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On the road with RNA interference: Missed stops and future directions in leveraging spray-induced gene silencing for sustainable fungal management in agriculture

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Spray-induced gene silencing (SIGS) has moved from proof-of-concept to one of the most compelling RNA-based routes for crop protection, yet its field deployment still rests on unresolved biological interfaces. In this review, we revisit SIGS against fungal pathogens by asking where exogenous double-stranded RNAs go, which organisms perceive them, and how delivery technologies may reshape their ecological footprint. We first examine double-stranded RNA (dsRNA) uptake as a limiting and still unevenly understood step: clathrin-mediated endocytosis is emerging as a recurrent entry route in fungi, whereas plant perception, transport across surface barriers, and systemic movement remain mechanistically obscure. We then discuss evidence that dsRNAs are not inert silencing triggers. Beyond sequence-specific RNA interference, they can activate pathogen-associated molecular pattern (PAMP)-like and stress–response pathways, including fungal high osmolarity glycerol (HOG) signaling, implying that dose, formulation, and exposure context may influence both efficacy and nontarget effects. This perspective is extended to the plant holobiont, where direct off-target silencing and indirect microbiome remodeling represent distinct but often conflated risk layers, particularly for endophytic and beneficial fungi exposed through systemic RNAi. Finally, we evaluate how nanocarriers, BioClay, chitosan particles, and artificial vesicles are being developed to protect dsRNAs from degradation, improve uptake and enable more precise delivery. We argue that the next phase of SIGS research must integrate molecular uptake biology, concentration-aware risk assessment, and scalable formulation design. Such integration will determine whether SIGS becomes merely another promising laboratory technology or a robust, ecologically informed platform for sustainable fungal disease management in agriculture.

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Introduction

RNA interference (RNAi) is a post-transcriptional gene silencing mechanism specific to eukaryotic cells first described by Fire et al., 1998 [1]. The mechanism involves the recognition of double-stranded RNA (dsRNA), which then regulates gene expression by degrading mRNA homologous to the dsRNA sequence. Over the years, RNAi has been studied for diverse agricultural purposes, especially in plant pest and pathogen management [2]. Two distinct RNAi-based strategies have been developed for plant protection: Host-induced gene silencing (HIGS), which relies on the transgenic expression of dsRNA within the plant to target pathogen genes, and spray-induced gene silencing (SIGS), which involves the exogenous application of dsRNA onto plant surfaces [3,4]. While HIGS requires stable genetic transformation, SIGS represents a non-transgenic alternative that enables transient gene silencing in pathogens through environmental RNA uptake [5].

SIGS relies on the topical application of dsRNA onto leaf surfaces, which then follows two distinct pathways: absorption by the plant or direct uptake by the pathogen. In the former case, the dsRNA has the potential to be

