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Review

A Combinatorial Nanobased Spray-Induced Gene Silencing Technique for Crop Protection and Improvement

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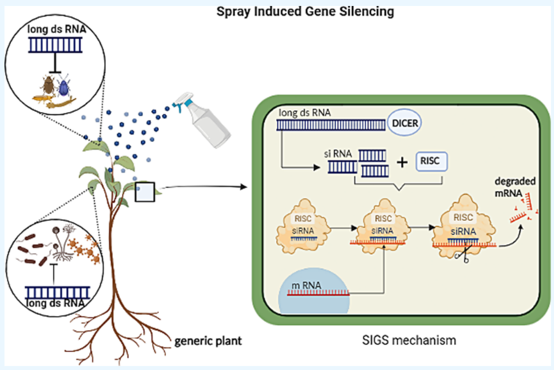
ABSTRACT: Recent research reports have shown that plant pests and pathogens have depleted the crop yield widely, which has led to an increased dependence on commercial pesticides and fungicides. Increased usage of these pesticides has also shown adverse effects on the environment, therefore many techniques have been implemented for solving the issue, some of which include using nanobioconjugates, RNA(i), which put into use double-stranded RNAs to inhibit gene expression. A more innovative and eco-friendly strategy includes spray induced gene silencing, which is being increasingly implemented. This review delves into the eco-friendly approach of spray induced gene silencing (SIGS) in combination with nanobioconjugates, which have been used concerning various plant hosts and their pathogens to provide improved protection. Furthermore, nanotechnological advancements have been understood by addressing the scientific gaps to provide a rationale for the development of updated techniques in crop protection.



1. INTRODUCTION

An estimation of the worldwide losses shows that plant pests and pathogens cause a significant reduction in production, and the strategies for pest management rely heavily on the use of chemical-grade pesticides, fungicides, and herbicides. Somehow, the disadvantages outweigh the advantages as many of these pesticides have a harmful effect, and around half of the pesticides are even washed away after application. So, for long-term food security, crop protection is deemed essential as global crop productivity is seriously threatened by oomycete and fungal diseases as stated by some researchers. For instance, the agent causing gray mold, *Botrytis cinerea*, damages fruits and vegetables to a large extent in the pre- and postharvest stages. A few more examples of disease-causing plant microbes are *Magnaporthe oryzae*, *Rhizoctonia solani*, *Xanthomonas oryzae*, and many more. Hence, to safeguard the crops against these diseases, new techniques are required for control and management.

Nanotechnology is a promising field of science that involves the manipulation of materials on a molecular and atomic scale. In crop protection, nanotechnology has the potential to revolutionize the way we grow and protect crops. Nanoparticles can be used as delivery systems for pesticides, fertilizers, and other agricultural inputs.[1] They can also be used to enhance plant growth, increase nutrient uptake, and improve soil quality. Furthermore, nanosensors can be used to monitor crop health and detect diseases before they become widespread. The use of nanoparticles in agriculture can be done in two ways: (a) the nanoparticles themselves and (b) the incorporation of pesticides in nanoparticle where the nanoparticles act as a carrier to give the desired result.

RNA interference (RNAi) is a phenomenon in which fungi communicate with the plant's pathogens, and this has been the subject of recent research wherein the fungi transmit short RNAs into the host plant to silence the genes involved in the immune system response of the host while plants send these small RNAs covered in extracellular vesicles to the pathogenic fungi to suppress the genes involved in virulence. Since its discovery, RNAi's potential to help plants develop resistance against a variety of pathogens has been under investigation widely.[2] Environmental RNAi is another phenomenon that was first observed in worms and later in fungi. This is also a gene-silencing method that is induced by the RNAs taken up from the environment.[3]

Researchers have stated that the exogenous application can effectively trigger the RNAi pathway by transporting the double-stranded RNA molecules onto the plant surfaces which focus on the genes of the insects feeding on the plants and

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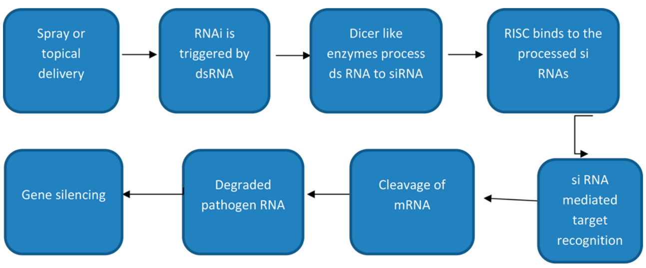
such infecting pathogens.[4] The application system for this RNAi delivery is also under investigation, and some researchers are believed to have come up with an idea to incorporate the RNAi technique for large-scale use.[5] The above-mentioned technique of RNAi includes host-induced gene silencing (HIGS), which is known as a modification technology that depends on the decision of an inverted repeat sequence in the plant genome, which also makes use of the RNAi pathway along with the appropriate vectors.[6,7]

