Critical Reviews in Biotechnology

ISSN: 0738-8551 (Print) 1549-7801 (Online) Journal homepage: www.tandfonline.com/journals/ibty20

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To cite this article: Gothandapani Sellamuthu, Amrita Chakraborty, Ramesh R. Vetukuri, Saravanasakthi Sarath & Amit Roy (2025) RNAi-biofungicides: a quantum leap for tree fungal pathogen management, Critical Reviews in Biotechnology, 45:5, 1131-1158, DOI: 10.1080/07388551.2024.2430478

To link to this article: https://doi.org/10.1080/07388551.2024.2430478

CRITICAL REVIEWS IN BIOTECHNOLOGY
2025, VOL. 45, NO. 5, 1131–1158
https://doi.org/10.1080/07388551.2024.2430478

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RNAi-biofungicides: a quantum leap for tree fungal pathogen management

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ABSTRACT

Fungal diseases threaten the forest ecosystem, impacting tree health, productivity, and biodiversity. Conventional approaches to combating diseases, such as biological control or fungicides, often reach limits regarding efficacy, resistance, non-target organisms, and environmental impact, enforcing alternative approaches. From an environmental and ecological standpoint, an RNA interference (RNAi) mediated double-stranded RNA (dsRNA)-based strategy can effectively manage forest fungal pathogens. The RNAi approach explicitly targets and suppresses gene expression through a conserved regulatory mechanism. Recently, it has evolved to be an effective tool in combating fungal diseases and promoting sustainable forest management approaches. RNAi bio-fungicides provide efficient and eco-friendly disease control alternatives using species-specific gene targeting, minimizing the off-target effects. With accessible data on fungal disease outbreaks, genomic resources, and effective delivery systems, RNAi-based biofungicides can be a promising tool for managing fungal pathogens in forests. However, concerns regarding the environmental fate of RNAi molecules and their potential impact on non-target organisms require an extensive investigation on a case-to-case basis. The current review critically evaluates the feasibility of RNAi bio-fungicides against forest pathogens by delving into the accessible delivery methods, environmental persistence, regulatory aspects, cost-effectiveness, community acceptance, and plausible future of RNAi-based forest protection products.

ARTICLE HISTORY
Received 6 July 2024
Revised 3 October 2024
Accepted 27 October 2024

KEYWORDS
Plant fungal pathogen; forest disease management; RNA interference; host-induced gene silencing; spray-induced gene silencing; host–pathogen interaction

Introduction

Pathogen outbreaks in large forest areas can negatively impact the species richness and socioeconomic value of forests [1]. Invasive pathogens are the primary cause of disease epidemics, irrespective of environmental variables and host heterogeneity at the individual or population level [2–4]. Environmental factors, such as drought, temperature, moisture, increased precipitation, snowfall, and windiness, might influence host vulnerability and disease epidemics [5, 6]. Moreover, biotic stresses (e.g., insect/fungus/oomycetes, insect/bacteria, or nematode/fungus complex interactions) rise annually [6–9]. Fungi (particularly ascomycetes and basidiomycetes) are the most recurrent phytopathogens causing disease in forest tree species [10, 11]. Oomycetes, which resemble fungi, can cause a variety of diseases, such as root rots, stem cankers, and foliar diseases, for example, outbreaks of severe decline and mortality by *Phytophthora* species: ash tree (*Fraxinus* species) [12], European beech (*Fagus sylvatica*) [13], Cork oak (*Quercus* species) [14, 15]. Endophytic fungi might be pathogenic to trees and cause a broad range of symptoms, such as bark cankers, blue stains, tissue necrosis, and dieback [16–18]. Recently, forest tree diseases caused by invasive fungi have increased in Europe [19, 20], triggering additional ecological and economic damage [21–23] (Figure 1). Interestingly, the dynamics of host–pathogen interaction under the influence of environmental factors in forests remain obscure, causing hindrance to sustainable pathogen management [26]. Sustainable disease management enhances forest resilience to pathogens and shields essential ecological services from forests [26–29]. So far, there are no reports on specific treatments to eradicate the fungal disease or manage invasive pathogens. However, recent studies have proposed classical forest resilience strategies for

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Supplemental data for this article can be accessed online at https://doi.org/10.1080/07388551.2024.2430478.

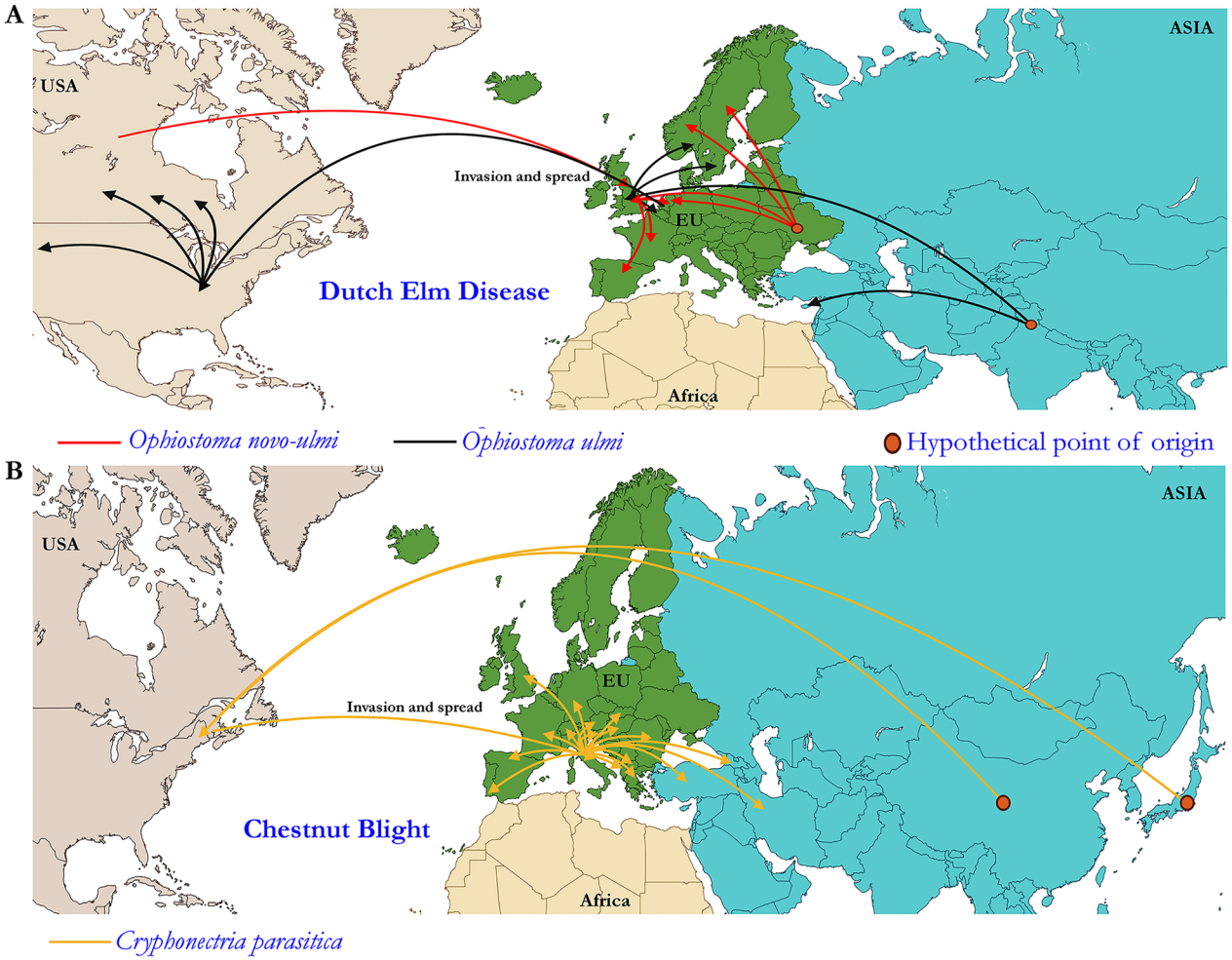

Figure 1. Invasive fungal pathogens threaten forest biodiversity. It is crucial to investigate the patterns and determinants of the underlying invasion to manage forest disease. Two examples of fungal pathogen invasion trajectory. (A) Dutch elm disease: Dutch elm disease (DED) was initially believed to have originated from the Himalayas (India). The native trees of the Himalayas, *Ulmus wallichiana*, are unaffected by the *Ophiostoma ulmi* and *Ophiostoma novo-ulmi*. *Ophiostoma ulmi* was first reported in Cyprus in 1658, in England in 1819, in Belgium in 1836, and in Sweden in 1950. From 1963 onwards, it spread to Norway and most European countries. In 1963, the disease was reported in North America, in Cleveland, Portland, and Ohio. It then spread from the Northern American region to Eastern Canadian provinces like Ontario in 1967, Manitoba in 1975, and Saskatchewan in 1981 [24]. Later, *Ophiostoma novo-ulmi* started a second round of infection among the elm trees in Britain in the late 1960s. It was believed to have originated from the Ukraine-Moldovan region and began spreading to other regions of the United Kingdom in 1967. Subsequently, the disease spread from England to other European countries such as the Netherlands, France, and Spain. The Netherlands also experienced the disease from Ukraine in 1975. Later, during the 1980s, the disease spread to more European countries, reaching Sweden in 1980 and Norway in 1981 [24]. (B) Chestnut blight: This disease is caused by the fungal pathogen *Cryphonectria parasitica*, first appearing in America in 1904 in New York. It was introduced to America through infested logs from China and Japan, where it was a harmless endophyte in Asian trees but became invasive in America and Europe. From North America, it spread to Italy in 1938 and other European countries. By the 1950s, the disease had spread to France, Spain, Portugal, Switzerland, and Slovenia, and later to other countries such as Turkey, Greece, Albania, Germany, Slovakia, Romania, Macedonia, and even to Iran to some extent [25].

managing primary and secondary infection [30]. A few studies have also reported eco-friendly biological control against forest fungal diseases and its integration into classical forest management practices [Box 1] [10, 31, 32]. Alternatively, systemic fungicides can release environmentally hazardous residues into the soil, affecting the phyllosphere and soil microbiota [33, 34]. In addition, intensive application of fungicides can increase the selection pressure, triggering resistance [34–36]. Besides, conventional tree breeding is more complex, time-consuming, and expensive than crops [37]. Hence, developing new approaches for sustainable and eco-friendly forest disease management is crucial. The review explores the available molecular approaches (i.e., RNAi) against fungal and oomycete pathogens and summarizes the roadmap for developing biofungicides.