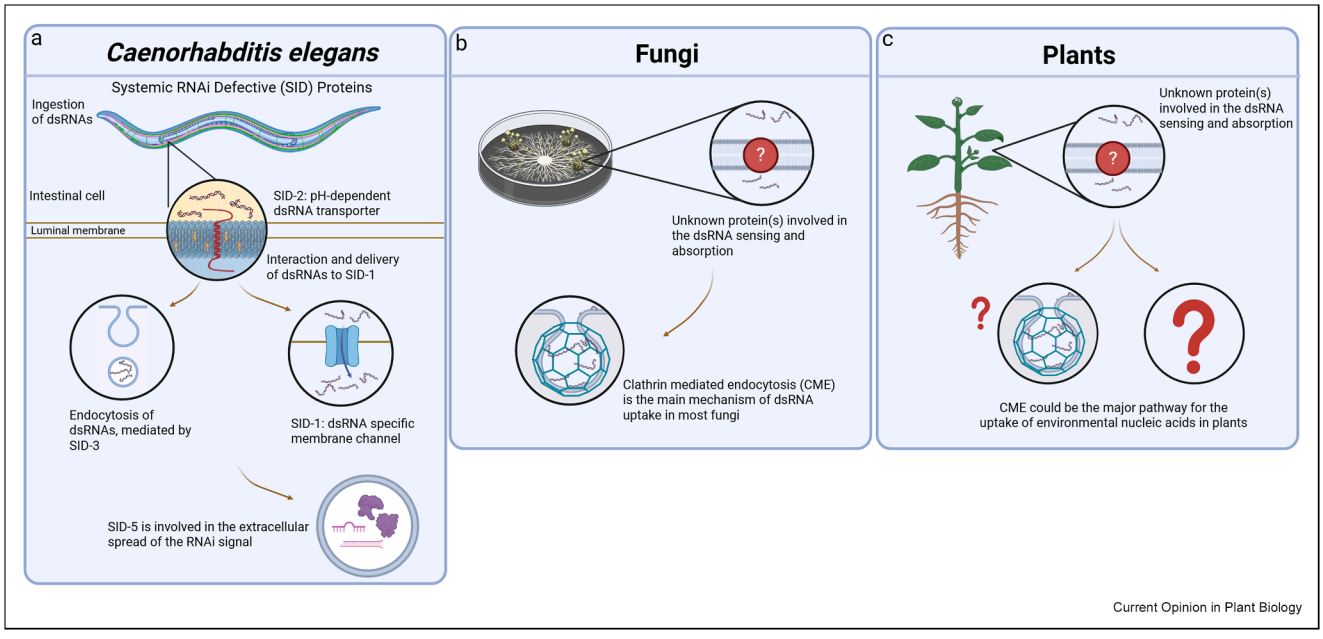
processed by the plant's RNAi machinery and translocated through the tissues, conferring systemic protection through a mechanism known as systemic RNAi [6–8]. This is particularly significant during infection, as the exchange of cellular material allows the host-derived RNAi signals to be transferred to the pathogen, resulting in direct gene silencing [9,10]. Alternatively, the pathogen may absorb the dsRNA directly from the leaf surface, triggering its own endogenous silencing pathways. In laboratory and greenhouse trials, SIGS applications efficiently manage fungal infections, which significantly contribute to crop losses in terms of quality and yield [11–14].

Unlike synthetic chemical methods, SIGS offers lower toxicity while maintaining lower fungicide resistance risks, although emerging evidence suggests that certain resistance mechanisms may arise [15–17]. SIGS is sequence-specific, and pathogens would need multiple mutations to develop resistance and avoid silencing [18]. Additionally, SIGS often targets pathogen essential genes for growth or virulence, challenging the ability to develop resistance without affecting pathogen survival or virulence. Consequently, factors such as dsRNA stability and the absorption efficiency in both plant and pathogenic fungi are critical determinants of SIGS performance [19,20]. Recent advances involve the formation of nanoparticle-RNAs complexes which can parallelly improve dsRNA stability while increasing absorption by the organisms [21,22]. While offering a more sustainable alternative to other chemical fungicides, SIGS presents potential risks to non-target organisms (NTOs), including other pathogens and beneficial members of the holobiont. In this review, we explore these critical aspects of SIGS in detail, addressing recent advancements in SIGS pathways, and synthesizing the most significant discoveries in the field.

Missing pieces of the puzzle: Cellular uptake of double-stranded RNA and non-canonical pathways

The absorption pathway of exogenous dsRNAs remains one of the most elusive aspects of RNAi, with most of the current knowledge derived from studies on nematodes and insects. Specifically, this absorption mechanism is fairly well characterized in *Caenorhabditis elegans* and comprehensively reviewed by Chen et al., 2021 [23] (Figure 1). SID-2, a single-pass transmembrane protein, senses the dsRNAs and delivers them to SID-1 or to the endocytosis pathway mediated by SID-3. SID-1 represents a dsRNA-specialized membrane channel that allows the dsRNA to escape the endosome and enter the cytoplasm, while SID-3 is a kinases ortholog protein expressed ubiquitously in most tissue types (Figure 1). Once the RNAi machinery is activated, a single

