed on research surrounding the highly damaging *Pseudomonas syringae* pathovars, *Erwinia amylovora*, *Xanthomonas* species, *Agrobacterium tumefaciens*, *X. fastidiosa*, and *Ralstonia solanacearum* species complex.

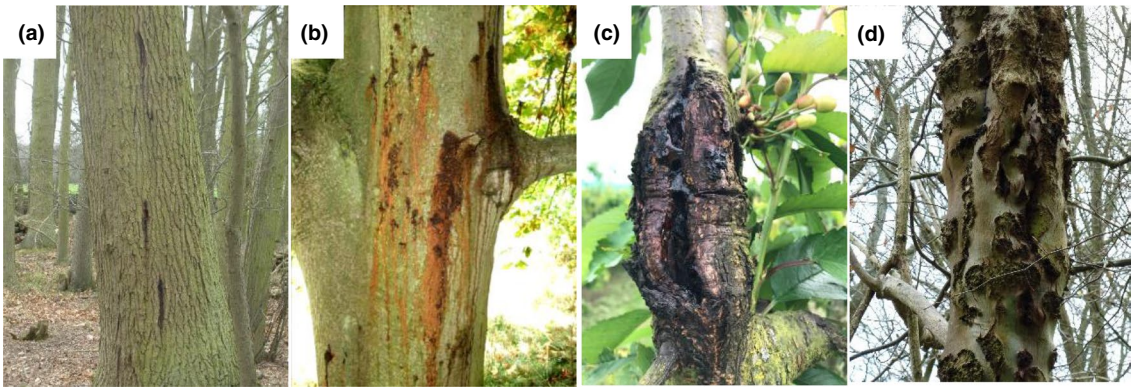

FIGURE 1 Common UK bacterial tree diseases. (a) Acute oak decline (caused by polybacterial consortium, for example, *Brenneria goodwinii*, *Gibbsiella quercinecans*, and *Rahnella victoriana*); (b) horse chestnut bleeding canker (caused by *Pseudomonas syringae* pv. *aesculi*); (c) cherry canker (caused by *Pseudomonas syringae* pvs *syringae* and *morsprunorum*); (d) ash bacterial canker (caused by *Pseudomonas savastanoi* pv. *fraxini*). We thank Oliver Booth, Federico Dorati, and Eric Boa for images (a), (b), and (d), respectively [Colour figure can be viewed at wileyonlinelibrary.com]

2 | PHAGES – VIRAL PARASITES OF BACTERIA

Phages are viruses that infect and replicate solely inside their specific host bacteria. Most phages isolated for biocontrol purposes belong to the *Caudovirales* order. All members of *Caudovirales* have a genome with double-stranded (ds) DNA that is packaged into an icosahedral capsid, and a tail structure, which binds to the bacterial host cell (ICTV, 2011). Within *Caudovirales*, phages belonging to three main families are most frequently used for biocontrol: *Siphoviridae*, which have long noncontractile tails; *Myoviridae*, which have long contractile tails; and *Podoviridae*, which have short noncontractile tails. Tailed phages also include jumbo phages, which have genomes larger than 200 kb. These phages have many more genes compared to smaller phages, as they have larger genomes due to larger capsids. This also enables them to be less dependent on the replication mechanisms of their hosts (Yuan & Gao, 2017).

Infections by *Caudovirales* can be virulent (lytic phages), where the viral DNA enters a host bacterial cell and replicates using its machinery to produce progeny virions, which are released when they lyse the host cell. Infections can also be temperate (lysogenic phages), where viral DNA integrates into the host genome to produce a prophage (Clokie et al., 2011). Prophages can be dormant and replicate along with their host cells or switch to the virulent cycle, leading to death of their host. Lysogenic phages also often encode several auxiliary genes that can provide benefits for the host bacteria (metabolism, virulence, degradation of toxic compounds) and positive selection for prophage-carriage (Fortier & Sekulovic, 2013). A third type of infection is called chronic infection, where lysogenic phages maintain ongoing infection (superinfective state), releasing virions from the host cell without lysing and killing it (Mai-Prochnow et al., 2015). Chronic infections are mainly caused by filamentous phages, which have a single-stranded (ss) DNA genome and are found in the *Inoviridae* family (ICTV, 2011). Filamentous phages are capable of reducing or increasing bacterial virulence in several plant-pathogenic bacteria, which could make their use as biocontrol agents challenging (Ahmad et al., 2014a, 2017; Yamada et al., 2007). As a result, most research thus far has focused on developing and testing lytic phages for plant pathogen control.

3 | PHAGES FOR TREATMENT OF BACTERIAL TREE DISEASES

Trees suffer a range of microbial diseases caused by fungal, oomycete, viral, and bacterial pathogens (Rabiey et al., 2019). Here we specifically focus on various bacterial diseases of trees in relation to existing phage biocontrol literature. As several diseases are localized to the vascular system of the trunk and limbs, phages are particularly attractive targets for systemic delivery through injection or sprays, potentially ensuring efficient dispersal throughout the tree vascular system. Several phages have been isolated and explored specifically in the biocontrol of bacterial diseases of trees, which are listed in Table 1.

3.1 | *Pseudomonas syringae* pathovars

Pseudomonas syringae is a species complex of flagellated, rod-shaped, gram-negative bacteria with over 60 plant-specific infecting pathogenic varieties (pathovars; pvs) (Xin et al., 2018). *P. syringae* pathovars can infect a range of various trees and shrubs (Scheck et al., 1997), including pv. *aesculi* (horse chestnut bleeding canker and leaf spot), pv. *actinidiae* (bacterial canker of kiwifruit, also reported on paper mulberry), pv. *actinidifoliorum* (leaf spots in kiwifruit), pv. *garcae* (halo blight of coffee), pv. *tabaci* (leaf spot of coffee), pv. *eriobotryae* (loquat canker), pv. *morsprunorum* (one of the causes of canker of stone fruit), pv. *mori* (bacterial blight of white mulberry), and pv. *persicae* (bacterial decline of stone fruit) (Alippi et al., 2013; Cunty et al., 2015; Green et al., 2009; Krawczyk & Łochyńska, 2020; Li et al., 2020a; Rodrigues et al., 2017; Scortichini et al., 2012; Vanneste et al., 2013; Young, 2014). The pv. *syringae* is linked to a broader host range and causes some of the most economically damaging tree diseases, including bacterial canker of stone fruits (the genus *Prunus*), pear (*Pyrus*), mango (*Mangifera*), lilac (*Syringa*), and citrus (*Citrus*) blast (Gutiérrez-Barranquero et al., 2019; Mirik et al., 2005; Scheck et al., 1997; Spotts & Cervantes, 1995). Diseases caused by *P. syringae* pathogens can be devastating to national economies and cause pandemics globally. For example, *P. syringae* pv. *actinidiae* (Psa) is a notorious pathogen that causes bacterial canker of the woody vine kiwifruit; its symptoms include dieback, cankers, leaf spots, and gum exudation. Psa was first isolated in Japan in 1984 (Takikawa et al., 1989). It began to emerge as an aggressive pathogen in Italy in 2008, and in New Zealand and Chile in 2010, and has since been reported in other countries including Portugal, Spain, France, and South Korea (Scortichini et al., 2012). The outbreak in New Zealand alone caused financial losses of up to NZ$930 million between 2010 and 2014 (Vanneste, 2017), illustrating the huge problems it poses for the economy. There are four characterized populations of Psa – the first identified population from Japan; a South Korean population; the pandemic-causing population found in Europe, New Zealand, and South America; and a small population found only in New Zealand and Australia (McCann et al., 2017). It has historically been treated with bactericidal sprays, many of which contain copper and streptomycin. However, bacterial resistance has begun to emerge and spread through pathogen populations via integrative and conjugative mobile genetic elements (Colombi et al., 2017). Although the gold kiwifruit variety that has been developed is resistant to the strains of Psa, it is not yet clear how robust the resistance will be in the long term. Thus, development of phage treatments against *P. syringae* pathovars warrants more study and could have significant potential to control the pathogen more sustainably and to tackle devastating economic losses.