In recent decades, RNA interference (RNAi), a potent gene-silencing process conserved in eukaryotes, has received extensive attention due to its potential applications in understanding functional genomics and expression studies (Figure 2). RNAi is triggered by inverse transcripts that are complementary to the target messenger RNA (mRNA) by the components of RNAi machinery, RNA-dependent RNA polymerase (RdRP), ribonuclease enzyme (Dicer [Dcr]), and the small RNA-binding protein [Argonaute (Ago)] [38–41]. It was first described as a transgene co-suppression of the endogenous gene in plants and fungi [42, 43]. Similarly, double-stranded RNA (dsRNA) injection causes silencing of highly similar target regions in nematodes [44]. Over the past two decades, RNAi mechanisms have been extensively used in functional genomic studies and pest control [41, 45–55]. The ability of dsRNA uptake and processing in pathogens or pests determines the effectiveness of RNAi [56–63]. Genetically modified (GM) protection techniques expressing small RNAs (siRNA) or dsRNAs were used against certain diseases to silence target genes in pests or pathogens, which is also known as host-induced gene silencing (HIGS) [64]. Alternatively, the transgenic technique that deals with *in vivo* expression of dsRNA is challenging for

Box 1. Non-molecular forest protection strategies.
Traditional forest disease management employs various strategies to prevent, reduce, and control disease outbreaks, including cultural, biological, and chemical techniques. Several management strategies, including cultural practices like sanitation and controlled burning, significantly reduce disease incidence by removing infected material and improving forest health. Alternatively, employed biological control methods, including introducing natural enemies and using disease-resistant tree species, offer environmentally friendly alternatives to chemical treatments. Different trapping systems capture forest pests, which are also vectors for pathogens on a mass scale, using pheromones or other attractive chemicals. The use of non-host chemicals and a push-pull strategy often work nicely. Advanced remote sensing technology may detect diseased or pest-attacked trees more efficiently from now and aid in forest protection. Using sniffer dogs to detect pests (i.e., bark beetle) that attacked trees has recently been initiated in Sweden and Czech Republic. In many forests, pesticides were used to save the fallen logs from wood-feeding pests (i.e., bark beetle) and pathogens (i.e., blue stain fungi). Integrated Disease Management (IDM) integrates these techniques, stressing frequent monitoring, threshold-based interventions, and promote different eco-friendly control options to improve disease management efficacy and sustainability.

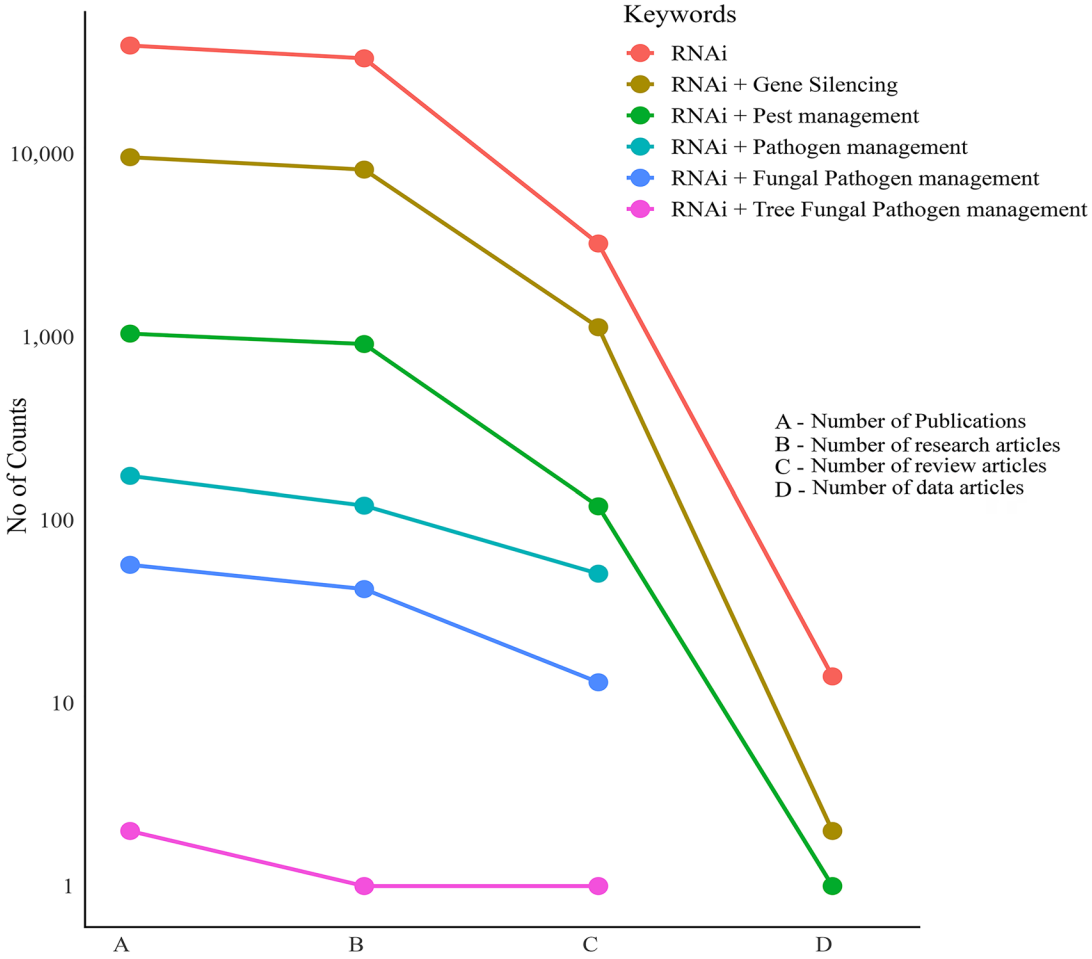

Figure 2. Web of Science search. Research and review articles published on RNAi-pest-fungal management in agroforestry over the past decade. Data was collected using specific keywords. The search was conducted on 11 March, 2024. The data showed minimal research work on RNAi-based tree fungal disease management.

Box 2. Sprayble dsRNA products become commercial.
RNA interference technology can potentially mitigate the challenges posed by forest pathogens. However, the dilemma in acceptance of dsRNA products by foresters, forest authorities and the public is well known. The current commercialization of RNAi crop protection products enhances the hope for deploying RNAi-based products in agroforestry. Registration of Ledporna (Calantha™-trade name) as a plant protection product against Colorado potato beetles [tagetting beetle proteasome beta type-5 subunit (PSMB5)] in USA on 22nd December 2023 can be celebrated as a landmark event for RNAi-based pest management research. The United States Environment Protection Agency (US-EPA) approves this sprayable dsRNA product. However, it is worth mentioning that the success of ledporna is based on two decades of rigorous research endeavors worldwide. Similarly, genetically modified (GM) maize expressing dsRNA targetting DvSnf gene [necessary for cellular vesicle transport] in western corn rootworm (WCR) is also commercialized under the trade name of SmartStax ®PRO in 2022. Such commercialization events showcase the positive societal outlook on dsRNA-based solutions. More sprayable dsRNA products against pests and pathogens will likely be launched shortly. Some of the EU-funded research projects such as Ecostack (Project number: 773554; Staking the Ecosystem Services: Mechanisms and Interactions for Optimal Crop Protection, Pollination Enhancement, and Productivity) and RATION (Risk assessment innovation for low Risk pesticides) that were launched in 2022 is endorsing such possibilities. The COST Action network, such as CA21134 "Towards zer0 pesticide agriculture: European network for sustainability (T0P-AGRI-Network)," also brings EU researchers, farmers and industries together for sustainable, eco-friendly pest and disease management in future.

trees and requires regulatory approval, which can take years. However, attempts were made to generate transgenic trees with beneficial traits in forests [65–72].

Alternatively, non-GM (non-recombinant) protection techniques hold promises for sustainable forest disease management. For instance, the spray-induced gene silencing (SIGS) method uses the direct application of dsRNA to host surfaces to silence target pathogen genes [65, 70, 72]. In this case, dsRNA must enter the target pathogens through direct or indirect uptake [73]. It is worth mentioning that non-transformative gene silencing can effectively control forest diseases if some limitations are alleviated. Recently, sprayable RNAi against insect pests has been receiving public and government approvals and becoming commercial [74] [Box 2]. In the following section, we discussed using the existing RNAi approach to combat fungal and oomycete disease in forests, its drawbacks, and the prospective for fostering resilient forests against various diseases caused by filamentous pathogens.

RNAi – a conserved regulatory mechanism for fungal endogenous genes

RNAi is a post-transcriptional gene silencing (PTGS) regulatory mechanism involving small interfering RNAs (siRNAs) and microRNAs (miRNAs) molecules that lead to mRNA degradation and degradation/translation blockage, respectively [41, 75–77]. This process is crucial for the innate immune response in many organisms, including fungi, and also regulates various aspects of cellular activities, vegetative growth, sexual reproduction, and many other traits [78, 79]. In fungi, RNAi has been demonstrated to regulate the expression of various genes, including those related to virulence and the production of secondary metabolites that contribute to host pathogenicity [80–82]. The RNA silencing phenomenon was first described in the fungus *Neurospora crassa*, where repetitive homologous transgene sequences were used to alter the genome and reversibly inactivate gene expression called quelling [43]. The model suggested that erroneous RNA generated directly from transgenes or as a result of the presence of transgenes might be employed as the template by quelling defective 1 (QDE1) with dual function as DNA-dependent RNA polymerase (DdRP) and RNA-dependent RNA polymerase (RdRP) that leads to the formation of dsRNA [56–59]. Subsequently, mRNA is degraded by converting dsRNA into small interfering RNA (siRNA) that makes RNA-induced silencing complexes (RISCs) by loading onto core components of the RNAi pathway proteins, QDE2 (Argonaute-Ago), DCL (Dicer) [83–86]. Evolutionarily conserved phylogenetic relationships showed distinct outgroups and separate clades among the plants, insects/mammals, and fungi based on the evolutionarily conserved Argonaute (AGO) and Dicer protein (retrieved from the NCBI and UniProtKB databases) in the RNAi pathway (Figures 3 and 4, Supplementary Table 1). The selected group of pathogenic fungi with available sequence information revealed a high level of conservation of the RNAi system (Table 1, Figures 3 and 4). It is worth mentioning that DCL1 and AGO1 were selected for phylogenetic analysis due to their central roles in the highly conserved miRNA pathways. However, such selection may induce some biases.

Transgene-induced co-suppression of host homologous mRNA has also been seen in various fungi, plants, and mammals [87–92]. The high efficacy of sex-induced silencing (SIS) was first discovered during the mating stage in *Cryptococcus neoformans*, but it was later demonstrated that the co-suppression mechanism functions during vegetative development [92, 93]. Similarly, during sexual development, targeted gene silencing revealed the existence of transgenic copies and significant expression of RNAi core components in *Aspergillus nidulans* [94]. Hence, RNAi can regulate stage and sequence-specific PTGS of homologous endogenous genes in fungal pathogens [70, 72, 95, 96].