Figure 1



Sensing and uptake of dsRNAs. The events preceding cellular uptake of dsRNAs remain largely unknown. In recent years, research has focused on the mechanisms of dsRNA sensing and uptake in *Caenorhabditis elegans* **(a)**, leading to the identification of the systemic RNA interference defective (SID) protein family. SID-1 functions as a dsRNA-specific membrane channel, while SID-2 is thought to mediate uptake through direct interaction and delivery of dsRNAs to SID-1 or via endocytosis. SID-1 subsequently facilitates the release of dsRNAs from endosomes into the cytoplasm. Additionally, SID-3, like SID-1, is essential for efficient dsRNAs import, and SID-5 is implicated in the extracellular spread of the RNA interference (RNAi) signal. In fungi **(b)** and plants **(c)**, the proteins responsible for the sensing and uptake of dsRNAs remain largely unknown. Only recently, a few studies identified a potential role for clathrin-mediated endocytosis in the internalization of dsRNAs in fungi, and possibly in plants as well. dsRNA, double-stranded RNA.

transmembrane domain protein, SID-5, supports the packaging of dsRNAs into exosomes for cell-to-cell export [23].

In insects and fungi, the dsRNA absorption pathway involves the clathrin-mediated endocytosis (CME) pathway; however, the full regulation mechanism is yet to be discovered [24–28]. In *Sclerotinia sclerotium,* the hyphal tip was identified as the most active region for uptake [28]. Liu et al., 2025 [29] addressed the same pathway in *Verticillium dahliae*. By labeling both single-stranded RNAs (ssRNAs) and dsRNAs with fluorescent markers, they assessed the active import of RNAs into fungal cells. Using chemical inhibitors and knockout of endocytosis-related genes, they confirmed CME as the major importing mechanism for RNAs.

Similarly, the understanding of the dsRNA uptake in plants remains largely unexplored. One of the main challenges is addressing the role of the different barriers (*i.e.*, cuticle, cell wall, and cell membrane) that the dsRNAs must surpass before entering the cytoplasm [30]. Plant cell sensitivity to dsRNAs uptake varies significantly across species. It is well recognized that the presence of peptide-based compounds, such as flg22 in BY2 tobacco cell culture, can enhance dsRNAs uptake [31]. flg22 is a 22 amino-acid-long derived peptide of bacteria flagella that is recognized by the plant as a pathogen-associated molecular pattern (PAMP) and, as such, can induce endocytosis and increase dsRNA uptake [30]. It is probable that flg22 increases dsRNA absorption by activating the receptors of the plant cells mimicking a response to a pathogen attack. This idea is corroborated by the fact that dsRNAs can be perceived by the plant as PAMPs and activate the immune system [32].

This aligns with recent discoveries by Zheng et al., 2025 [33], which reveal that dsRNA not only triggers fungal RNAi, but also activates fungal stress response pathways. Indeed, dsRNAs exert a PAMP-like effect in the fungus, activating the high-osmolarity glycerol (HOG) pathway that regulates fungal development, virulence, and pathogenesis [34]. This stress response was also demonstrated to be independent of dsRNA sequence or length but dependent on high dsRNA concentrations [33]. He et al., 2023 [35] provided the first evidence in *Arabidopsis* that vesicle-mediated fungal small-RNAs can enter plant cells via CME, thereby suggesting CME as a major pathway for the uptake of environmental nucleic acids in plants. This groundbreaking discovery opens new avenues for future research, particularly in understanding the cross-kingdom exchange of small RNAs and dsRNAs. While the mechanisms underlying dsRNA sensing and uptake, particularly in plants, remain elusive, there is a better understanding of the molecular canonical pathway of RNAi within a cell once dsRNA is absorbed [36,37].