Frampton et al. (2014) isolated 24 phages from New Zealand orchards. All phages were found to be lytic, stable under storage conditions, and a subset of them were capable of also lysing *Pseudomonas viridiflava*, the causative agent of kiwifruit tree blight. Importantly, the isolated Psa strains from this study were found to be highly related to those found in Europe, suggesting that phage treatments

TABLE 1 Recent development in using phages as biological control of bacterial tree diseases

Pathogen	Host and disease	Current control method	Other biological control methods	Bacteriophages with biocontrol potential	Reference
Agrobacterium tumefaciens	Crown gall disease of dicotyledous plants	Hygiene practices and use of the antagonistic *Agrobacterium radiobacter* (*Rhizobium rhizogenes*) K84 and K1026 bacterial strains		Atu_ph02, Atu_ph03	Attai et al. (2017)
				Jumbo phage Atu_ph07	Attai et al. (2018)
Erwinia amylovora	Fire blight of Rosaceae, especially apple and pear	Copper spraying and antibiotics	Antagonistic bacteria such as *Pantoea agglomerans* (*Erwinia herbicola*) strain Eh1087	PEa phages, particularly PEaI(H)	Ritchie (1979)
				fEa116C	Schnabel and Jones (2001)
				L1, M7, S6, Y2	Born et al. (2011)
				ΦEa1337-26, ΦEa2345-6	Boulé et al. (2011)
				φEa104, φEa116	Müller et al. (2011)
				phiEaH2, phiEaH2	Dömötör et al. (2012), Meczker et al. (2013)
				phiEa2809	Lagonenko et al. (2015)
				phiEaP-8	Park et al. (2018)
				ΦEaH2A, ΦEaH5K, ΦEaH7B	Schwarczinger et al. (2017)
				ΦEa46-1-A1, ΦEa21-4	Parcey et al. (2020)
Pseudomonas syringae pvs *syringae* and *morsprunorum*	Bacterial canker of *Prunus* species	Copper-based pesticides	Garlic (*Allium sativum*) extract and the antagonistic bacterium *Bacillus* sp., *Moringa oleifera* and *Azadirachta indica* plant extracts	MR phages, particularly MR16	Rabiey et al. (2020)
				φ6	Pinheiro et al. (2019, 2020)
Psuedomonas syringae pv. *actinidiae*	Bacterial canker of *Actinidia* spp.	Removal of infected material and spraying with copper-based pesticides and streptomycin	Endophytic *Pseudomonas* sp. from mānuka (*Leptospermum scoparium*)	φPsa17	Frampton et al. (2015)
				φPSA2	Di Lallo et al. (2014)
				PPPL-1	Park et al. (2018)
				Jumbo phage Psa	Wojtus et al. (2019)
				φXWY0013, φXWY0014, φXWY0026	Yin et al. (2019)
				PN05, PN09	Ni et al. (2020)
Pseudomonas syringae pv. *aesculi*	Bleeding canker of *Aesculus hippocastanum* and leaf spot of *A. indica*	Removal of infected material; no chemical controls available currently in the UK		2S, RC5CS	James et al. (2020)
Xanthomonas arboricola pv. *juglandis*	Walnut blight	Copper spraying	Various antagonistic bacterial strains and prohexadione-Ca	P1–P26	Romero-Suarez et al. (2012)
				Xaj2, Xaj24	Dömötör et al. (2016)
				*f*20-Xaj, *f*29-Xaj, *f*30-Xaj	Retamales et al. (2016)

(Continues)

TABLE 1 (Continued)

Pathogen	Host and disease	Current control method	Other biological control methods	Bacteriophages with biocontrol potential	Reference
Xanthomonas arboricola pv. *pruni*	Bacterial spot of stone fruit	Copper spraying, antibiotics, and pruning	An antibiotic metabolite of a *Pseudomonas aeruginosa*, nonpathogenic *Xanthomonas campestris* strains	F8	Saccardi et al. (1993)
Xanthomonas axonopodis pv. *citri*	Asiatic citrus canker	Copper spraying, antibiotics, and insecticide to control the spread of its vector, the Asian leafminer	Native antagonistic *Bacillus* sp.	Filamentous phage XacF1 Cp1, Cp2 Jumbo phage XacN1	Ahmad et al. (2014a) Ahern et al. (2014) Yoshikawa et al. (2018)
Xylella fastidiosa	Pierce's disease of grape, phony peach disease, citrus variegated chlorosis, almond leaf scorch, coffee leaf scorch, olive quick decline syndrome	Copper spraying, zinc, antibiotics, antagonistic bacteria, neonicotinoids to prevent spread of insect vectors (only in USA) and extensive pruning	Use of *Zelus renardii* (leaf hopper assassin bug); exploration of bacterial strains, a microbial metabolite, plant extracts and an entomopathogenic fungus by the BIOVEXO project (https://bbi-europe.eu/projects/pdf/BIOVEXO)	Sano, Salvo, Prado, Paz	Ahern et al. (2014), Das et al. (2015)
Ralstonia solanacearum, *R. pseudosolanacearum*, and *R. syzygii*	Bacterial wilt of *Eucalyptus*, potato, tomato, aubergine, mulberry, tobacco, and banana	Chemical control	*Bacillus thuringiensis*, *Bacillus cereus* and *Pseudomonas* spp. rhizobacteria, *Bacillus amyloliquefaciens*	NJ-P3, NB-P21, NC-P34, NN-P42 Lysogenic ϕRSM phages M5, M8	Wang et al. (2019) Yamada (2013) Ramírez et al. (2020)

developed in either Europe or New Zealand could be applied in both locations. Another study by Frampton et al. (2015) further explored the genome of one of the isolated phages, φPsa17, capable of infecting a range of Psa strains including Japanese and South Korean isolates. Phage φPsa17 was identified as a T7-like virus, confirming its lytic status and feasibility as a biocontrol agent. Phages with lytic activity against Psa populations from South Asia have also been isolated, including environmentally stable phage PPPL-1, isolated in South Korea, and φXWY0013, φXWY0014, and φXWY0026 phages isolated in Shanghai (Park et al., 2018; Yin et al., 2019). This indicates phages can be isolated for each geographically distinct Psa population, which can be an important consideration if national regulatory rules prevent the use of non-native biocontrol phages.