Cross-kingdom RNAi: RNA molecules on the move

Recent advances in cross-kingdom RNAi have significantly deepened our understanding of plant–pathogen

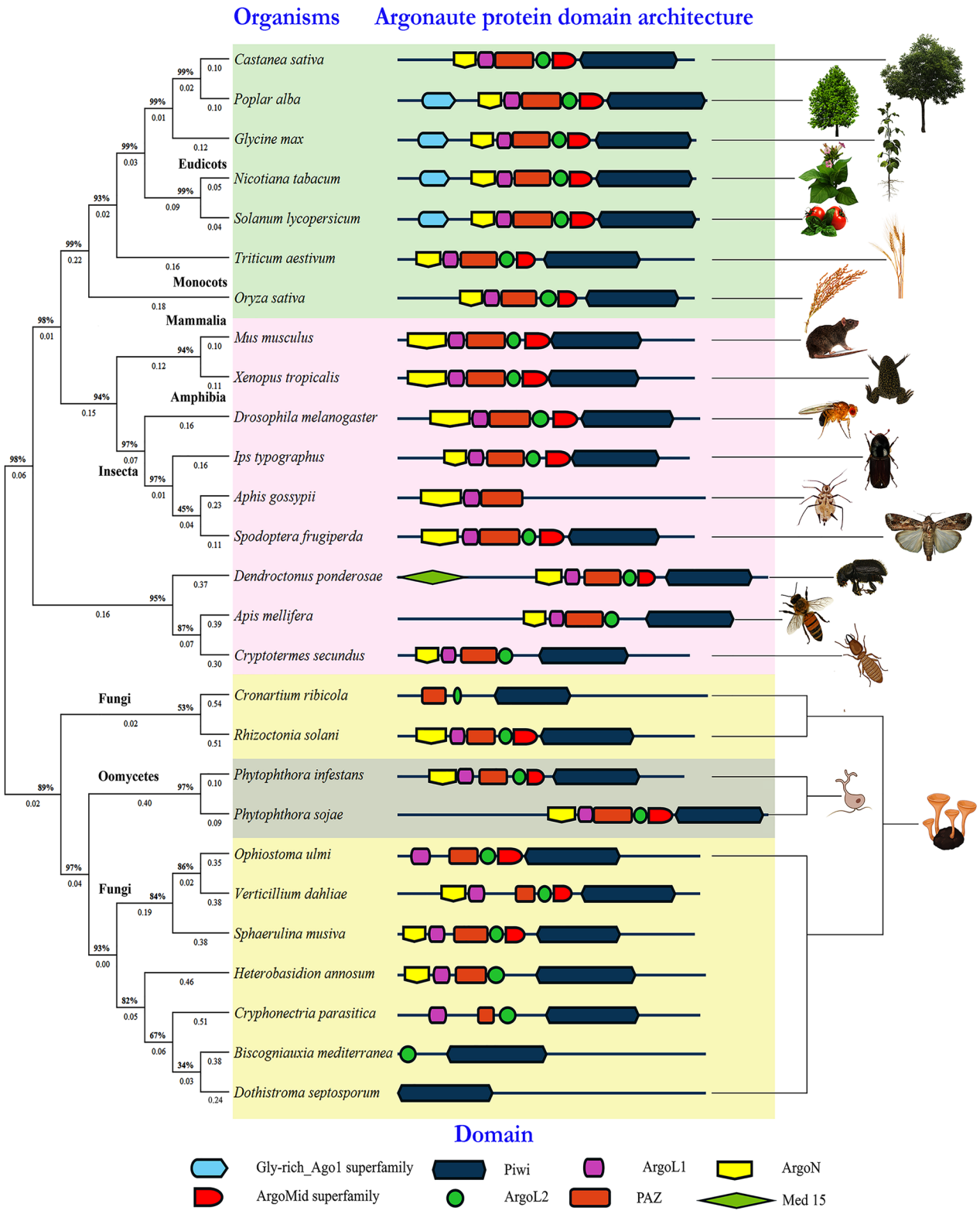

Figure 3. Conservation of RNAi mechanisms in different organisms. Phylogenetic and structural analysis is based on selected Argonaute proteins (AGO) (RNAi core gene) among plants, insects/mammals, and fungi. The separate clustering showed distinct evolutionary trajectories. The accession numbers for AGO proteins are presented in Supplementary Table 1.

interactions, revealing the complex bidirectional communication mediated by RNA molecules. Host-derived dsRNA or siRNA can transfer between plants and pathogens, influencing gene expression on both sides [97–102]. For instance, plant-derived siRNAs can inhibit pathogen virulence, as observed in tomatoes

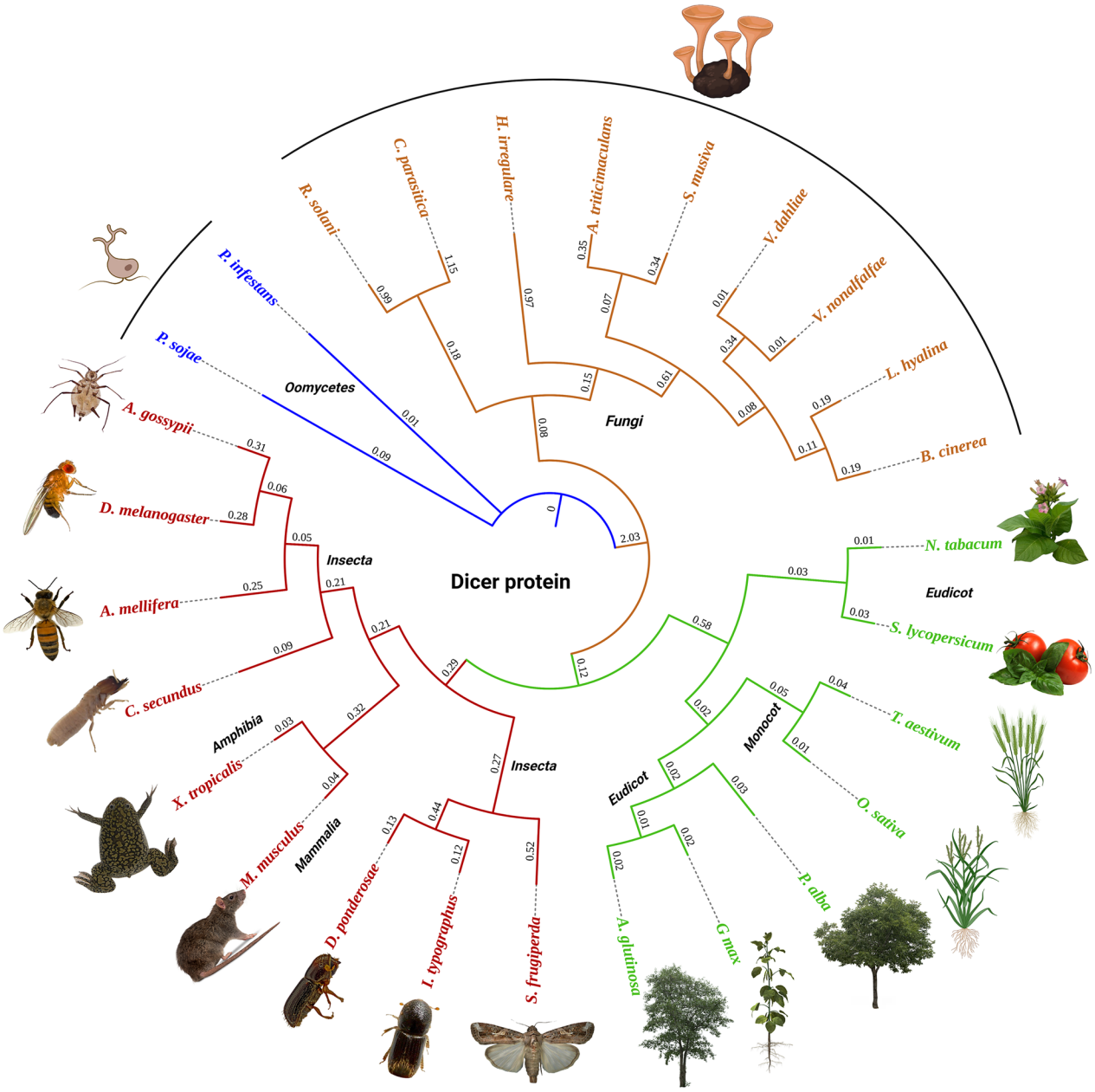

Figure 4. The phylogenetic tree representing the evolutionary relationships between various species is constructed using selected Dicer proteins (DCL), a core gene in the RNAi pathway, from plants, insects/mammals, and fungi. The distinct clustering patterns highlight the unique evolutionary trajectories of these groups. The accession numbers for the DCL proteins used in the analysis are provided in Supplementary Table 1.

against *Botrytis cinerea*, cotton against *Verticillium dahliae*, and wheat against *Fusarium graminearum* [103–105]. Alternatively, in the host–fungal pathogen interaction model system, *Arabidopsis thaliana* and *B. cinerea*, fungal-derived siRNA can enter host cells and hijack host *AGO1* to hinder host immunity and stimulate infection [106]. Analogous mechanisms might be involved in *Picea abies* with artificial infection of *Ophiostoma* (Figure 5: Panel-1). Likewise, it has also been observed in animal pathogen–parasite interactions and insect-pathogenic fungi interactions [107–109].

The transfer of dsRNA and siRNA underlines the bidirectional nature of cross-kingdom RNAi. It is worth mentioning here that recent reports have already documented the movement of mRNA molecules of plants to fungal pathogens to influence the expression of virulence genes [72, 101]. For instance, pathogens like *B. cinerea* deploy siRNAs to suppress host immunity, while host plants such as *Arabidopsis* secrete exosome-like extracellular vesicles to deliver siRNAs that silence fungal virulence genes [110]. Conversely, *B. cinerea* and *V. dahilae* deliver siRNAs

Table 1. List of RNAi core machinery genes in the pathogenic fungi.