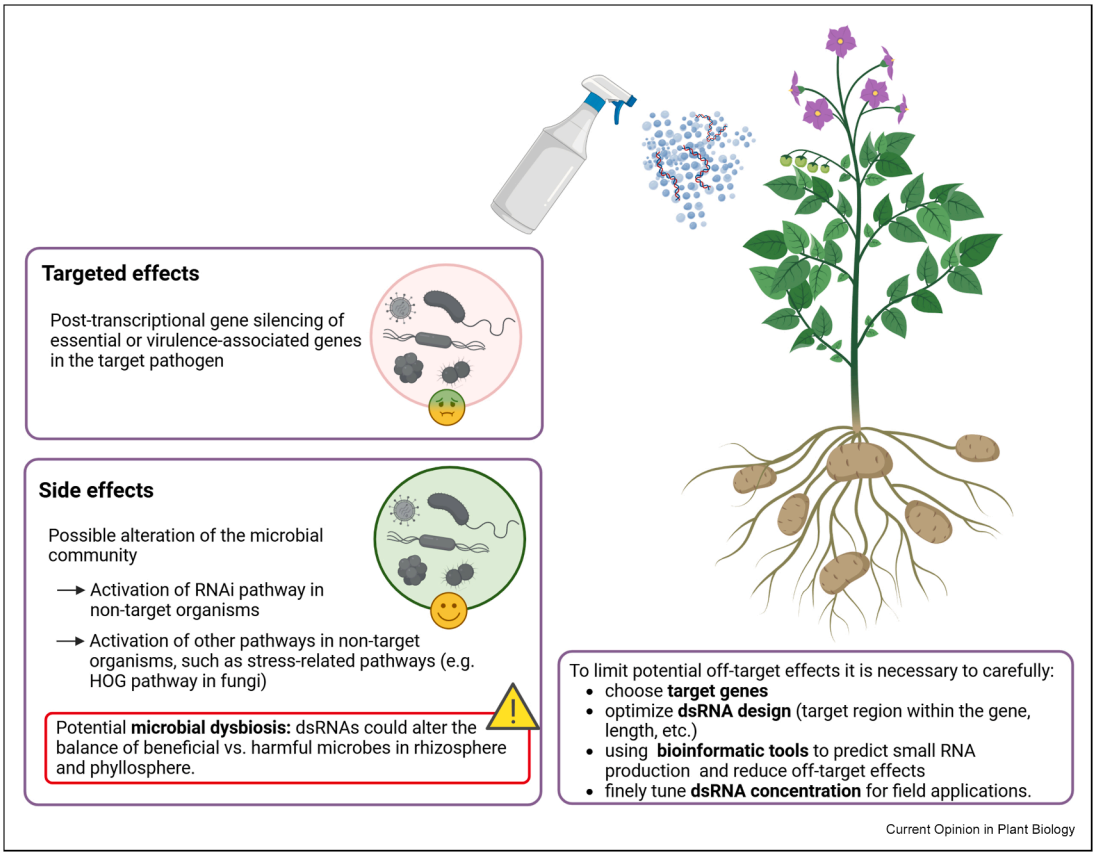
Silent casualties: The impact on non-target organisms

Some fungi can absorb RNAs from the environment, but uptake efficiency varies [38]. Well-studied fungal pathogens like *Botrytis cinerea*, *S. sclerotium*, *Rhizoctonia solani*, *Aspergillus niger* and *V. dahliae* exhibit strong dsRNA uptake, whereas others, such as *Colletotrichum gloeosporioides*, show no RNA uptake, and only minimal uptake occurs in the nonpathogenic fungus *Trichoderma virens* [19]. This uptake efficiency can strongly influence one of the most critical aspects of RNAi application in agriculture, namely its potential impact on non-target organisms, particularly beneficial microbes that contribute to plant health (Figure 2). It is worth noting that in SIGS applications, the ability of both pathogenic and beneficial fungi to absorb dsRNA can influence silencing efficacy. However, since most beneficial microorganisms reside in the soil and within the plant, they are less likely to be directly affected by SIGS. On the other hand, the systemic RNAi cascade in plants may inadvertently lead to unintended gene silencing in beneficial fungi within the holobiont [39,40].

An experimental evaluation of off-target effects confirmed that *Botrytis cinerea*-specific dsRNAs targeting the *BcBmp1* gene (a Fus3/Kss1-type mitogen-activated protein) did not affect the growth of *Trichoderma harzianum* or *Fusarium oxysporum* [40]. More recently, Nerva et al., 2024 [41] evaluated off-target effects following SIGS applications on woody tissue, comparing the impacts of pathogen-specific dsRNAs and non-specific GFP-dsRNAs on both target and non-target fungal species. This study demonstrated that sequence-specific dsRNAs significantly reduced the prevalence of target fungal pathogens in grapevine, with variations depending on plant genotypic features. Notably, it was shown that dsRNAs, regardless of sequence specificity, substantially impacted both fungal and bacterial communities, suggesting that even highly specific dsRNAs may have indirect effects on the microbial population.