Psa phages have also seen success when used in integrated pest management (IPM). Ni et al. (2020) used phages PN05 and PN09, either in a cocktail or individually, along with the antibacterial essential oil carvacrol. The combination of both phages and carvacrol produced the greatest inhibitory effect of Psa, resulting in complete inhibition after eight hours of application, although regrowth was observed at low carvacrol concentrations. The combination was also successful at clearing and preventing biofilm formation, suggesting the use of Psa phages in conjunction with other naturally occurring bacterial inhibitors could be an effective control method for established infections.

Several *P. syringae* pathovars including pvs *morsprunorum*, *persicae*, and *syringae* (Pss) cause bacterial canker of fruit trees. Species in the genus *Prunus* are particularly susceptible, including cherry, peach, plum, and apricot, and bacterial disease can cause significant economic losses. In the United Kingdom, bacterial canker of cherry is estimated to cause up to 75% loss of trees per year (Spotts et al., 2010), and there are few suitable treatments due to lack of chemical bactericides or resistant tree cultivars. While copper compounds have been used previously, they are often ineffective against internal bacterial populations and can be phytotoxic to certain tree species (Kennelly et al., 2007). Despite the lack of efficient pathogen control, only two studies have focused on phage treatments against Pss in the past 20 years, after an initial period of phage research (Crosse & Garrett, 1966; Pinheiro et al., 2019; Rabiey et al., 2020). The well-researched phage φ6, which is known to lyse *P. syringae* pv. *phaseolicola*, was tested as a potential biocontrol against Pss (Pinheiro et al., 2019). As well as lysing many Pss strains, φ6 could also lyse two Psa strains (Pinheiro et al., 2020). This finding suggests that this phage could potentially be used to control multiple *P. syringae* diseases due to broader host range. Another study by Rabiey et al. (2020) isolated several novel phages against Pss and *P. syringae* pv. *morsprunorum* (Psm) pathovars from cherry orchards in England. They carried out a detailed analysis on 13 out of the 70 isolated phages, which were found to be potentially suitable for biocontrol due to their stability in storage and lytic life cycle. Phages showed varying degrees of efficacy in reducing bacterial population densities immediately or several weeks after application. Overall, phage cocktails combining either all or some of the phages caused the highest reduction of Pss and Psm abundances on infected cherry twigs. Interestingly, phage

MR16 was most successful at reducing Pss abundances close to zero on cherry leaves after 5 weeks of treatment. While further field trials are necessary, phages tested in both studies show promise in treating bacterial canker of fruit trees. In the future, more detailed analysis of phage host ranges should be performed to test if they could be used against other Pss strains of different crops.

Finally, a study has been performed on phages infecting *P. syringae* pv. *aesculi* (Pae), which causes bleeding canker of European horse chestnut (*Aesculus hippocastanum*). First isolated in India on Indian horse chestnut (*A. indica*) as a leaf spot pathogen, Pae was identified in Belgium, the Netherlands, and the United Kingdom in 2002. It appears to be spreading easily as its range has rapidly extended both west into Ireland and east into central Europe (Green et al., 2013; Pirc et al., 2018). It is now believed to be endemic in Britain, with 70% of trees analysed in a 2007 survey showing symptoms (Forestry Commission, 2008). No treatment exists to tackle Pae infections other than the removal of infected material. However, as the disease progression can be slow, some trees can recover or become resistant (Forest Research, 2020). Some success in treating Pae with phages was observed by James et al. (2020). Twenty-two phages were isolated from both healthy and infected trees in the south of England; all were capable of infecting five Pae strains, and none were able to lyse plant-beneficial bacteria. Four phages were further characterized, and while all could inhibit Pae growth, bacterial resistance and regrowth occurred after an average of 16 h, indicative of rapid evolution of phage resistance. More research is thus required to test if phages can control Pae in the long-term, or if this is limited by potential phage resistance evolution.

3.2 | *Erwinia amylovora*

Erwinia amylovora is a species of rod-shaped, gram-negative bacteria. It causes fire blight on apple, pear, and various other Rosaceae species. The disease affects the fruits, blossoms, shoots, and branches of an infected tree. Advanced infection leads to necrosis, turning infected areas black and scorched, hence the name fire blight. *E. amylovora* is believed to have evolved in the wild in North America and was first identified in New York in the 1780s (Denning, 1794). It then spread to Europe, Egypt, central Asia, and Oceania over the 20th century by import of infected plant material. By 1998, all EU countries except Portugal had observed fire blight infections (CABI, 2019). If an orchard becomes infected with the disease, an entire year's harvest can be lost. For example, an outbreak in Connecticut, USA, in 2000 led to the loss of $42 million and the removal of 350–450,000 apple trees (Douglas, 2020). Current treatments include copper compounds and streptomycin, although more environmentally friendly methods, such as breeding plant resistance, SAR inducers, antagonistic bacteria, and recently phages, have been developed (Norelli et al., 2003).

Of all the bacterial tree diseases, phage treatments against fire blight are the most extensively studied (Table 1) (Roach, 2011; Roach et al., 2013). From the seven commercially available agricultural phage treatments, two are treatments developed against fire blight: AgriPhage-Fire Blight in the United States and Erwiphage in Hungary. AgriPhage-Fire Blight is promoted for use on organic orchards of the United States and is said to lyse *E. amylovora* within 30 minutes of infection. However, it cannot be applied at the same time as copper or iron pesticides, and must be sprayed every 2 weeks (Certis, 2019). Erwiphage and its successor Erwiphage PLUS are composed of two parts: the phages and a UV-protective solution. They must be applied after sunset and like AgriPhage, cannot be applied in combination with copper compounds (Erwiphage, 2020). Erwiphage contains two siphoviruses, PhiEaH2 and PhiEaH1, that were isolated from soil in Hungary, and subsequent genomic analysis has shown that they are not highly related to one another (Dömötör et al., 2012; Meczker et al., 2013). However, the specific phages in AgriPhage-Fire Blight have not been disclosed, preventing genomic comparisons with Erwiphage.

The extensive research of *E. amylovora* and its phages allows informed decision making when choosing novel phages for further research as biocontrol. *E. amylovora* produces an acidic capsular exopolysaccharide (EPS) named amylovoran that provides protection against plant defence mechanisms and enables nutrient uptake (Nimtz et al., 1996). Some phages, especially podoviruses, enter *E. amylovora* by degrading amylovoran with an EPS depolymerase, and hence their infectivity depends on the presence of amylovoran (Kim & Geider, 2000; Müller et al., 2011; Roach et al., 2013). The efficacy of *Erwinia* phages can thus depend on the amount and type of EPS produced by its host. Roach et al. (2013) determined that *Myoviridae* phages preferred hosts producing low EPS, whereas *Podoviridae* phages preferred hosts producing high EPS. *Myoviridae* phages also preferred hosts that produced levan, another EPS produced by *E. amylovora*, which facilitates sucrose metabolism and acts as a potential virulence factor (Bogs & Geider, 2000; Gross et al., 1992).