There are several disadvantages, one being the production of transgenic lines, which is seen to be a time-consuming process. Many investigative procedures have also suggested that HIGS cannot be easily adopted for testing various targets in a limited amount of time, and therefore to overcome these probable disadvantages, spray-induced gene silencing (SIGS) came into play. It is an approach by which the pathogen infection is constrained by the topical administration of the double-stranded RNA (dsRNA) or single-stranded RNA (sRNA) molecules onto the plants to suppress pathogen virulence-related genes. Successful results have been observed by researchers for the control of *F. graminearum* infection on barley where the spray application of noncoding ds RNA targeted three crucial fungal CYP51 genes required for ergosterol synthesis, and the results demonstrated effective inhibition of the disease.[8]

These aforementioned techniques are believed to provide a sustainable and eco-friendly alternative to heavy pest management systems. In this Review, we will discuss the role of a double-stranded RNA nanobioconjugate via SIGS describing the general mechanisms of action for spray-induced gene silencing. This Review also highlights the targeted crops against the pathogens in addition to the future perspectives of SIGS in crop protection and disease management.

Chart 1. Flowchart of the Spray Induced Gene Silencing (SIGS) Technique



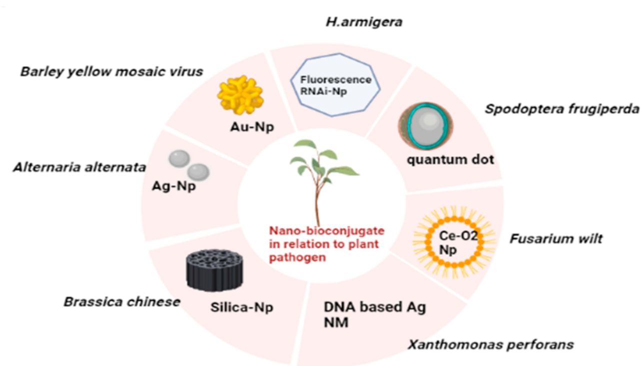


Figure 1. Illustration of the various nanobioconjugates in crop protection.

2. THE RNAI-BASED NOVEL APPROACH IN AGRICULTURE

Research to change crops for better performance is underway in numerous plants, and there has been an increased use of gene silencing in plant biotechnology, which incorporates the genome editing tools as an added advantage. The mechanism of gene silencing was discovered by American scientists Andrew Fire and Craig Mello, who found that injecting the dsRNA into *C. elegans* triggered the silencing of the genes with sequences similar to that of the dsRNA, which was further referred to as RNA interference. This method is considered the primary step for the development of pathogen control or management. The first demonstration was done when scientists developed potato virus Y-resistant plants expressing the RNA transcripts of a viral proteinase gene. RNA interference (RNAi) is a powerful tool for gene regulation and has been utilized in various fields including agriculture.

The RNAi-based approach has become increasingly popular in the agricultural industry as it provides a novel way to control pests and diseases and enhance crop productivity. The technique involves the introduction of small interfering RNAs (siRNAs) into plants that target specific genes responsible for undesirable traits, which can lead to the suppression of disease-causing pathogens or increased resistance to environmental stressors such as drought or heat.[9,10]

One unique aspect of using RNAi which has been demonstrated is the ability to mitigate increased resistance to the whole plant because these molecules have greater mobility through the plant's system, which gives an astounding result of a crop not being genetically regulated but being protected by the small RNA molecules.[11] More recent studies take us through the topical application of these dsRNAs whose formulation has been improved to meet the variable needs of the farmers. The methods of application are still under research, but some articles state the uses being foliar sprays, treatment of seeds, microlevel injections, and many more. The RNAi-based approach offers an environmentally friendly alternative to traditional chemical pesticides and herbicides, making it an attractive option for sustainable agriculture. RNA interference is being increasingly considered to be nature's defense mechanism against the molecular parasites which affect genome stability.[1]