Pathogens/diseases	Ago (QDE-2)	DCL	DdRP	RdRP (QDE-1)
Cryphonectria parasitica (Chestnut blight)	GQ250184 (Ago1) GQ250185 (Ago2) GQ250186 (Ago3) GQ250187 (Ago4)	DQ186990 (DCL1) XM_040918288 (DCL2)	AY485619	HF912382
Lachnellula hyalina (Canker disease)	XM_031147658	XM_031149076 (DCL2)	PRJNA576247	XM_031151682
Cronartium ribicola (White pine blister rust)	PRJNA190829 PRJNA352055			
Heterobasidion annosum (Root rot pathogen)	PRJNA362289 PRJNA250418 GSE30230			
Heterobasidion irregulare (Root rot pathogen)	PRJNA46703 PRJNA245138 PRJEB8921			
Ophiostoma ulmi (Dutch elm disease)	PRJNA173023		DQ368645	PRJNA173023
Nectria coccinea var. Faginata (Beech bark disease)	PRJNA994555		HQ840395	PRJNA994555
Rhizoctonia solani (Rhizoctonia Root Rot)	XM_043321326 (Ago1) XM_043327027 (Ago2)	XM_043321487	XM_043327164	XM_043331779
Biscogniauxia mediterranea (Charcoal canker)	PRJNA570004 PRJNA567201 PRJNA567202 PRJNA571054		GQ844765	PRJNA570004 PRJNA567201 PRJNA567202 PRJNA571054
Fusarium circinatum (Pitch canker)	PRJNA817734 PRJNA257359		MW402656	NC_076564
Dothistroma septosporum (Red band needle blight)	PRJNA584708	PRJNA584708	PRJNA584708	OX464993
Verticillium dahliae (Verticillium wilt)	XM_009659642 (Ago1)	XM_009651848 (dcl1) XM_009655850 (DCL2)	XM_009653248	XM_009657112
Botrytis cinerea (Botrytis bunch mold)	XM_024697207 XM_001546167	XM_024696562 (DCL1)	MG714858	PRJNA264284
Rhizina undulata (Rhizina root disease)	PRJNA372875		JX943748	PRJNA372875
Sphaerulina musiva (Septoria Canker)	XM_016907097	PRJNA681597	XM_016909586	PRJNA681597
Botryosphaeria dothidea (Beech tar crust)	PRJNA782403 PRJNA81801 PRJNA450131			MZ073729
Biscogniauxia nummularia (Beech tar crust)	PRJNA331563 PRJNA442727	PRJNA331563 PRJNA442727	KY624236	PRJNA331563 PRJNA442727

into plant cells to silence host immunity genes, highlighting the bidirectional nature of cross-kingdom RNAi [111]. Beyond pathogens, RNAi communication extends to beneficial microorganisms, with root endophytes like *F. solani* potentially transferring RNAi signals to host plants, influencing gene expression and conferring beneficial traits [112]. Additionally, miRNA-based approaches, including artificial miRNAs (amiRNAs), could be employed to control fungal pathogens by silencing critical fungal genes through cross-kingdom RNAi, where miRNAs produced in plants can transfer across species boundaries and silence target genes in fungal pathogens. For instance, in response to *V. dahliae* infection, cotton plants increase the production of miRNA (miR166 and miR159), which are transferred to the pathogen to silence key virulence genes. Specifically, these miRNAs target and inhibit the expression of *V. dahliae* genes encoding *Ca2+-dependent cysteine protease* (Clp-1) and *isotrichodermin C-15 hydroxylase* (HiC-15), both essential for fungal virulence [113]. Alternatively, in wheat, the stripe rust pathogen *Puccinia striiformis* f. sp. *tritici* (Pst) uses a microRNA-like RNA (milRNA), Pst-milR1, to suppress plant defenses by targeting the wheat *pathogenesis-related 2* (PR2) gene. Silencing Pst-milR1 enhances wheat resistance, indicating its role in cross-kingdom RNAi events [114]. Furthermore, amiRNA has demonstrated potential in managing *Phytophthora infestans*, the pathogen responsible for potato late blight. By targeting the crucial RXLR effector *Avr3a* gene, amiRNA constructs effectively reduced pathogen load and Avr3a levels in transgenic potatoes [115]. However, a recent study computationally predicted miRNAs from 13 fungi and demonstrated their targets in *F. oxysporum* and chickpea [116]. In these interactions, endogenous miRNAs from plants can be transferred into invading fungal pathogens, inhibiting vital fungal genes related to virulence or

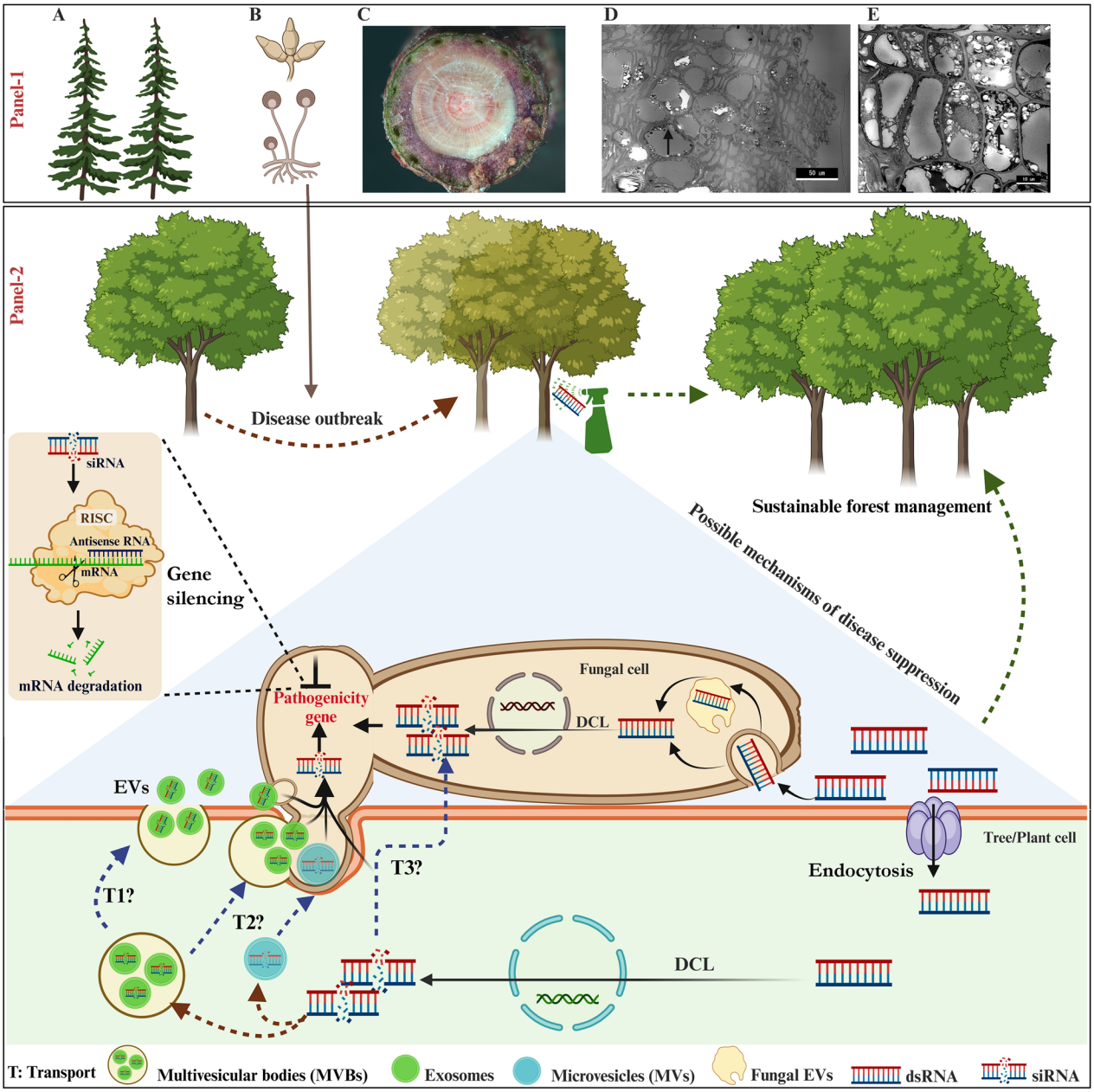

Figure 5. A model depicting possible mechanisms of cross-kingdom RNAi between host and pathogen. Panel 1: Disease development of *Picea abies* stem infested by *Ophiostomatoid* fungi. A: healthy *P. abies*; B: infestation with *Ophiostomatoid* fungi; C: *P. abies* stem image of 65 days after infection; D and E; transmission electron microscopy (TEM) images of infected stem showed fungal hyphal growth accumulation in cavities (black arrows in D–E). Panel 2: Possible ways of disease suppression through potential pathways for siRNA uptake. Interaction of a plant cell with a fungal pathogen during plant RNAi-mediated (dsRNAs) gene silencing (spray-induced and/or host-induced). Exogenous applied dsRNAs can be taken directly into the fungal cells (through actively growing hyphae) on the plant surface through endocytosis (i.e., clathrin-mediated cellular uptake). Alternatively, dsRNAs enter the plant cells *via* different mechanisms, such as endocytosis or absorption through the cuticle. The plant generates short RNAs (siRNAs) as part of the natural defensive response and transports dsRNA to fungi probably through multiple ways such as multivesicular bodies (MVBs) (T1); haustorium uptake of plant extracellular vesicles (EVs) (T2); or siRNA absorption through other unknown transporters (T3) or mechanisms. The plausible alternate or unknown ways of dsRNA entry to pathogenic fungi are represented by question marks (?). After entering the fungal pathogen, the siRNAs are released from the vesicles, which initiates target gene silencing in the pathogen and, eventually, causes the death of fungi.

survival. Such findings provide a new direction in future plant–pathogen interaction research. This approach might also be effectively applied in managing tree fungal pathogens, offering a sustainable and targeted method for disease control in forestry. Additionally, recent research has identified the significant role of

miRNA_17532 in cross-kingdom RNAi between wheat and the biocontrol fungus *Clonostachys rosea*. miRNA_17532 is transported from wheat into *C. rosae*, where it modulates the expression of key fungal genes, enhancing the biocontrol agent's ability to promote plant health [117]. This interaction illustrates how wheat can fine-tune its relationship with beneficial fungi, improving the efficacy of *C. rosae* in biological control. This finding underscores the broader potential of miRNAs in not only pathogen suppression but also in enhancing beneficial plant–microbe interactions in sustainable agriculture and forestry practices.

RNAi against phytopathogenic fungi in agroforestry: current status

RNAi *via* host-induced gene silencing (HIGS) or spray-induced gene silencing (SIGS) is effective against several phytopathogens and insects [64, 118]. Small interfering RNA molecules (siRNA/dsRNA) targeting immune-responsible genes translocate to plant hosts from pathogens to make plants susceptible to pathogens [106]. Conversely, the host plant can also translocate siRNA/dsRNA to the pathogen through specific transport mechanisms like extracellular vesicles during host–pathogen interaction to induce essential (pathogenicity and defence) gene silencing (Figure 5: Panel-2) [97, 111]. Several studies demonstrated that HIGS and SIGS efficiently control phytopathogenic fungi, including *Fusarium, Aspergillus, Puccinia, Rhizoctonia, Verticillium*, and other fungal pathogens (Table 2). Pathogen suppression occurs in different ways *via* HIGS and SIGS. For example, stable expression of the *chitin synthase 3b* (*Chs3b*) gene in wheat reduced mycotoxin accumulation in grains and increased resistance [121]. A similar trend was observed for other pathogens in banana [153, 154] and tomato plants [155, 156]. Stable transgene expression in the host reduced the target endogenous transcript level, which indicates the translocation of siRNA from host to pathogen cells. Analogously, virus-induced gene silencing (VIGS) can be deployed against forest pathogens. For instance, *1,3-glucanosyltransferase* (*GTF1* and *GTF2*), *mitogen-activated protein kinase* (*MAPK*), *MAPK-PUZ*, and *protein kinase A* (*PKA*) genes were silenced by the barley stripe mosaic virus (BSMV) resulting decreased haustorium production and hyphal development in wheat infested with *Blumeria graminis, P. triticina, P. striiformis* respectively [119, 133, 137, 138]. Bean pod mottle virus (BPMV)-mediated gene silencing was also documented in soybeans [131, 157]. A recent review comprehensively discussed forest virome (including VIGs) as a source to manage forest pathogens [158]. However, identifying suitable viruses for downstream VIGs applications demands dedicated studies on tree-specific mycoviruses in different forest types belonging to different geographical regions and a greater understanding of mycovirus-host balance in 3D forests with contrasting microhabitats.