Furthermore, several *E. amylovora* phages have been identified that are able to lyse other closely related bacterial species. Some phages are able to lyse *Erwinia pyrofoliae*, a pathogen of Asian pear trees (*Pyrus pyrifolia*), which causes blackshoot blight (Lehman et al., 2009; Park et al., 2018). Phage treatments involving such broad host range phages would be beneficial as they have a potential to reduce two different diseases. However, broad host specificity could also lead to complications if phages can infect beneficial, nontarget bacteria. For example, many *Erwinia* phages are able to lyse strains of *Pantoea agglomerans* (Born et al., 2017; Lehman et al., 2009), which can be commensal or even beneficial, such as the plant growth-promoting strain P5 (Shariati et al., 2017). As some *P. agglomerans* strains can be used to treat fire blight due to their antagonism towards *E. amylovora* (Nagy et al., 2012; Stockwell et al., 2010), care must be taken to use phages whose host range does not include beneficial bacteria.

3.3 | *Xanthomonas* spp.

Xanthomonas is a genus composed of rod-shaped, gram-negative bacteria that produce xanthomonadin, a yellow pigment that aids

epiphytic survival in high light intensity (Poplawsky et al., 2000). Over 35 species are found in this genus, capable of infecting more than 400 plant hosts (Timilsina et al., 2020). Several species in this genus are causative agents of tree diseases, including *X. arboricola* pvs *juglandis* (walnut blight), *corylina* (hazelnut blight), *populi* (bark necrosis of poplar), and *pruni* (bacterial spot of stone fruit and almond); and *X. axonopodis* pvs *citri* (Asiatic citrus canker) and *eucalyptorum* (leaf blight of eucalypt) (Ferraz et al., 2018; Fischer-Le Saux et al., 2015; Garita-Cambronero et al., 2018). Of these pathogens, only three—*X. arboricola* pvs *juglandis* and *pruni* and *X. axonopodis* pv. *citri*—have been subjected to phage research.

X. arboricola pv. *juglandis* (Xaj), the causative agent of bacterial walnut blight, is present on every continent (Giovanardi et al., 2015). It can infect catkins, flowers, leaves, fruits, and shoots, and fruit infection is the most economically devastating as it can lead to low fruit quality (Pereira et al., 2015). Its spread is exacerbated by increased rainfall, particularly in spring, when rain occurs soon after bud emergence (Lindow et al., 2014). Copper compounds have been primarily used for disease treatment, but there is a decrease in efficacy due to the emergence of copper-resistant strains in many walnut-producing regions (Higuera et al., 2015; Ninot et al., 2002; Pereira et al., 2015). Thus, various phages have been isolated to explore potential for new treatments. McNeil et al. (2001) isolated Xaj phages in New Zealand and observed that the phages were capable of surviving in short-term storage at 4°C but had low efficacy in lysing more than one Xaj strain. Three more studies have since been performed that isolated and characterized various phages from Chile, Hungary, and New Zealand (Dömötör et al., 2016; Romero-Suarez et al., 2012). Romero-Suarez et al. (2012) identified phages capable of lysing multiple strains of Xaj but were unable to persist in long-term storage. Dömötör et al. (2016) isolated phages Xaj2 and Xaj24, which could lyse a range of Xaj strains and had a high lytic ability. However, none of the Xaj phage studies have been tested in field trials. This is especially important as Xaj populations are highly affected by rainfall, which could also impact phage biocontrol efficacy.

A novel patented biopesticide containing a mixture of phages targeting Xaj was produced by the University of Chile. Tobacco leaves inoculated with the phage mixture showed either no or very few disease symptoms. In a trial using walnut branches, the necrosis damage of the untreated group was 75%–100%, compared to the phage-treated group whose necrosis damage was much lower, in the range of 0%–25%. Phages were stable under different temperatures and storage conditions, and formulations were added to the phage mixture to protect them from UV and solar radiation. The patent information is available online (Ormazabal & Guajardo, 2017), but it is not clear if these phages have yet been commercialized.

X. arboricola pv. *pruni* (Xap) is another pathovar that has been subject to phage research. Xap causes leaf spot and cankers on species in the *Prunus* genus, and infected fruits can have spots, necrotic tissue, or drop early (Garita-Cambronero et al., 2018). The disease is found on every continent, though the degree of severity varies (Garita-Cambronero et al., 2018). Leaf and fruit spot in peach trees in northern Italy was a particular problem during the 1990s. Zaccardelli et al. (1992) collected several phages from this region that were able to lyse several Xap strains, including strains that infect plum trees. Phage F8 with a wide host range was further explored as a potential biocontrol agent. Spraying F8 phage onto nectarine fruits before bacterial inoculation prevented disease symptoms in 92.12% of cases. F8 survival was significantly lower in the field trials than in the laboratory, and further reduced by several commercial pesticides. Additional field trials in a peach orchard by Saccardi et al. (1993) showed that weekly application of phage F8 caused a 55% reduction in diseased fruits in an orchard with low disease pressure. However, no further studies or commercialization of F8 phage has been reported since.

X. axonopodis pv. *citri* (Xac) causes Asiatic citrus canker on various citrus plants including orange, lemon, grapefruit, pomelo, and lime (Gottwald, 2000). The disease is particularly prevalent in regions with high rainfall and warm temperatures, with the pathogen causing leaf spotting and lesions, fruit rind blemishes, defoliation, shoot dieback, and fruit drop. It is tackled mostly via removal of infected trees or through the use of resistant varieties, for example, Valencia oranges and mandarins that are grown in areas where the disease is most prevalent. Although the disease most probably originated in South-east Asia, it has now been found in South America and the United States (Gottwald et al., 2002). Several novel Xac phages had been isolated in Japan, and Cp1 and Cp2 were the first identified Xac phages after isolation in 1967. These phages can infect nearly every strain of Xac isolated in Japan and are extensively used for phage typing of Xac strains (Ahmad et al., 2014b). The filamentous phage XacF1 has also been studied due to a broad host range (Ahmad et al., 2014a). Due to its chronic infection cycle, it did not lyse but it significantly reduced the growth rate, xanthan production, swimming, and swarming motility of infected Xac cells. It also significantly reduced the severity of disease symptoms of infected trees compared to the non-XacF1 control. Recently, the jumbo phage XacN1 was isolated; it has a large genome of almost 385 kb and a wide host range against Xac strains, suggesting its potential use for phage biocontrol against citrus canker (Yoshikawa et al., 2018).

3.4 | *Agrobacterium tumefaciens*

A. tumefaciens is a rod-shaped, gram-negative bacterium that causes crown gall disease in dicotyledonous plants. Incorporation of a small segment of *A. tumefaciens* DNA, named T-DNA, into the genome of the host plant results in the formation of tumours named galls on the roots and stem base. The disease is most damaging to tree nurseries, as young trees are more susceptible to infection and those infected are usually unsellable. Trees and shrubs are particularly susceptible to infection, including stone and pome fruit trees, willow trees, grape vines, and apple trees (Kado, 2002). Control of crown gall has included specific hygiene practices and use of the antagonistic *Agrobacterium radiobacter* K84 strain and its engineered strain K1026 (Penyalver et al., 2000). *A. radiobacter* K84 produces

an antiagrobacterial compound called agrocin 84. Several strains of *A. tumefaciens* are unaffected by, or can become resistant to, agrocin 84, and therefore there is a need for novel biocontrol against crown gall disease (López et al., 1989; Süle & Kado, 1980).