3. NANOMATERIAL-BASED RNAI DELIVERY FOR PEST AND CROP MANAGEMENT

Present agricultural advancements, crop protection strategies, insect and pathogenicity control, increased production, crop improvement, and quality, have reached new heights due to the involvement of materials that deal with nanotechnology. In this modern era of science and technology, bionanotechnology has delivered numerous eco-friendly approaches for fabricating novel nanobased materials for correct usage in the agricultural sector. Insect pests and other bacterial, fungal, as well as viral pathogens are the major areas of concern when we talk about cultivation-affecting diseases, loss of yield, and economic downfall. Focusing on exogenous applications and various modes to deliver dsRNA/RNAi for gene silencing against pests and pathogens, nanoparticles can be an effective tool. Nanomaterials 1−100 nm in size hold more than enough potentiality, and based on today's scenarios, researchers are

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highly interested in studying them because of their stability, specificity, reactivity, shape, size, and many more properties.[12]

Silver, gold, copper, zinc, and titanium nanoparticles can be synthesized as nanopesticides to destroy plant-affecting insects and, therefore, are widely applied for plant disease resistance. To construct such a nanopesticide, the insoluble active ingredient is fused with the inorganic coating of nanoparticles or conjugated with the polymers. Likewise, silica-made nanoparticles are generally used for making nanofertilizers and pesticides.[13] Chaud and his team have demonstrated cluster-based metallic nanomaterials comprising thiophanate methyl, encapsulation containing trichlorfon and acetochlor, and micelle cum nanoemulsion-based particles filled with pyrethrin and citronella, types of nanopesticides.[14] For dispatching dsRNA to its targeted gene, nanomaterials are encapsulated and, therefore, act as nanocarriers that showcase high specificity and create a shield from further enzymatic or other chemical degradation. Not only this but a systematic easy uptake of dsRNA also takes place by the pest and pathogens to proceed with the silencing of further post-transcriptional mechanisms.[15] After the entry of inorganic nanoparticles, metal like zinc, copper, gold, and silver disrupt the pathogen's cell wall or membrane, initiate ROS accumulation and free radicals, intercalate in between the DNA material of the pathogen, and bind to the thiol group of such proteins resulting in deactivation.[16] Furthermore, liposome-mediated gold nanoparticles, guanidine-made polymers, and nanocarriers with detergent formulations have portrayed a defensive capacity against nucleolytic degradation, quick penetration and passing of RNAi through the insect cell walls, rapid uptake, and prevention.[17]

Per studies, it was observed that the nanocarrier mode of the RNAi gene dysregulating method to prevent pests and microbes like the African malaria mosquito, yellow fever mosquito, bean yellow mosaic virus, cucumber mosaic virus, *Rhizoctonia solani*, *Fusarium oxysporum*, *Xanthomonas oryzae*, and so on is a successful utilization.[12,18,19] The tomato leaf miner is another disease-causing insect, which can be decreased using nanobioconjugates as a remedy for the ailment.[20]

3.1. Types of Nanomaterials and Their Conjugates Used. While synthesizing a dsRNA consisting of nanomaterial, the stability is achieved by the formation of electrostatic interactions occurring between the cationic charged particles of nanomaterials and the phosphate group of dsRNA. Hence, this nanoparticle/dsRNA complex bears a net positive charge and can easily embed onto negatively charged cell membrane surfaces as well as other cellular entities. Soon endocytosis takes places, and the prior encapsulation of the nanomaterial prevents it from further nuclease and chemical degradations.[21] Emerging nanomaterials and their conjugates are showing possible impacts in the agricultural sector with a variety of nanomaterials in use. Ag@dsRNA@GO DNA-based silver nanomaterials can diminish the consequences of *Xanthomonas perforans* in tomato plants. Nanomaterials made of TiO_2 also portray antibacterial properties against *X. perforans* as well as other spot disease-causing microbes. Along with that, cerium-made nanoparticles applied in tomato plants are quite powerful against *Fusarium wilt* disease-causing fungi.[22,23]

Fungal diseases encountered by crops like corn, wheat, barley, tomato, mustard, and so were treated using RNAi nanocarriers to their corresponding targeted genes like ACTT2, ACTTS2, aflR, MLO, MAPK, Dicer-like 1, Dicer-like 2, FcGls1, PAC1, etc.[24] Fluorescent-based RNAi-NPs are delicately designed with a cationic polymer as its outer shell and a polyphenylene dendrimer as the inner shell consisting of fluorescent perylene-3,4,9,10-tetracarboxydiimide (PDI) as the chromophore that can obstruct *H. armigera* growth. Due to its core and outer shell components, they can fluently channel across the live cells of insects and pathogens, thus displaying extensive efficacy for the delivery of dsRNA.[25,26]