This observation may be linked to earlier findings that dsRNAs can trigger broad, non-specific responses in both fungi and plants, potentially leading to the activation of diverse biosynthetic pathways, such as the production of defense-related metabolites in plants, which in turn could alter the surrounding microbial communities [33]. This interpretation provides a plausible explanation for the observed indirect effects, a relevant aspect that, to our knowledge, has not been previously addressed in the literature. While protecting plants from target pathogens is essential, it is equally important to assess the indirect effects on host-associated microbiome dynamics, which warrant further investigation. Indeed, most of the current research is centered on the direct off-target effects of

Figure 2



Direct and indirect effects of dsRNAs on the plant-associated microbial population. One of the most underexplored aspects of RNA interference (RNAi) technology is the potential for off-target effects on non-target organisms in the surrounding environment. While numerous studies have investigated the efficacy of dsRNA treatments against target pathogens, only a limited number have addressed their impact on non-target microorganisms, thereby raising concerns about unintended ecological consequences. It has been demonstrated that dsRNA applications can not only trigger RNAi pathways in non-target organisms, but may also activate stress-related responses, leading to microbiome alteration and possibly function. This disruption is particularly concerning, as it may not only alter microbial populations, but also impair the plant's capability to absorb nutrients, increase plant susceptibility to other pathogens, and disrupt beneficial symbiotic relationships. As spray-induced gene silencing (SIGS) strategies move toward large-scale applications, further research is essential to elucidate the specific effects of dsRNAs on non-target species and to ensure their safe use in agricultural ecosystems. dsRNA, double-stranded RNA.

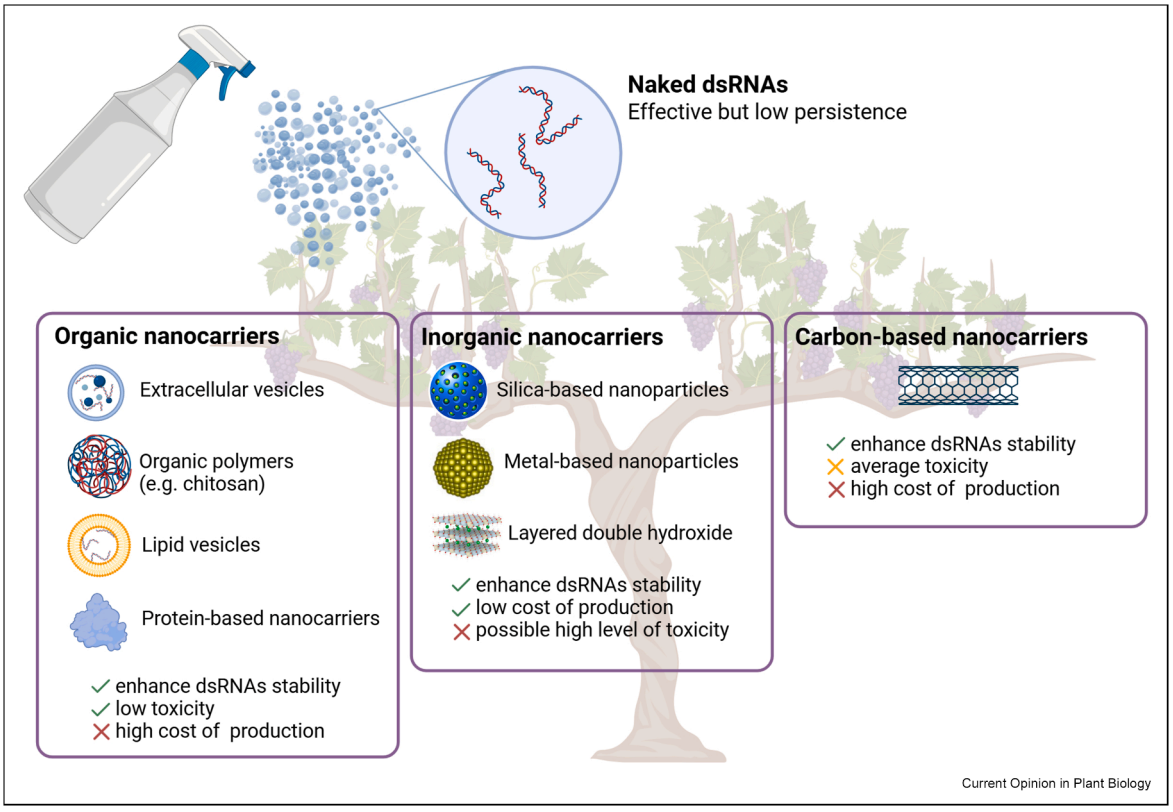
the SIGS treatments, overlooking the potential for indirect off-target effects, which represent a significant gap in our understanding. However, we contend that uncovering these mechanisms is not only important but also essential as it may reveal critical insights into the broader ecological implications of dsRNA-based approaches.