A. tumefaciens phage research began in the 1960s, though many of the isolated phages were found to be lysogenic (De Ley et al., 1972; Expert & Tourneur, 1982; Korant & Pootjes, 1970). One of the first highly virulent phages isolated was PB21 in 1967 (Stonier et al., 1967). Though capable of producing large clear plaques and inhibiting tumour initiation, it was unable to reduce *A. tumefaciens* infection inside galls (Stonier et al., 1967). Isolation of *A. tumefaciens* phages for biocontrol has only recently restarted. Several phages have had their genomes characterized, such as the unique flagellatropic phage 7-7-1, T4-like Atu_ph04, and the lysogenic T7-like Atu_ph08, and the myophage Milano, enabling further knowledge of *A. tumefaciens* phages and comparisons with other phages (Attai & Brown, 2019; Kropinski et al., 2012; Nittolo et al., 2019). Phages Atu_ph02 and Atu_ph03 caused significant reduction of tumour formation on potato discs compared to the non-phage-treated control, suggesting their potential use in phage cocktails (Attai et al., 2017). The isolation of jumbo myophage Atu_ph07 has also shown promise in biocontrol (Attai et al., 2018); characterization of its genome showed it is probably wholly lytic, and it was capable of lysing a wide host range of *A. tumefaciens* strains without lysing other related species. Recent studies also suggest that these phages could encode potentially powerful antimicrobials. For example, expression of Atu_ph02 and Atu_ph03 phage-encoded peptidoglycan hydrolase (PPH) within bacterial cells has been shown to lead to rapid cell lysis, cell elongation, and branching (Attai et al., 2017).

3.5 | *Xylella fastidiosa*

Xylella fastidiosa is a xylem-limited, gram-negative bacterium with various subspecies such as *multiplex*, *fastidiosa*, and *pauca*. It causes a vast number of diseases in various hosts. Pierce's disease of grape is one such disease, leading to leaf scorch and drop, fruit wilt, and reduced growth of vines (Jackson, 2000). Another is olive quick decline syndrome, which produces similar symptoms resulting in dieback and tree death (Fahrenkamp-Uppenbrink, 2016). It has been estimated that in Italy alone, *X. fastidiosa* has caused €1.5–5.2 billion of losses for olive production (Schneider et al., 2020). Other important diseases caused by *X. fastidiosa* include phony peach disease; leaf scorch of oak, elm, plane, and mulberry trees; plum leaf scald; leaf scorch of coffee; and citrus variegated chlorosis. The bacterium favours warm, arid climates and was prevalent mainly in the Americas until 2013, when it arrived in mainland Europe via Apulia, Italy. There have since been outbreaks across France, Portugal, and Spain (European Commission, 2020). The disease is generally spread via movement of infected plant material, and insect vectors that feed on xylem-sap, such as the meadow spittlebug *Philaenus spumarius* in Europe (Cornara et al., 2019).

There are currently no treatments to eradicate *X. fastidiosa*, and the main management practice is to prevent further spread. This involves copper or zinc spraying, use of antibiotics, removal of infected trees and nearby susceptible hosts, and suitable crop management (EFSA Panel on Plant Health, 2016). Phage research has shown some success in the treatment of *X. fastidiosa*. Ahern et al. (2014) isolated four virulent phages named Sano, Salvo, Prado, and Paz in Texas, USA, and used them as a cocktail to treat infected grapevines 3 weeks postinfection. No further symptoms developed after a week from phage application (Das et al., 2015), and phages were found distributed throughout the grapevine phyllosphere with no observation of phage-resistant isolates in planta. Another study explored the ability of a *X. fastidiosa* insect vector, the glassy-winged sharpshooter (*Homalodisca vitripennis*), to spread the phage Paz (Bhowmick et al., 2016). The phage was applied to stems on which the insects fed on. While the insect could ingest phages in low amounts, they were unable to transfer phages between plants. More research on these phages, their viability during storage, and methods of application, is thus needed for the development of viable biocontrol method for this devastating pathogen.

3.6 | *Ralstonia solanacearum* species complex

R. solanacearum is a gram-negative, rod-shaped bacterium with a single flagellum, and predominantly found in soil (Peeters et al., 2013). It is considered as a species complex, containing a vast number of subgroups that are widespread across tropical, subtropical, and temperate zones. It is currently divided into three species: *R. solanacearum*, *R. pseudosolanacearum*, and *R. syzygii* (Safni et al., 2014). Wide geographic distribution reflects its wide host range, which includes key solanaceous crops like potato, tomato, and aubergine (eggplant), as well as tobacco. *R. solanacearum* is also capable of infecting many key tree species in South America, Asia, and Africa. Bacterial wilt of mulberry has been reported as one of the most destructive bacterial diseases (Ji et al., 2008). *R. solanacearum* and *R. pseudosolanacearum* cause bacterial wilt of *Eucalyptus* trees in the Americas, and Asia and Africa, respectively (Carstensen et al., 2016; Coutinho & Wingfield, 2017). Infection of trees aged 2–4 years is most common, and results in tissue reddening and wilting, and dropping of leaves. Stress is a common exacerbator of disease symptoms, especially if trees have been poorly planted and have root knotting or other injuries (Alfenas et al., 2006; Coutinho & Wingfield, 2017). The disease can cause up to approximately 80% growth reductions, leading to reduced pulp production and considerable economic losses (Ferreira et al., 2017). It has recently been reported that *R. solanacearum* causes bacterial wilt in ironwood trees in Guam (Ayin et al., 2019). The third species, *R. syzygii*, is mostly found in South-east Asia. It has three subspecies: *syzygii*, which causes Sumatra disease of *Syzygium* (cloves); *indonesiensis*, which infects multiple *Syzygium* species, as well as potato, tomato, and pepper; and *celenesensis*, which causes

blood disease of banana (*Musa* spp.) (Safni et al., 2018). Sumatra disease of clove is highly destructive in Indonesia and can infect trees over 10 years old causing leaf discolouration and drop, branch dieback, and secretion of bacterial exudate (Bennett et al., 1985). Blood disease by *R. syzygii* subsp. *celebesensis* is observed as reddish discolouration of infected *Musa* plants and secretion of red-brown bacterial exudate when cut. *R. syzgii* subspp. *celebesensis* and *syzgii* can spread via insect vectors, while significant spread is also caused by contaminated farm equipment, water, or soil. Less is known of spread of the subsp. *indonesiensis*. In Latin America, the Caribbean, and the Philippines, bananas are affected by *R. solanacearum* phylotype II race 2 strains, which cause moko disease (Pardo et al., 2019). It is similar to blood disease of banana found in Indonesia, and causes bacterial wilt, bacterial exudation from vascular tissues, and fruit rot. Certain strains can also cause exudation from the male inflorescence, allowing the disease to then be spread by insect vectors (Fegan & Prior, 2006). The bird of paradise plant (*Strelitzia alba*) has also been found to be host for the *Ralstonia* species *R. pickettii* (Polizzi et al., 2008). The infected trees had brown/black leaf stripes and necrotic lesions, and overall, 10%–15% of the tree population were infected in glasshouses in Sicily.