RNAi studies on forest pathogens

Native and invasive pathogens threaten preserving forest biodiversity, habitat loss, and ecosystem services [22, 159, 160]. The list of invasive forest fungal pathogens (IFPs) is on the rise in Europe and worldwide under climate change, putting pressure on ecosystems and threatening biodiversity loss [23, 161, 162]. Small RNAs can offer a new paradigm for managing the notorious native and invasive pathogenic fungi [163]. The current status of forest fungal pathology provides an assessment of RNAi performance in the forest saprophytic pathogen *Ophiostoma* spp. For example, suppressing the melanin pathway gene *polyketide synthase* (*PKS1*) with an RNAi decreased pigmentation in *O. piceae* and *O. floccosum* [164]. The first study inhibited pectinase activity and virulence in *O. novo-ulmi* by targeting the pathogenicity gene (*endopolygalacturonase* [*ePG*]). [165]. Several studies focused on finding genes that have a role in pathogenic fungi virulence, fitness, and cell functioning processes have continued utilizing gene disruption to interfere with gene expression precisely [166–168]. However, to date, forest pathogens have demonstrated virulent gene expression in: *Ophiostoma australiae*, *O. tasmaniense*, and *O. undulatum* [169], *O. novo-ulmi* [170, 171], *O. ulmigenome* [172], *Armillaria ostoyae* [173], *Heterobasidion annosum* [174], *Cryphonectria parasitica* [175]. Furthermore, the functional annotation of pathogenicity and virulence effect during host–pathogen interaction has been reported in *O. novo-ulmi* [147, 170]. A recent study demonstrated the effectiveness of SIGS in controlling *F. circinatum*, the causative agent of Pine Pitch Canker disease [150]. This highlights the potential of RNAi-based approaches for managing forest pathogens. Future research must focus on gene expression and pathogenicity to further elucidate the mechanisms of fungal virulence. In addition, novel dsRNA delivery methods have been developed for forest pest management, which holds potential for application against forest pathogens [60, 176]. The stability of dsRNA in the host (woody plant) environment has been investigated *via* trunk injection, which systematically translocates functional siRNA and hpRNA to various tissues while avoiding host endonuclease activity [177, 178]. When dsRNA/siRNA is absorbed through septal pores and vesicles, it can

Table 2. List of HIGS (GMO) and SIGS (non-GMO) applications against pathogenic fungi.

	Pathogen	Host	Target gene	Method of siRNA production/ vector/host	Outcome	Ref
GMO (HIGS)	*Blumeria graminis*	Barely and Wheat	BgGTF1 BgAvra10 GTF1 or GTF2	HIGS-pIPKb007:BgGTF1 pIPKTA30N:BgAvra10 VIGS-barley stripe mosaic virus	Silencing the effector gene, which resulted in reduced fungal development	[119]
	Fusarium graminearum	Barely Wheat Stiff brome	FgCYP51 chitin synthase 3 (FgChs3) protein kinase FgCYP51	HIGS-pLH6000-Ubi:CYP51 HIGS-pHAU-HygB-Chs3 HIGS-FgSGE1 HIGS-pCAMBIA1300	Depletion of ergosterol and the accumulation of sterol precursors, which imbalance and alter plasma membrane structure and function	[120] [121] [122, 123] [124]
	Phytophthora infestans	Potato	PiGPB1, PiCESA2, PiPEC, PiGAPDH	HIGS-pGWB1:PiGPB1, pGWB1:PiCESA2, pGWB1:PiPEC pGWB1:PiGAPDH	PiGPB1 targeting the G protein β-subunit (PiGPB1) restricted disease progress	[125]
	Botrytis cinerea	Arabidopsis Potato & Tomato	Bc-DCL1 and Bc-DCL2 BcTOR	HIGS-pHellsgate:BcDCL1 and DCL2 HIGS-BcTOR-RNAi	Significantly inhibits gray mold disease in both HIGS and SIGS	[111] [126]
	Verticillium dahliae	Arabidopsis Cotton	Bc-DCL1 and Bc-DCL2 Vd-hygrophobins1 (VdH1)	HIGS-pHellsgate-DCL1 and DCL2 pBI121-VdHi	Significantly inhibits development and disease in both HIGS and SIGS	[111] [105]
	Sclerotinia sclerotiorum	Soybean Rapeseed	Ssoah1 SsPG1, SsCBH, and SsOAH1 ABHYRDOLASE-3	pBPMV-OA VIGS-Bean pod mottle virus (BPMV) pMDC83	Decreased fungal infection and disease symptoms	[127] [128] [129]
	Bremia lactucae	Lettuce	Bl-Cellulose Synthase (Bl-CES1)	pGSA1165:blCes1	Inhibition of cell wall biosynthesis gene and reduced growth and inhibition of sporulation	[130]
	Uromyces appendiculatus	Dry bean	Ua-chitinase (Ua-Chi)	VIGS-Bean pod mottle virus pBPMV:UaChi	Significant reduction of infection	[131]
	Aspergillus flavus	Maize	AflC	pAGM4723-AflC250-Bacteria induced	Reduction in aflatoxins content with reduced fungal growth	[132]
	Puccinia triticina	Barley	PtMAPK, a cyclophilin (PtCYT1), and a calcineurin regulatory subunit (BtCNB).	VIGS-Barley stripe mosaic virus (BSMV)-induced RNAi pγ42:PtMAKP pγ42:PtCYT1 pγ42:PtCNB	Suppressed disease phenotype and a reduction in endogenous transcript	[133] [134]
	Botrytis cinerea	Strawberry	BcDCL1/2	HIGS-pHellsgate–Bc-DCL1/2	Significantly enhanced tolerance	[135]
	Phakopsora pachyrhizi	Soybean	Acetyl-CoA acyltransferase (ATC) Glycine cleavage system H protein (GCS_H) 40S ribosomal protein S16 (RP_S16) Conidiation-related protein 6 (CRP_6) Putative 3-hydroxy-3-methylglutaryl-coenzyme A reductase (PHR) Hypothetical proteins (Un_1, Un_2, Un_3)	VIGS-pBPMV-IA-V2	Significant reduction in pustule number	[136]
	Puccinia striiformis	Wheat	PsCPK1 PsFUZ7	barley stripe mosaic virus (BSMV)-(HIGS) pMCG161:PsCPK1 barley stripe mosaic virus (BSMV) pAHC25:PsFUZ7	Significant reduction in the length of infection hyphae and disease phenotype	[137] [138]

(*Continued*)

Table 2. Continued.

	Pathogen	Host	Target gene	Method of siRNA production/ vector/host	Outcome	Ref
Non-GMO (SIGS)	*Fusarium graminearum*	Barely	FgCYP51 And FgAGO, FgDCL	SIGS-MEGAscript Kit-*In vitro* synthesis	Depletion of ergosterol and the accumulation of sterol precursors which	[139] [120]
		Wheat	FgChs7, FgGls and FgPkc		imbalance and alters plasma membrane structure and function	[140] [123]
	Botrytis cinerea	Fruits (tomato, strawberry, and grape), Vegetables (lettuce and onion), Flower petals (rose), Chickpea, Tomato Rapeseed, red bell-peppers, cherry, grapes, Strawberry	Bc-DCL1 and Bc-DCL2 vacuolar protein sorting 51 (BcVPS51) dynactin (BcDCTN1) and suppressor of actin (BcSAC1) virulence-related genes (BcVDS)	SIGS-MEGAscript Kit-*In vitro* synthesis SIGS-AgroRNA (Genolution, Seoul, Korea) SIGS-Genolutions (Korea)	Significantly inhibits gray mold disease	[111] [141] [135] [142]
	Verticillium dahliae	*Arabidopsis*	dynactin (VdDCTN1) and suppressor of actin (VdSAC1)	SIGS-MEGAscript Kit-*In vitro* synthesis Soil/root treatment	Significantly inhibits development and disease	[141]
	Fusarium asiaticum	Wheat	Myo5	SIGS-MEGAscript Kit-*In vitro* synthesis	Cell wall defects, life cycle disruption and virulence reduction	[143]
	Sclerotinia sclerotiorum	Rapeseed	toxin biosynthesis (SS1G_01703), ROS response (SS1G_02495), and cell cycle regulation (SS1G_09897), ribosomal biogenesis (SS1G_07873) and mitochondrial protein import (SS1G_06487)	SIGS-MEGAscript Kit-*In vitro* synthesis	Decreased fungal infection and disease symptoms	[95]
	Austropuccinia psidii and Coleosporium plumeriae	Syzygium jambos	β-tubulin (β-TUB), translation elongation factor 1α (EF1-a), acetyl-CoA transferase (ATC), cytochrome P450 (CYP450), mitogen-activated protein kinase (MAPK), glycine cleavage system H (GCS-H), 28S ribosomal RNA, and three haustorial targets (HAUS01136, HAUS01215, and HAUS12890)	SIGS-MEGAscript Kit-*In vitro* synthesis	Significant reduction of pustule development.	[144]
	Aspergillus niger	Tomatoes, apples and grapes	AnVPS51, AnDCTN1, AnSAC1	SIGS-MEGAscript Kit-*In vitro* synthesis	Significantly inhibited plant disease symptoms,	[141]
	Rhizoctonia solani	Rice	RsCTN1, RsSAC1	SIGS	Significantly inhibited plant disease symptoms,	[141]
	Magnaporthe oryzae	Rice	MoDES1	SIGS-MEGAscript Kit-*In vitro* synthesis	Growth reduction with conidia morphological deformity	[145]
	Plasmopara viticola	Grapevine	PvDCL1/2	SIGS-MEGAscript Kit-*In vitro* synthesis	Significant mycelial reduction	[146]
	Botryotinia fuckeliana	Strawberry	chitin synthase class III (BfChs3a &b) and DICER-like proteins (BfDCL1 and BfDCL2)	SIGS-MEGAscript Kit-*In vitro* synthesis	Inhibition of growth	[147]
	Phakopsora pachyrhizi	Soybean	Acetyl-CoA acyltransferase (ATC) Glycine cleavage system H protein (GCS_H) 40S ribosomal protein S16 (RP_S16)	SIGS-MEGAscript Kit-*In vitro* synthesis	Significant reduction in rust symptom	[136]
	Phytophthora infestans	Tomato	Guanine-nucleotide binding protein β-subunit (PiGPB1) Haustorial membrane protein (PiHmp1) Cutinase (PiCut3) Endo-1,3(4)-β-glucanase (PiEndo3)	SIGS-MEGAscript Kit-*In vitro* synthesis	Significant reduction of late blight disease	[148]
	Phytophthora capsici	Tobacco	Necrosis and ethylene-inducing peptide 1-like proteins 2 and 6 (NLP2 and NLP6)	SIGS-MEGAscript Kit-*In vitro* synthesis	Significantly suppress infection	[149]
	Fusarium circinatum	Pinus	FcVDS: Vacuolar protein sorting 51 (Vps51), Dynactin complex (DCTN1) Suppressor of actin (SAC1) FcPTP: 2A phosphatases (Pp2a, Sit4, Ppg1) Phosphatase 2 A-associating protein (Tap42) FcCHS: Chitin synthases (Chs1, Chs2, Chs3b) β-1,3-Glucan Synthase (Gls1)	SIGS-T777T plasmid_Bacterial expression	Significant inhibition of the pathogen virulence	[150]
	Fusarium oxysporum	Tomato	RNA-dependent RNA polymerase 1 (RDR1)	SIGS-RDR1 and RDR2	Significant reduction of wilt	[151]
	Penicillium italicum	Citrus	PiDCL1/2	SIGS-MEGAscript Kit-*In vitro* synthesis		[152]

migrate locally and systemically through plasmodesmata and phloem to fungal cells as bidirectional cross-kingdom RNAi during host–pathogen interaction [100, 179]. The commercialization of SIGS is a gigantic leap for RNAi-based pest and pathogen management products [74]. However, further studies are needed for possible application methods against pathogens, target genes, and non-target effects on other beneficial microbes (Figure 6).