Improving spray-induced gene silencing efficiency through double-stranded RNAs formulation and encapsulation

The success of SIGS relies not only on molecular design but also on environmental stability and pathogen uptake. While Mosquera et al., 2025 [20] emphasize that future design efforts must prioritize highly specific dsRNA sequences to maximize silencing efficiency at minimal concentrations, these structural advances must be paired with innovative delivery methods to replace traditional agrochemicals. Consequently, researchers are turning to advanced carriers, such as nanoparticles and lipid-based vesicles to shield dsRNA from degradation and enhance delivery efficiency [42–44] (Figure 3).

Recent studies highlight the role of extracellular vesicles (EVs) in RNA transport, not only within plant cells but also across kingdoms, facilitating targeted gene silencing [45]. Extracellular vesicle-mediated RNAi (EV-RNAi) is emerging as a promising and environmentally friendly approach for plant protection [46]. Artificial nanovesicles (AVs) further improve RNA stability by shielding from degradation and enhancing delivery to target cells. These vesicles can be engineered to mimic natural EVs, allowing precise targeting of

Figure 3



Stabilization of dsRNAs. To enhance the delivery of double-stranded RNAs (dsRNAs), particularly in organisms where uptake mechanisms remain poorly understood, various strategies have been explored to stabilize naked dsRNAs. These stabilization approaches generally involve encapsulation and can be classified into three main categories based on the chemical composition of the nanoparticles: organic, inorganic, and carbon-based nanocarriers. Among these, organic nanocarriers are particularly promising due to their low toxicity and minimal environmental impact. However, current methods for their isolation and cargo loading are often labor-intensive and low-efficient, requiring further optimization. While these nanocarrier systems hold considerable potential for RNAi-based plant protection, the development of cost-effective, scalable, and straightforward production processes is essential to facilitate the broader implementation of spray-induced gene silencing (SIGS) applications in agricultural settings. RNAi, RNA interference.

pathogens. For example, Qiao et al., 2023 [44] synthesized artificial nanovesicles using cationic lipid formulations for antifungal RNAi applications against *B. cinerea*, extending dsRNAs protection to 10 days in tomatoes and grape berries and 21 days on grape leaves. However, challenges remain, including efficient RNA loading into vesicles and large-scale production. Further research is needed to optimize RNA loading techniques and enhance scalability. Alternative approaches to nanocarriers for RNA molecules include BioClay, which incorporates layered double hydroxide (LDH) particles to achieve controlled dsRNA release [47], and various nanoparticles, such as chitosan [48], alginate-chitosan nanoparticles [33], carbon quantum dots (CQD) [49], lipofectamine [50,51], and liposomes [52].

While nanoparticle-based applications have yet to be fully validated in agricultural settings, the techniques described show significant promise. By tailoring these methods to specific agronomic contexts, they offer new pathways for sustainable crop protection. Some of these techniques show considerable promise. For instance, companies such as Monsanto/Bayer are developing product lines like BioDirect, which utilize dsRNAs targeting *D. virgifera*. However, detailed information regarding the specific formulation strategies, particularly the integration of siRNA/dsRNA with nanomaterials or co-formulants to enhance plant uptake and RNAi efficacy, remains largely unavailable in the public domain [53]. The involvement of major agricultural biotechnology companies nevertheless highlights the recognized potential of encapsulation-based delivery strategies and further underscores their relevance and prospective viability for large-scale agricultural implementation. A commercially available and approved (in a few countries) product is Calantha, made by

GreenLight Biosciences. This is a bio-insecticide based on RNAi that contains Ledprona with dsPSMB5 as an active ingredient against Colorado potato beetle *Leptinotarsa decemlineata*. It is formulated as a simple, aqueous-based solution without cationic polymers. Although specific formulation details are not disclosed, the product is designed to ensure a shelf life of up to two years within the distribution channel, allow for uniform application, facilitate ease of handling and mixing, and maintain the efficacy of Ledprona [54]. Products of this nature present new opportunities in the agricultural sector, providing additional tools for combating plant pathogens.