Infection of trees by *R. solanacearum* can be difficult to control as it can exist in the soil without the need for a host and occasionally spread by numerous insect vectors (Coutinho & Wingfield, 2017). Most in planta phage biocontrol studies of *Ralstonia* have involved non-tree crops such as tomato, potato, and tobacco (Álvarez & Biosca, 2017). For example, Wang et al. (2019) found that laboratory experiments predicted phage infectivity in greenhouse and field experiments leading to up to 80% reduction in bacterial wilt disease incidence in tomato. In addition to lytic phages, several lysogenic phages have been identified, including filamentous phages that have been shown to cause considerable changes in *Ralstonia* virulence and protection from infection (Yamada, 2013). Promisingly, a recent study found that phages isolated in Colombia were able to control moko disease on banana (Ramírez et al., 2020). A cocktail of two phages, M5 and M8, reduced the growth of *R. solanacearum* strain UA1591 to undetectable levels in laboratory tests. Moreover, no wilting symptoms were observed in young, infected banana plants treated with the cocktail, while no-phage control plants and those infected with only one phage died within 30 days. While phage biocontrol of *Ralstonia* has been less studied with trees, some success has been achieved using *Bacillus thuringiensis*, *Bacillus cereus*, and *Pseudomonas* spp. rhizobacteria in suppression of eucalyptus Ralstonia wilt (Ran et al., 2005; Santiago et al., 2015). The application of endophytic *Bacillus subtilis* Lu144 effectively reduced bacterial wilt disease incidence in mulberry (Ji et al., 2008). Previous work has also shown that phage efficiency can be increased in combination with beneficial *Bacillus amyloliquefaciens*, leading to better control of *R. solanacearum* in tomato (Wang et al., 2017). While phage biocontrol shows promise in controlling *Ralstonia*, more work is needed focusing on tree-specific *R. solanacearum* and *R. syzygii* species.

4 | CHALLENGES OF PHAGE BIOCONTROL IN TREE DISEASE MANAGEMENT

Development of efficient and functionally robust phage biocontrol against plant-pathogenic bacteria pose several challenges (Buttimer et al., 2017). These range from technical challenges associated with product storage and application methods in field conditions to a lack of understanding of the underlying ecology and evolution of phage–pathogen interactions in complex plant-associated microbiomes. Due to long generation times and often seasonal harvesting, tree-pathogenic bacteria create unique problems for disease management that need to be considered when developing phage biocontrol applications.

4.1 | Isolation of safe, efficient, and pathogen-specific phages

Phages can be easily isolated against tree-pathogenic bacteria from various environmental sources, and several studies have characterized the specificity of phages across different pathogen isolates or pathovars (Balogh et al., 2010). At the same time, it is equally important to test that employed phages do not target beneficial bacteria present in the complex plant-associated microbiomes. Such tests have been conducted previously with *R. solanacearum* to show that pathogen-specific phages were unable to infect 400 culturable tomato rhizosphere bacterial isolates (Wang et al., 2019) or 13 other phytopathogenic bacteria or 46 unidentified environmental bacteria (Álvarez et al., 2019). Similarly, AgriPhage-Fire Blight commercial phage product has been reported to be highly specific in killing only *E. amylovora* on apples and pears, with no claimed effect on surrounding microbial communities (Certis, 2019). However, this is not always the case, as has been reported with *Erwinia* phages, which have a relatively broad host range, being capable of infecting commensal bacteria (Lehman et al., 2009). In the case of trees, the composition and diversity of the associated microbiome is likely to change over the years and seasons (Koskella & Taylor, 2018). If phages would be applied only during certain periods of the tree life cycle, such as prior and during the harvest of fruits, phage safety testing could also focus on the associated plant microbiomes present during these periods. However, as year to year variation is likely, phage effects on a wider collection of plant-associated bacterial strains might be needed. High host specificity could also be a problem, especially if the targeted pathogens show high intraspecies diversity or local adaptation. In an ideal case, phages specific to several genotypes of pathogenic bacterial species should be used as they should show broader killing activity across different locations. However, it would be important to understand how multiple phages can interact with target bacteria to determine whether there might be any detrimental effects of phage infection (e.g., competition for

attachment, infection, or replication; Brockhurst et al., 2017) that reduces the efficacy of biocontrol.

4.2 | Phage survival during storage and field application

Phage survival during storage is another important factor that could limit their use and should be extensively tested upon isolation of suitable phages (Golec et al., 2011; González-Menéndez et al., 2018; Łobocka et al., 2017). Rabiey et al. (2020) found that −20°C was the best temperature for long-term storage of phages isolated against Pss and Psm, with up to 10-fold higher survival and viability attained with higher starting phage titres after 6 months of storage. Phage survival during their storage is important as it has been seen that it can significantly affect Pss pathogen control with phage φ6 (Pinheiro et al., 2019). Phages can also be highly sensitive to changing environmental conditions and their lytic activity can be reduced or abolished by high temperatures, ultraviolet (UV) radiation (Carstens et al., 2019; Pinheiro et al., 2019), lack of moisture, extreme pH, aeration, salinity, and other stressful environmental conditions (Álvarez et al., 2019). The viability of phage φ6 against Pss was decreased by exposure to UV-B radiation, exposure to solar radiation, and high temperatures (Erwiphage, 2020; Pinheiro et al., 2019; Samuni et al., 1984). Though these factors alone do not make phages unsuitable for biocontrol, they could considerably limit their application and use by reducing phage densities in the field. The commercially available Erwiphage PLUS phage treatment developed against *E. amylovora* is composed of a UV-protective solution that protects phages against UV radiation (Erwiphage, 2020). Also for this reason, Erwiphage PLUS must be applied after sunset (Erwiphage, 2020). The size of larger trees might also make it difficult to apply phages evenly over the foliage, and hence phage application via the tree vascular system might potentially offer a more practical approach.

4.3 | Resistance and phage–bacteria coevolution

While phage resistance has been shown to rapidly rise in laboratory conditions (Clokie et al., 2011), there are fewer studies on phage resistance evolution in natural environments, including plants. Seminal studies have shown that phages and bacteria are locally adapted in natural soils (Vos et al., 2009) and can coevolve following fluctuating selection dynamics in soil microcosms (Gómez & Buckling, 2011). Moreover, phage–bacteria coevolution has been documented in the phyllosphere of horse chestnut trees, where phages were shown to specialize towards bacteria from their sympatric population, that is, the tree they had been isolated from relative to other trees (Koskella et al., 2011). Rapid evolution of phage resistance could reduce the long-term efficiency of phage biocontrol unless phages are able to keep up with their host by coevolving. While few experiments have documented phage resistance evolution in the context of plants (Koskella & Brockhurst, 2014; Wang et al., 2017, 2019), this is relatively understudied in the case of tree-pathogenic bacteria. Phage resistance evolution could also have some unexpected benefits in terms of incurring fitness costs for the evolved pathogen genotype. These costs are often due to the alteration or loss of a phage-binding site (receptors), for example in the bacterial lipopolysaccharide layer, pilus, flagella, or capsid (Labrie et al., 2010). Such changes have been shown to affect pathogen fitness as observed by reduced pathogen growth and virulence in *R. solanacearum* (Wang et al., 2019) and *P. syringae* pv. *tomato* (Meaden et al., 2015). Recently, James et al. (2020) reported that evolved phages were better at controlling the populations of Pae and could thus potentially retain their infectivity over longer periods of time during field applications. Phage ability to coevolve and retain their infectivity would be especially useful in the case of tree-pathogenic bacteria, which have opportunity to persist with their host plants much longer compared to seasonal smaller crop plants. This would be expected to lead to patterns of coevolution not only between but also within individual trees, which should be considered when developing phage biocontrol against tree pathogens.