Feasibility of RNAi against forest pathogens

Transgenic-based approach

The transgenic approach, where the host plant acts as a delivery system to produce dsRNA or siRNA by transgene constructs (hairpin construct-hpRNA) to induce silencing of an essential target gene, is also known as host-delivered RNAi (HD-RNAi) [97–99, 119, 180–183]. In 2010, the first *in vivo* report of RNAi-mediated silencing during the host–pathogen interaction with *F.*

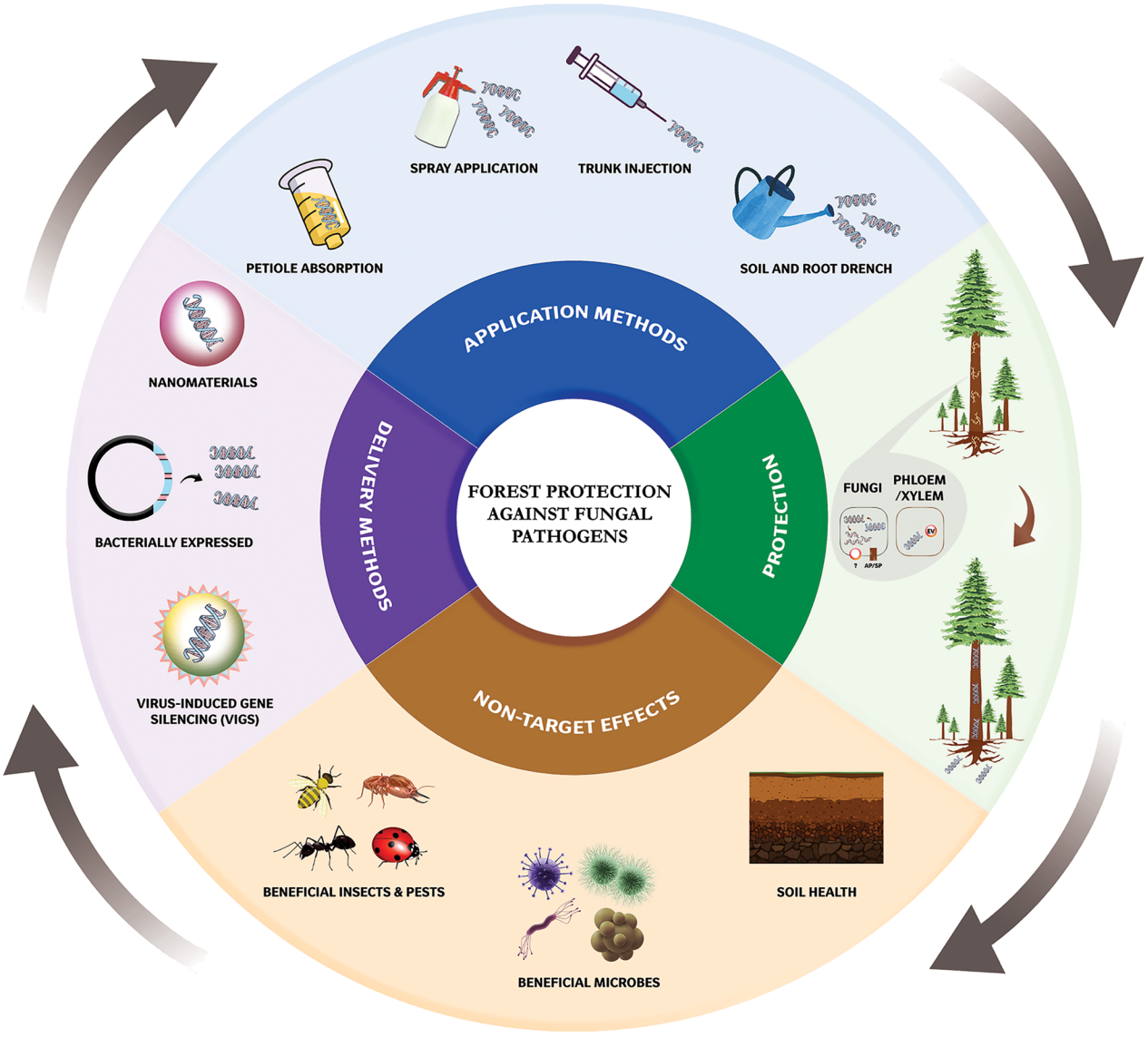

Figure 6. Proposed modes of dsRNA delivery and application methods for the woody plants against fungal pathogens. (A) Delivery methods: showing the economical delivery methods for large-scale production for environmental applications with enhanced stability *via* nano-coating. (B) Application methods: dsRNA application method for woody plant tissues through the root, stem, and leaves (petiole). (C) Tree protection: the dsRNA uptake in fungi cells can be directly from plant surface or through plasma membrane transporters [apoplast (AP) or symplast (SP)] and extracellular vesicles (EV) or some other mechanisms yet to be discovered. dsRNA processes through the RISC complex to degrade target genes from pathogenic fungi, aiding forest disease management. C). Non-target effects: the impact of dsRNA exposure on beneficial forest insects, pests, and soil microbes must be evaluated for developing RNAi-based fungal protection products.

verticillioides was produced, demonstrating the potential of HD-RNAi for disease control [184]. Despite the prevalence of dsRNA or siRNA translocation between species, how these RNAs move through extracellular spaces in different organisms is not fully understood. However, evidence supports the ability to transfer siRNA and dsRNA signals within or across different types of organisms, illuminating this phenomenon [99, 181, 183, 185]. In both fungi and plants, the uptake and transport of RNA molecules are primarily mediated by clathrin-mediated endocytosis (CME), involving the formation of clathrin-coated vesicles facilitating RNA uptake in both directions fungus-to-plant and plant-to-fungus. A recent study has shown that *B. cinerea* secretes siRNA *via* extracellular vesicles (EVs), which are internalized by plant cells through the CME pathway. These siRNAs hijack the plant's Argonaute protein 1 (AGO1) to suppress host immunity. Notably, mutations that disrupt this clathrin-mediated pathway in *Arabidopsis* enhance resistance to *B. cinerea* [186]. In contrast, dsRNA/siRNA uptake and transport in insects and nematodes are facilitated by transmembrane channels known as Systemic Interference Defective (SID) [187–189]. However, fungi lack SID channels, suggesting alternative mechanisms for dsRNA/siRNA transport, such as through EVs through CME, nutrient acquisition *via* specific transporters, passive diffusion across the cell wall, or other transmembrane channels [64, 190, 191]. For example, Cai et al. (2018a) demonstrated that siRNA was transferred from the *Arabidopsis* host plant to the necrotrophic fungus *B. cinerea via* extracellular vesicles, particularly exosomes [97]. HD-RNAi has been successfully applied for disease management in crops such as cotton [192], banana [154, 193], potato [125], maize [194, 195], wheat and barley [196], and rapeseed [129]. Nevertheless, the transgenic approach has some limitations in the agricultural and forestry sectors, such as transformation techniques (vary depending on the target crop or tree species), environmental safety, and societal acceptance of genetically modified organisms (GMOs) [197, 198]. Hence, an alternative approach to GMOs is of higher necessity for crop or tree disease management applications.

Non-transgenic-based approach

External application of dsRNA against target genes, also known as SIGS, is a novel emerging non-GMO approach that enhances resistance against pathogens. SIGS can provide safe and powerful plant protection against fungal pathogens by targeting genes vital to fungal pathogenicity [97, 143, 145, 148, 199]. Externally applied dsRNA translocates to fungal cells by bidirectional cross-kingdom RNAi, conferring plant disease resistance [111, 179, 200]. A key mediator of this process is EVs, which play a critical role in plant–microbe interactions by transporting dsRNA cargo between the plant host and fungal pathogens [191]. EVs, secreted through multivesicular bodies (MVBs), carry defence-related molecules, such as siRNAs and RNA-binding proteins (RBPs), that can suppress pathogen virulence. In *Arabidopsis*, various subtypes of EVs have been identified, each playing distinct roles in plant defence and inter-organism communication. Notably, TETRASPANIN (TET)-positive EVs, similar to mammalian exosomes, increase abundance during fungal infections such as *B. cinerea* [110]. Recent research has highlighted the importance of TETRASPANIN8 (TET8) in the transport of glycosyl inositol phosphoceramides (GIPCs) from the Golgi apparatus to the plasma membrane, functioning as a sphingolipid carrier. The deletion of TET8's C-terminal tail significantly disrupts GIPC transport and EV biogenesis [201]. Other EV subtypes, including PEN1-positive, EXPO-derived, autophagy-related EVs, and pollensomes, contribute to various processes, indicating a specialized system for EV-mediated functions in plants with unique biogenesis pathways, cargo, and transport [202, 203]. For instance, in several studies, sunflower (*Helianthus annuus*) EVs enriched with cell wall remodeling enzymes and defence proteins have been shown to inhibit the virulence of *S. sclerotiorum* [204]. Interestingly, no significant disease reduction or target gene silencing was observed when EVs were obtained from barley (*Hordeum vulgare*) sprayed with a dsRNA targeting the CYP51 gene in *F. graminearum* [205, 206]. Such findings indicate that the target specificity of the RNA-binding proteins [Argonaute 1 (AGO1), RNA helicases (RHs) and annexins (ANNs)] in plant EVs influences the outcome of host–plant interactions with pathogens [207]. Hence, the composition of RNA-binding proteins and their involvement in determining the outcome of plant–pathogen interactions through EVs must be explored case-by-case.

Furthermore, direct application of dsRNA on the surface of fruits, vegetables, and flowers significantly inhibits pathogens (e.g., *B. cinerea*) [111]. The high efficiency of RNAi was demonstrated in *Magnaporthe oryzae*, with a maximum uptake of dsRNA *via* the plant tissues and increased longevity of silencing efficiency when sprayed on the abrading surface compared to the regular surface [145]. Similarly, other research on the SIGS-biofungicide successfully demonstrated against phytopathogens, including: *F. graminearum* [139], *M. oryzae* [145], *Phytophthora infestans* [148], *B. cinerea* [208, 209], *Austropuccinia psidii,* and

Coleosporium plumerias [144]. Although SIGS has potential pathogen growth reduction, other factors negatively influence RNAi activity, like RNA stability and durability in the natural environment.