Conclusions

SIGS has emerged as a promising and sustainable strategy in modern agriculture, offering an effective and environmentally friendly alternative to conventional chemical fungicides for plant pathogen control. By minimizing environmental impacts and reducing the likelihood of resistance development, SIGS potentially represents a transformative shift in crop protection approaches. A critical unresolved issue limiting the potential application of SIGS application, is the attainment of a comprehensive understanding of the sensing and absorption mechanism of dsRNAs. While partial insights have been gained regarding fungal absorption pathways, these mechanisms remain largely unknown in plants. Our limited understanding of how plants absorb environmental dsRNAs and our partial knowledge of this process in fungi severely restricts the direct application of RNAi against phyllosphere pathogens. This knowledge gap also hinders the potential use of SIGS for targeting wood-inhabiting pathogens via systemic RNAi. Most of the current research is mainly focused on conjugating, combining, or encapsulating dsRNAs with diverse materials, organic (vesicles, chitosan) and inorganic (clays, etc.). We believe that focusing research on elucidating the molecular mechanisms of dsRNAs absorption, including the involved pathways and receptors, will readily translate this fundamental understanding into more precise and efficient encapsulation techniques for field applications.

While the issue of non-target organism effects has been raised by several researchers, only a few studies have specifically assessed this in fungi. The recent discovery that dsRNAs can activate the fungal HOG pathway underscores the potential for unintended effects at high dsRNA concentrations. Fungi appear to be the most susceptible plant-associated microorganisms to this effect, as no RNAi pathways have been identified in prokaryotes to date. However, the complex topic of non-target organisms is intrinsically wide-ranging. It encompasses organisms that can be impacted from the soil or the environment, as well as those that can be affected by the SIGS effect through systemic RNAi within the plant, such as endophytes. It is evident that important aspects of SIGS and RNAi, particularly concerning their practical application in agriculture, still require greater attention and deeper investigation by the scientific community. Addressing these aspects represents a crucial step towards the successful and sustainable agricultural application of SIGS in the future.

Declaration of competing interest

The authors declare that they have no known competing interests.

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Data availability

No data was used for the research described in the article.

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** of outstanding interest

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The authors provided a comprehensive overview of spray-induced gene silencing (SIGS) for the control of plant pathogenic fungi, elucidating the more complex aspects of its application. Their thorough analysis is expected to inform and support future research in this field by highlighting both the potential benefits and the limitations of this technique, thereby promoting its effective and responsible use.

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For the first time, the authors identified a sequence-nonspecific, PAMP-like effect of dsRNA in Magnaporthe oryzae, providing a potential explanation for a major unresolved question in the field of dsRNA applications in fungi. Previous studies have highlighted the variability in treatment efficacy, suggesting the involvement of additional mechanisms, though without clarifying their nature. This study reveals that dsRNA not only activates RNA interference (RNAi) but also induces the canonical fungal stress response pathway, high osmolarity glycerol (HOG), thereby emphasizing the importance of dsRNA concentration in eliciting these effects.

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In their study, the authors interestingly investigated whether their Botrytis cinerea-specific dsRNA could also affect other fungal organisms, including beneficial species such as Trichoderma harzianum and pathogenic ones like Fusarium oxysporum. Through this approach, they demonstrated both the efficacy of their treatment and its selectivity, showing that it did not disrupt the surrounding fungal community balance.

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The authors combined grafting with the application of double-stranded RNAs (dsRNAs) to enhance plant resistance to fungal pathogens. They demonstrated that the scion genotype modulates the rootstock's ability to respond to fungal inoculation and also influences the rootstock-associated microbiota, leading to changes in the fungal community composition. While confirming the effectiveness of dsRNAs against target fungal species, they also revealed, for the first time, alterations in non-target microorganisms—highlighting critical considerations that deserve further investigation.

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