5 | IMPROVING PHAGE TREATMENTS OF BACTERIAL TREE DISEASES

5.1 | Increasing phage efficiency

Phage efficiency could be limited by narrow host range of a phage, thereby reducing its infectivity on different genotypes or pathovars of one given bacterial pathogen species (Frampton et al., 2015; James et al., 2020). One relatively straightforward way to circumvent this would be to use phages in combination as cocktails. Phage cocktails enable the lysis of a wider range of host bacterial strains by a selection of narrow host range specialist phages. While being more efficient at reducing pathogen densities, application of phage cocktails can also constrain the emergence of phage resistance, increasing the amount of time the treatment is effective (Rabiey et al., 2020; Wang et al., 2019). Benefits of phage combinations have been reported with several plant-pathogenic bacteria. For example, Rombouts et al. (2016) observed varying success when applying a cocktail of six KIL phages to treat leek blight caused by *P. syringae* pv. *porri*. Their results found that the timing of phage application was an important factor for leek blight treatment, and field trials were successful only when phage cocktails were sprayed onto the plants a day after the field was first infected, followed by respraying every 2 weeks. An opposite pattern was found in a study focusing on phage treatment of rice blight caused by *Xanthomonas oryzae* pv. *oryzae*. Here, phage treatment was more effective when sprayed on preinfected plants (83.1% decrease of disease severity) compared to when sprayed on plants postinfection (decrease between 28.9% and 73.9%; Ogunyemi et al., 2018). The success of phage combination treatments will thus depend on when and how they are applied in the field, which should be considered with trees that are likely to experience several phage treatments during their life span. As a result,

phage cocktails might be renewed by adding new phages to retain high efficacy over years.

Phage efficiency could be further increased using experimental evolution or genetic engineering. For example, phages can be experimentally coevolved to become more infective to their host bacteria, which has been shown to lead to increased phage infectivity on the ancestral host genotype (Friman et al., 2016; Rabiey et al., 2020). Similarly, phage host range could be increased by evolving phages in the presence of susceptible and resistant bacterial host genotypes, to allow new rare phage mutants capable of infecting resistant hosts to emerge (Sant et al., 2021). Genetic engineering of phages and their enzymes has also been proposed as a potential way to improve phage infectivity. This can be achieved via various techniques, such as bacteriophage recombineering of electroporated DNA (BRED), homologous recombination, in vivo recombineering using lambda phage, and CRISPR-Cas gene editing (Pires et al., 2016). In addition to infectivity, phages could be engineered to better resist environmental stress, including high temperatures, low and high pH, and UV radiation (Huss & Raman, 2020), which could increase their survival during the application. Phage genes could be even transferred to plants to attain more resistant cultivars. For example, Borejsza-Wysocka et al. (2008) extracted the phage phi-Ea1h *dpo* gene, a depolymerase that degrades the capsule of *E. amylovora*, and inserted it into apple, with the resulting engineered plants showing a clear reduction of disease symptoms when infected with *E. amylovora*, that is, necrotic shoot length reduction from 94% to 48%–51%. Another study used the amylovoran-degrading depolymerase gene *dpoL1-C* from phage L1 to create a recombinant *E. amylovora* phage Y2 (Born et al., 2017) that was more successful at lysing the pathogen than its parental phage. The recombinant phage also retained its parental host range and latency period but suffered a cost in terms of lower burst size. While these results are promising, more research on phage evolution, achieved either experimentally or via engineering, is required with tree-pathogenic bacteria.

5.2 | Improving the success of phage application

Phages can be applied as solutions, sprays, or powder format, which allows addition of protective agents that can prolong phage survival under field conditions (Żaczek et al., 2015). Use of phage formulations with protective compounds can increase phage persistence in the environment, especially in regard to protection from UV radiation. Three protective formulations for *X. campestris* pv. *vesicatoria* phages were explored by Balogh et al. (2003). Formulations of skim milk, flour, and sucrose were shown to significantly prolong phage survival on tomato leaves in both high UV light levels and temperatures in comparison to nonformulated phages (Balogh et al., 2003; Iriarte et al., 2007). However, the application method of phages can significantly affect their biocontrol efficacy (Figure 2). Phages can be introduced onto plants by spraying, drenching of the soil, or by coating the seeds and tubers (Iriarte et al., 2012; Rahimi-Midani & Choi, 2020). In addition to directly inoculating phages in the plant rhizosphere or phyllosphere, phages can be introduced via field irrigation channels. Moreover, phage concentration and application frequency and timing (before or after bacterial infection) can influence phage treatment success in the field (Álvarez et al., 2019; Carstens et al., 2019; Holtappels et al., 2020; Iriarte et al., 2007; Kering et al., 2019; Żaczek-Moczydłowska et al., 2020). In the case of the LIMEstone1 phage product developed against *Dickeya solani* (the causative agent of potato soft rot), phage-sprayed potato seed tubers had a 13% higher yield than the untreated control plants in a field trial (Adriaenssens et al., 2012). However, seed tubers that were immediately planted into the soil after spraying had more infection symptoms, suggesting that phage-treated tubers need time to dry before sowing (Adriaenssens et al., 2012). AgriPhage-Fire Blight product developed against *E. amylovora* is being used as a preventive treatment to protect growing leaf tissue or as a curative treatment when plants are subject to heavy disease pressure (all bloom stages) and when the first disease symptoms are visible (United States Environmental Protection Agency, 2020). Smaller crop plants have also been treated by injecting phages directly into the xylem (Askora et al., 2009; Yamada et al., 2007). Such phage application could also potentially be feasible for trees and help deliver the phage systemically throughout the tree vascular system along with the water flow, though a combined phage injection and physiological analysis with different trees is needed to understand whether phages can be dispersed internally or not. Moreover, it is currently unclear how long inoculated phages are able to persist in association with trees. While environmental sampling suggests that phages can be stably isolated from horse chestnut tree leaves (Koskella et al., 2011), it is not clear if inoculated biocontrol phages can overwinter and survive in the absence of, or at very low, host bacterial densities. Long-term monitoring of biocontrol phage survival is thus required to explicitly study if phages can form a long-term association with treated trees, spanning multiple seasons, or if phages need to be inoculated more regularly to attain a high level of protection.