Enhancing forest protection: advantages of non-transgenic vs. transgenic RNAi

The non-transgenic spray-induced gene silencing (SIGS) approach presents several compelling advantages over traditional transgenic RNAi methods, particularly in the context of forest protection. Unlike transgenic RNAi, which involves genetic modifications of host plants to produce RNAi molecules, SIGS bypasses the need for genetic alterations, thus avoiding the complexities and regulatory challenges associated with GMOs [210–212]. This method allows for the direct application of dsRNA to various forest species, which is challenging to engineer genetically [213]. SIGS delivers rapid, on-demand protection against fungal pathogens, with observable effects shortly after application, which is critical for managing outbreaks and reducing damage. Additionally, SIGS is environmentally friendly; dsRNA naturally degrades without leaving environmental footprints, thus reducing the ecological impact compared to chemical fungicides, with unintended effects primarily confined to species closely related to the target or sharing the same niche [214]. The adaptability of SIGS facilitates responsive, tailored treatments that can be adjusted as new fungal threats emerge, offering a dynamic and sustainable strategy for managing forest diseases.

Challenges of dsRNA-based forest disease management strategy

Despite several studies, forest disease management is still challenging for many reasons, including inadequate coverage and inconsistent findings of specific management strategies to improve forest disease resistance. The research findings pinpoint numerous significant challenges that must be alleviated before developing RNAi-based solutions against forest fungal pathogens.

dsRNA delivery: trunk injection, encapsulation, and SIGS

Considering forest trees are complex, integrating dsRNA into them can be difficult. It might be challenging to ensure that the dsRNA/siRNA reaches the target cells or tissues inside the tree and is efficiently taken up. Trees have protective barriers, such as bark and waxy cuticles, that might inhibit dsRNA absorption [215]. The external application of dsRNA molecules through trunk injection has delivered the desired outcomes (Table 3) [220]. It demonstrated effective dsRNA delivery in citrus and grapevines by root drenches and stable injections for 57 days. Similarly, confocal microscopy studies displayed that the application of dsRNA *via* ash root and petiole absorption significantly improved emerald ash borer mortality, implying the ability of the plant vascular system for effective dsRNA transport [221]. Other hardwood tree roots, including white oak and loblolly pine, displayed dsRNA absorption and translocation in various tree tissues [222, 223]. A recent study highlighted the effectiveness of SIGS in managing *F. circinatum*, the pathogen responsible for Pine Pitch Canker disease [150]. However, these studies were not conducted on mature trees, demanding further experimental corroboration.

Furthermore, trunk injection in apple trees and grape vines reduced RNA accumulation at 3 and 10 days [177]. Nevertheless, the optimal dsRNA concentration and dosage must be calculated. A low dosage may not effectively reduce disease, but a high concentration may negatively affect tree physiology. Trunk injection in apple trees requires a higher concentration of dsRNA to be deemed physiologically active [177, 178]. For the preparation of dsRNAs using *in vitro* transcription techniques, commercial products (kits) are required. So far, commercial kits for dsRNA production have been extremely costly. Alternatively, bacterially produced dsRNA provides a more sustainable and cost-effective technique for large-scale

Table 3. External application of dsRNA in woody plants.

Trees studied	dsRNA application	Gene studied	dsRNA concentration	Outcome	Ref
Malus domestica *Malus pumila*	Trunk Injection	GFP & *T. urticae* gene	GFP-500ug *T. urticae* gene-1 g	Accumulation of dsRNA was improved.	[216, 217] [178]
Vitis vinifera	Trunk Injection/ petiole absorption	GFP	GFP-500ug	Accumulation of dsRNA was increased.	[177]
Citrus sinensis	Soil/Root Drench Application/Trunk Injection	Vitellogenin-A1-like (Vg) and Juvenile hormone acid O-methyltransferase-like (JHAMT)	1mg-200mg	Significant decrease in target gene expression.	[218] [219]
Pinus radiata	SIGS	FcVDS: FcPTP FcCHS	300 ng	Significant inhibition of the pathogen virulence	[150]

dsRNA manufacturing [209, 224–226]. The dsRNA delivery *via* tree-associated virus will have potential but may suffer public acceptance [227].

Several studies have demonstrated that using synthetic inorganic materials as carrier molecules with ion exchange abilities, biocompatibility, biodegradability, and high intracellular delivery efficacy increases RNAi efficiency [228]. Recently, the possibility of a nanobiotechnological approach to enhance RNAi efficiency was reviewed for long-term crop protection [229, 230]. For instance, the Bio-clay (layered double hydroxide-LDH)-coupled dsRNA complex enhanced RNA durability for up to four weeks on plants, prolonging its activity against *B. cinerea* [142]. A recent study on artificial nanovesicles (AVs) [cationic lipid formulations] demonstrated the extended duration of dsRNA protection [231]. Another nanocomplex, Chitosan/SPc complex (CSC), enhanced dsRNA uptake, stability, and longevity against *Rhizoctonia solani* [62]. Studies demonstrated that peptide complex (cell-penetrating peptide:dsRNA) targeting chloroplast cells rapidly translocated dsRNA and induced gene silencing [232]. It is worth mentioning here that the efficiency of dsRNA uptake differs between microorganisms and cell types [141]. Nevertheless, nanoparticle-coated dsRNA is protective from environmental conditions, host extracellular RNases, and other ribonucleases and increases dsRNA uptake, thereby providing more extended protection against pathogens, enhancing the feasibility of RNAi-based forest disease management (Figure 6). Several limitations must be acknowledged when considering the application of SIGS and trunk injection approaches against fungal pathogens. First, not all fungi possess the RNAi machinery necessary for RNA interference; for example, *Ustilago hordei* encodes this machinery, while *U. maydis* lacks RNAi components [233]. Second, the ability of fungi to take up exogenous dsRNA varies; *Colletotrichum truncatum* can internalize dsRNA [234], whereas *C. gloeosporioides* cannot [141]. Additionally, some fungi and oomycetes, such as *Phytophthora*, encode RNAi suppressors that can inhibit the RNAi process [235]. These factors should be carefully considered before employing RNAi-based strategies to combat fungal pathogens, as they could significantly limit the effectiveness of the approach.

Mycoviruses can also serve as carriers for dsRNA targeting pathogenic fungi. They transmit naturally throughout fungal populations through various processes, including hyphal anastomosis, spore transmission, and horizontal gene transfer [236, 237]. This natural dispersal ability can be exploited to deliver dsRNA to pathogenic fungi efficiently. Mycovirus research has also discovered numerous novel mycoviruses that can be explored during the development of dsRNA-mediated forest fungal pathogen management strategies [158]. Mycoviruses can be engineered to carry dsRNA sequences targeting specific genes in forest fungal pathogens. Upon infection of the fungal host, these dsRNA sequences can trigger RNAi machinery within the host, leading to the degradation of complementary RNA molecules and subsequent silencing of target gene expression [238]. A well-known example of hypoviruses, a specific group of mycoviruses, is *Cryphonectria hypovirus* 1 (CHV1), which successfully treat chestnut blight disease caused by *Cryphonectria parasitica* [25]. This inter-kingdom virus transmission approach associated with genetic elements (e.g., viral vectors) has immense potential for biotechnological applications in dsRNA delivery, and gene editing in fungi. It minimizes off-target effects for practical applications in fungal disease management, delivering two-tier specificity arising from host selectivity and dsRNA target specificity. However, the biosafety aspects and societal acceptability of this promising technology demand careful consideration and experimental corroboration.

Environmental fate: biosafety regulation for RNAi-based bio-fungicides

Requirement for regulatory approval: a key step for environmental application

The use of dsRNA in managing forest diseases must be accompanied by regulatory approval and monitoring. However, obtaining approval and overcoming environmental regulations may be time-consuming and expensive. It can differ across territories, principally accentuating the process or the end product due to associated risks and the need to manage toxicity levels to non-target organisms (NTOs) effectively [239, 240]. Biosafety guidelines for RNAi technology are distinct by country and location. According to the European Food Safety Authority (EFSA), transgene expression of siRNA/dsRNA in GM plants, known as transient RNAi, has become essential for addressing safety and ecological risk assessment strategies in the application of current regulatory systems [241–243]. No particular category exists for these dsRNA/siRNA-based products in Europe, and the same regulatory system that governs the licensing of traditional synthetic fungicides/pesticides also applies to RNAi-based products [244].

Environmental and safety considerations: highest priority

When considering the RNAi approach in forest disease management, it is essential to thoroughly assess

environmental and safety considerations to ensure effective and safe implementation. Regulatory authorities must evaluate information on dsRNA molecules, such as their intended target genes, method of action, possible off-target effects, and environmental persistence. Recent bioinformatic tools provide an excellent opportunity and information to screen the target gene and evaluate the plausible off-target effects [245–247]. Such information is vital to ensure the safe and responsible use of RNAi-based forest protection and address possible environmental and human health issues. Captivatingly, the RNAi technique does not always entail a transgenic expression of target dsRNA; deploying topically applied RNAi-based products or SIGS for managing tree fungal diseases may obtain higher public acceptance [248] [Box 2]. Regulatory outlines for topical RNAi-based products (SIGS) in agroforestry are still emerging worldwide. Despite this, massive research has been performed on topically applied RNAi-based bioproduct developments for their promising benefits and eco-friendliness. Spray-based dsRNA products offer benefits over commonly used crop protection methods using chemical pesticides. Even a mixture containing reduced amounts of pesticide and dsRNA formulation works well, decreasing the pesticide contents from the environment [249]. Nonetheless, the environmental fate and stability of the dsRNA in conjugates or naked form deployed in SIGS and their effect on beneficial soil or plant-associated symbiotic microorganisms must be assessed. Precisely, the external application of dsRNA molecules must undergo dedicated studies dealing with the environmental fate of dsRNA, degradation time, and uptake by the NTOs [250]. However, in order to improve dsRNA stability and efficiency, extra carrier molecules must stay in the environment for an extended period, which demands further research into their cytotoxicity and degradation [250–253]. To address off-target effects in RNAi, nanocarrier-wrapped dsRNA molecules must be carefully designed and selected, assuring specificity and avoiding sequence similarity with undesired targets [254]. These siRNA/dsRNA molecules may have off-target effects on NTOs, potentially interrupting their regular biological functions. A minimal level of sequence homology is associated with the specificity of siRNAs for a specific mRNA. According to previous findings [255], precise sequence similarity between an mRNA and the dsRNA typically leads to suppression of the targeted mRNA. However, the degree of phenotypic repression varies depending on the mRNA targeted. The expression of the two target genes, *V-ATPase A* and *actin-2*, which exhibited the highest degree of similarity (17–21 nucleotide matches) when exposed to *Euschistus heros*-specific dsRNA in parasitoid wasp *Telenomus podisi*, was observed and was shown to be unaffected by the *E. heros*-specific dsRNA [256]. Despite the high variability in sequence between the two compounds, it is nonetheless possible to silence a gene [257]. While dsRNA requires to be transformed into siRNA to initiate RNAi, their off-targets require varying amounts of consecutive matching nucleotides to occur in sequence. It is crucial to evaluate the types of siRNA that can be formed from a particular dsRNA and their putative target assessment to avoid potential risks [245]. These risks include sequence-specific effects, such as unintended gene silencing, and sequence-unspecific impacts, such as immune system stimulation, RNAi core machinery saturation, and epigenetic modifications [258–260]. For instance, dsRNA may trigger immune responses in NTOs, negatively affecting their fitness and leading to broader ecological consequences. In addition, introducing external dsRNA could saturate the endogenous RNAi core machinery, impairing miRNA-mediated gene regulation, which is critical for proper development and physiological functions [260]. Studies have also demonstrated that dsRNA can induce RNA-directed DNA methylation (RdDM), potentially leading to the *de novo* methylation of cytosines in various sequence contexts (CG, CHG, CHH), as well as histone modifications. Notably, these epigenetic changes may be heritable, with DNA methylation being trans-generationally maintained in the progeny, even in the absence of the original dsRNA trigger [258, 261]. Therefore, bioinformatic criteria used for dsRNA design should account for the possibility of inducing such unintended epigenetic effects. Various computational techniques and algorithms [dsCheck (http://dscheck.rnai.jp/), Genome-wide Enrichment of Seed Sequence matches (GESS) (https://www.flyrnai.org/gess/), siDirect 2.0 (http://siDirect2.RNAi.jp/)] are available to anticipate potential off-target effects and help optimize RNAi molecule design by analyzing the siRNA species that can be derived from a particular dsRNA sequence [262, 263]. However, despite these *in silico* approaches, experimental validation and extensive characterization are required to confirm the specificity of RNAi compounds and reduce the risk of off-target effects on NTOs [264, 265]. The possible dsRNA side-effects on other beneficial tree microbiota (i.e., ectomycorrhizal fungal mutualists) in forest habitats (i.e., soil environment), which play a significant role in ecosystems, must be considered when employing dsRNA-based management mechanisms [266]. Dedicated bioassay studies evaluating off-target effects on NTOs must be conducted case-to-case [221, 260, 267, 268].

Furthermore, to enhance the efficacy and uptake of dsRNA fungicides, various formulations have been explored, including LDH nanoclays, chitosan,

polyethyleneimine (PEI), star polycations, artificial nanovesicles (AVs) [cationic lipid formulations], and peptide complex (cell-penetrating peptide:dsRNA) [62, 142, 231, 232]. These formulations can improve the stability of dsRNA in the environment and facilitate cellular uptake in target organisms. However, while these enhancements may increase RNAi efficiency, they also raise important risk assessment considerations. For instance, increased stability could lead to prolonged environmental persistence, raising the potential for unintended interactions with NTOs [230, 269]. Likewise, improved uptake mechanisms might inadvertently enhance the exposure of NTOs to dsRNA, heightening the risk of off-target effects. Therefore, a balanced evaluation of both the benefits and risks associated with these formulations is essential for the sensible application of dsRNA-based fungicides.

The ecological risk can also be assessed by tracking the fate of dsRNA molecules in the forest soil environment. Interestingly, microorganisms can degrade dsRNAs by in-house ribonucleases [270]. Furthermore, regardless of texture, pH, clay content, or other soil characteristics, studies demonstrated that soil extracts spiked with dsRNA (0.3–37.5 µg/g) had degraded the dsRNA and corresponding biological activity was undetectable after two days following application to soil [271]. In addition, two dsRNAs against identical sequences with distinct lengths (100 bp and 968 bp) and structures (linear and hairpin) were found to have indistinguishable kinetics and degraded quickly in the soil environment, suggesting that dsRNA size, structure, and sequence do not appear to influence degradation kinetics [272]. Considering the costs and benefits of RNAi-based products in contrast to other commercial fungicides requires investigating the relative degradation rates of these compounds in diverse environments on a case-to-case basis [273]. Since developing new fungicides takes a long time and significant financial resources, caution must also be taken to prevent the emergence of fungicide resistance [274]. Mutations in the target sequence can lead to RNAi resistance, particularly when the RNAi fungicide consists of a single 20-nt siRNA. The likelihood of resistance development needs to be evaluated, and mitigation strategies must be formulated before applying RNAi-based products against forest fungal pathogens [275, 276].

Societal dilemma with adopting molecular tools (i.e., RNAi) and their environmental applications

Currently, our society is not fully open to accepting molecular biological products. After the COVID-19 pandemic, the situation worsened a bit. To promote public acceptance of RNAi-based strategies for controlling forest diseases, researchers and foresters should actively engage with state forest enterprises or individual forest owners or stakeholders to address any confusion and concerns through direct communication and cooperation [277, 278]. In order to precisely represent the trade-offs that society is ready to accept and adopt new generations of fungicides, it is crucial to integrate their views on risks and benefits [279]. Reduction of pesticide use and long-term environmental benefits, species-specificity, biosafety, and cost efficiency might be the key factors that make society accept RNAi-based strategies. We may be motivated to trust and adopt new technologies if we are well-informed about them and their precise mode of action [280]. Developing communication plans to increase the societal acceptability of RNAi-based strategies is fundamental. The societal acceptability of SIGS might open new avenues for RNAi-based forest pathogen management practices [74] [Box 2].

Future prospects and concluding remarks

According to recent discoveries, the exchange of RNA-silencing signals *via* RNA cross-kingdom communication in different plant systems underlines the importance of mobile RNA as a crucial regulatory molecule modulating host-fungal pathogen relationships. Numerous plant EVs have been utilized to deliver siRNA/dsRNA to fungus; however, how fungi uptake plant EVs is still unknown. Further studies on the processes of siRNA/dsRNA translocation are obligatory. Nonetheless, several SIGS or related studies have exposed that plants can absorb and often distribute dsRNA systemically [221, 281]. However, the duration for which dsRNA/siRNA might persist on the forest trees has yet to be evaluated. Other exogenous approaches that might be tested in forests include trunk injection and root/soil adsorption. Addressing public concern about RNAi-based products and forest environmental impact is crucial. Any possible short or long-term impacts on the forest environment, including interactions with other animals/beneficial insects (i.e., pollinators)/microorganisms (i.e., soil microorganisms) and their ecological consequences, must be experimentally evaluated on a case basis. While dsRNA-based forest disease management strategies imply promise, resolving many obstacles is essential for successful deployment (Figure 6). An approach based on scientific parameters to validate RNAi-biofungicides is elementary to preparing the most appropriate risk assessment protocols [282].

Acknowledgements

The "EXTEMIT-K," No. CZ.02.1.01/0.0/0.0/15_003/0000433 and "EVA 4.0", No. CZ.02.1.01/0.0/0.0/16_019/0000803 financed by OP RDE funded AR, AC and GS. Additional financial support was obtained from the Excellent team grants to AR and AC from FLD and CZU. SS obtained support from the Internal Grant Agency (IGA, 2024-2025) FLD, CZU. R.R.V. acknowledges support from FORMAS (2019-01316), NOVO Nordisk Foundation (0074727), SLU Centre for Biological Control, and The Crafoord Foundation. GS also acknowledges COST Action (CA20110) Grant (E-COST_GRANT-CA20110-0721b157) for supporting short term training at Prof. Lionel Navarro lab in Paris, which helped in understanding some of the concepts presented in this review article. The authors would also acknowledge the anonymous peer reviewers who gave valuable comments on the earlier versions of the manuscript. Figures 1 and 3–6 were prepared using biorender.com.

Authors' contributions

AR and GS conceived the idea. GS prepared the first draft of the manuscript. GS, SS and AR prepared the figure. GS prepared the tables. AR, AC and RRV thoroughly edited the manuscript. All authors have read and approved the final manuscript.

Glossary

RNA interference: RNA interference (RNAi) is a biological process that involves silencing gene expression by degradation or inhibiting the translation of specific messenger RNA (mRNA).

Bio-fungicides: Bio-fungicides are biological compounds such as plant extracts or naturally occurring microorganisms employed to manage fungal diseases.

Pathogen outbreaks: Pathogen outbreaks refer to sudden increases in the occurrence of infectious diseases caused by microbes within a specific population, geographic area, or ecosystem.

Argonaute: Argonaute (Ago or QDE2) proteins are a class of proteins found in eukaryotic organisms that typically contain conserved domains, including PAZ (Piwi-Argonaute-Zwille), MID (middle), and PIWI (P-element-induced wimpy testis) domains that play a central role in the RNAi.

RNA-dependent RNA polymerase: RNA-dependent RNA polymerase (RdRP) belongs to the larger class of enzymes called template-directed nucleic acid polymerases that catalyze the synthesis of RNA molecules using an RNA template.

DNA-dependent RNA polymerase: DNA-dependent RNA polymerase (DdRP) is a complex multisubunit RNA polymerase and essential for the synthesis of RNA molecules using a DNA template.

Dicer: Dicer (Dcr) is a type III ribonuclease widely recognized as a key enzyme in the RNAi pathway. The primary function of Dicer is to cleave long dsRNA molecules, or precursor RNA molecules with stem-loop structures, into shorter fragments (20–25 bp). These small RNA fragments, generated by Dicer, serve as guides for the RNA-induced silencing complex (RISC).

Host-induced gene silencing: Host-induced gene silencing (HIGS) is genetically modified to express double-stranded RNA (dsRNA) molecules targeting specific genes of invading pathogens by harnessing RNAi mechanisms.

Spray-induced gene silencing: Spray-induced gene silencing (SIGS) is non-transformative and involves the application of dsRNA molecules directly onto plant surfaces to induce RNA interference (RNAi) in target pests or pathogens.

Virus-induced gene silencing: Using viral vectors containing a target gene fragment, virus-induced gene silencing (VIGS) generates dsRNA that initiates RNA-mediated gene silence. It is one of the reverse genetics methods for gene function research.

Cross-kingdom RNA interference: Cross-kingdom target genes are silenced by RNA interference (RNAi) processes, which are caused by the transfer of RNA molecules (siRNAs or miRNAs) between organisms belonging to different groups (e.g., plants to pathogens and vice versa).

Virulence: Pathogenicity, or the ability of microbes to cause disease, is strongly associated with virulence. Highly pathogenic microbes have a greater potential to cause severe illness or death in infected hosts.

Extracellular vesicles: The tiny, membrane-bound vesicles cells produce in the extracellular space are called extracellular vesicles (EVs). Exosomes, microvesicles, and apoptotic bodies are examples of classical EVs. These entities are distinguished by their formation and are crucial for intercellular communication, cell signaling, and the translocation of biomolecules between cells.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

We acknowledge funding for *Open Access publishing facilitated by Czech University of Life Sciences Prague (CZU)/Ceska Zemedelska Univerzita v Praze, as part of the Taylor & Francis - CzechELib Transformative Agreements*.

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