5.3 | Combating phage resistance evolution

While phage resistance is likely to evolve as a result of strong phage selection, this could be potentially limited by using a combination of multiple phages, which decreases the likelihood of acquiring multiple independent phage resistance mutations (Wright et al., 2018). Moreover, evolving a broad range of resistance to multiple different phages can be relatively more costly, leading to high magnitude fitness trade-offs with growth or other bacterial traits associated with competitive fitness (Wang et al., 2019; Wright et al., 2018). Preadapting phages against pathogens could also not only increase the phage infectivity, but also allow phages to infect most frequently emerging mutants, forcing bacteria to evolve via alternative evolutionary trajectories. Recent findings also suggest that phages could be used to steer pathogens to become less virulent (Gurney et al., 2020). While this can be driven by resistance evolution to lytic phages, it could also result from infections by lysogenic phages that

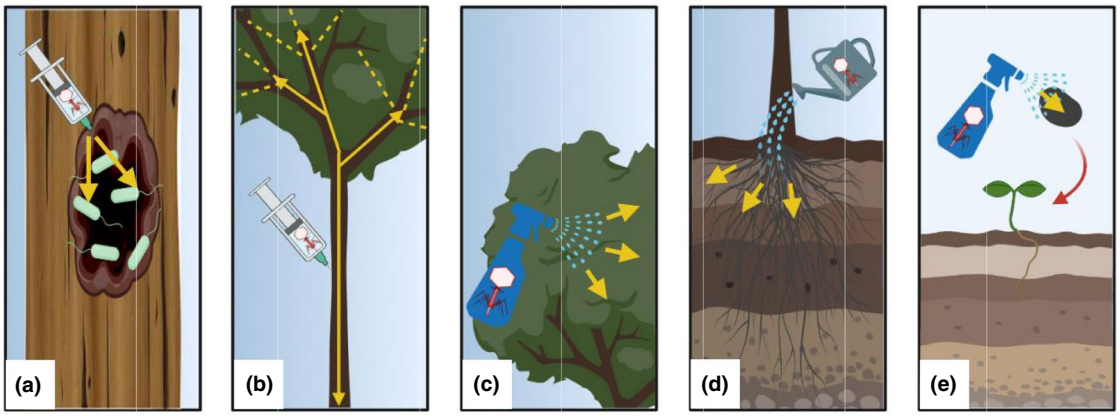

FIGURE 2 Potential application approaches for the use of phage to treat bacterial diseases of trees. (a) Injecting phage into tree bark should only target-specific bacterial host; (b) injecting xylem with phage could lead to more systemic spread of phages throughout the tree; (c,d) spraying leaves and drenching roots could target local bacterial infections in the phyllosphere and rhizosphere; (e) seed coating might help protect seedlings during very early infection. Figure created with BioRender.com [Colour figure can be viewed at wileyonlinelibrary.com]

could rewire bacterial gene regulation when inserting into the bacterial genome. This would make the use of lysogenic phages more attractive in plant pathogen control (Huss & Raman, 2020). Currently, very little is known about the underlying genetic mechanisms of phage resistance with most of the tree-pathogenic bacteria. This is a clear shortcoming, as phage resistance could rise quickly when repeatedly exposed to phage treatments throughout the tree lifetime.

5.4 | Embedding phage biocontrol in integrated pest management

Phage treatments could also be used in conjunction with other biological control agents in an integrated pest management (IPM) approach, which instead of pathogen eradication focuses on minimizing the crop losses in a way that is both economic for the farmer/grower and sustainable for the environment (compulsory in the EU since 2014; ECPA, 2019). One of the first studies on phages in IPM looked at combining *Xanthomonas axonopodis* pv. *vignaeradiatae* phages with streptomycin to treat leaf spot of mungbean (Borah et al., 2000). The combination was successful, lessening seedling infection and increasing seed germination. Importantly, this treatment used a far lower concentration of streptomycin and phage compared to when they were used on their own. In another study, Obradovic et al. (2005) tested various combinations of the commercial *X. campestris* pv. *vesicatoria* AgriPhage product with two plant growth-promoting (PGPR) bacterial strains (*Bacillus pumilus* and *Pseudomonas fluorescens*) and two antagonistic *Pseudomonas* species (*P. syringae* and *P. putida*). While the use of PGPRs and antagonistic bacteria, both alone and in combination with AgriPhage, produced poor results, AgriPhage used in combination with SAR inducers (harpin and acibenzolar-S-methyl) resulted in significant disease reduction. It was suggested that the presence of phages on leaves in combination with acibenzolar-S-methyl reduced populations of a *P. syringae* pv. *tomato* race 3 strain of the pathogen to levels that did not induce a visible hypersensitive response. Moreover, recent findings have demonstrated that using phages in a combination with pathogen-suppressing *B. amyloliquefaciens* bacteria can lead to better protection of tomato due to evolutionary trade-off between being resistant to phage or antimicrobials produced by *B. amyloliquefaciens* (Wang et al., 2017). Together, current evidence suggests that IPM could improve phage efficiency in combination with other bacteria, and that the presence of native tree microbiota could potentially shape pathogen responses to phage treatments.

6 | CONCLUSIONS

Phages are promising biocontrol agents that have the potential to replace damaging copper pesticides and antibiotics either alone or in combination with other control methods through IPM. One of the key benefits of phages is their high host specificity, leaving nontarget microbes present in tree microbiota probably unaffected. They are also classified as organic disease control agents due to their naturally high abundance in nature. However, there are still many unknowns, especially regarding the ecological and evolutionary effects of phage treatments in the rhizosphere and phyllosphere. Specifically, phage resistance evolution could pose a significant problem with long-lived plants that probably require repeated phage treatments. Additionally, phages could become inactivated by UV light and extreme temperatures under field conditions. Current research on phage biocontrol of tree diseases is patchy, and thus far, most of the research has focused on *E. amylovora* and Psa, which are highly aggressive tree pathogens. Two successful commercially available phage treatments against *E. amylovora* have been produced, and dozens of phages against Psa have been isolated, giving hope that a phage treatment will soon be produced commercially to combat the current rampant kiwifruit canker infections. Other tree pathogens, including Pss, Pae, and *X. fastidiosa*, have only been subject of a small number of phage studies, despite causing devastating diseases. There are also existing and emerging tree diseases that have not yet been studied for phage biocontrol, for example *P. avellanae* (causing

canker and decline of hazelnut), *P. savastanoi* pv. *savastanoi* (causing olive knot disease), and *Brenneria goodwinii*, *Gibbsiella quercinecans*, and *Rahnella victoriana* found in oak bleeding cankers associated with acute oak decline (Brady et al., 2017; Buonaurio et al., 2015; Scortichini et al., 2003). While considerable work exists on development of phage therapies against *R. solanacearum*, most of this work has focused on non-tree strains. In the future, phage research needs to be furthered to involve more pathovars specific to tree diseases. Potential problems arising due to phage resistance evolution could be avoided using phage cocktails or preadapted phages, while combining phages with PGPR could also offer another avenue to increase phage treatment efficiency. The safety of phage treatments could be validated in direct experiments involving tree microbiota, while phage survival in the field could be improved using protective formulations and more efficient inoculation methods. Even though trees pose unique challenges for phage biocontrol due to their large size and relatively long lifespan, most of the existing concepts developed with small short-lived crops could be easily transferred to phage biocontrol of tree-pathogenic bacteria.

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Data sharing is not applicable to this article as no new data were created or analysed.

ORCID

Emily R. Grace https://orcid.org/0000-0002-7409-9571
Mojgan Rabiey https://orcid.org/0000-0001-8463-9087
Ville-Petri Friman https://orcid.org/0000-0002-1592-157X
Robert W. Jackson https://orcid.org/0000-0002-2042-4105

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