Nanocarriers like star polycation (SP) can efficiently travel through the subsequent transdermal layers and, therefore, deliver dsRNA/siRNA by both oral and topical routes, in the case of insects. Oriental fruit flies, green pea aphids, and other pests get reduced when SPs are applied using a spray technique for disease prevention.[16,19,27] Consequently, chitin synthase-producing genes of *Anopheles gambiae* get downregulated when treated with RNAi gene regulatory tools using chitosan-based nanomaterials. Vigorous studies also suggested carbon quantum dots, silica-made nanoparticles, liposomes, fluorescent nanoparticles, and granulated polymers would be beneficial in hindering *Spodoptera frugiperda*, *A. aegypti*, and *O. furnacalis* development.[16,28,29]

Layered double hydroxide (LDH) nanosheets can also be termed as "bioclay" or "nanoclay" generally made of bentonite, hectorite, saponite, laponite, and more, which act as a nanocarrier for RNAi delivery. They are aluminosilicates which are sprayed over the plants for disease control and crop protection.[30,31] This particular mode of dsRNA application over plants and pathogens protects the RNAi from UV radiation and provides better stability and leaf attachment, fast cellular engulfment, and spreading of dsRNA; as a result, it safeguards the plant from various diseases.[32] Silver nanoparticles are quite strong against enormous phytopathogens like *Alternaria alternata*, *Pyricularia oryzae*, and *Cladosporium cucumerinum* whereas *Phoma destructive*, *Curvularia lunata*, *Rhizoctonia solani*, etc. can be killed using nanopesticides made of copper nanomaterials (Figure 1).[33]

4. SPRAY INDUCED GENE SILENCING

By altering the genetics of the host plants to express single-stranded RNAs which target genes relevant to pathogen virulence, a technique called host induced gene silencing is being increasingly used in plant defense measures.[34] The application of HIGS is limited because of the effective methods for plant transformation. Host induced gene silencing falls under the processes of genetically modified organisms (GMO), and many consumers resist the idea of GMO products, which has left researchers searching for non-GMO crop protection strategies. This has led to the development of a new and improved strategy known as spray-induced gene silencing (SIGS). It is a non-GMO alternative to HIGS as spray-induced gene silencing prevents pathogen infection by topical application or foliar spraying of the double-stranded RNA or single-stranded RNA molecules onto plants to silence the virulence genes transiently.

As SIGS is not dependent on plant transformation, it is thought to be applicable for all of the crops when some of the critical requirements are met, which includes the functionality and sequence information concerning the targeted genes of the pathogen.[35] Following this, the targeted genes of the pathogen should be expressed as the RNAi effect is mostly post-transcriptional. Potential prospects for SIGS include insects that chew as they can easily take up the sprayed dsRNAs which are known to invade the cuticle of some insect species.[36]

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Table 1. SIGS Mediated Protection against Pathogens Affecting Different Crops

SL no.	plant species	microbial pathogens	type	disease caused	target genes	references
1.	rice (*Oryza sativa*)	*Magnaporthe oryzae,Rhizoctonia solani,Xanthomonas oryzae*	fungi	rice blast	*DES1, MoAP1*	Ray et al., Sarkar et al.
			bacteria	rice sheath blight	*DCTN1+SAC1, PG*	Halder et al.
				rice leaf blight		
2.	potato (*Solanum tuberosum*)	*Phytophthora infestans*, potato virus Y, potato leaf roll virus, potato spindle viroid	fungi	late blight	*StDND1, StDMR1, StDMR6*	Sun et al., Sundaresha et al., Petrov et al.
			virus	potato mosaic, leaf rolling	*StTCP23*	Jiang et al.
3.	barley (*Hordeum vulgare*)	*Fusarium graminearum, M. oryzae*	fungi	head blight	*CYP51A, CYP51B, CYP51C*	Schlemmer et al., Sang et al.
		Fusarium asiaticum		barley blast	*β2Tub*	Cagliar et al., Das et al.
		Sclerotinia sclerotiorum		cereal head blight		Dubrovina et al.
				stem rot		Bilir et al.
4.	thale cress (*Arabidopsis thaliana*)	*Botrytis cinerea*	fungi	gray mold	DCL-1, DCL-2	Kuo et al., Machado et al.
		S. Sclerotiorum, F. graminearum		stem rot		Bilir et al., Ray et al.
				head blight		

Research provides some successful implementations of this technique. For instance, in the case of barley leaves, the double-stranded RNA is designed to target the fungal cytochrome, which inhibits the fungal pathogen and the inhibition of *B. cinerea* on *Brassica napus* with the help of this spray.[8,37]

4.1. Mechanism of Spray Induced Gene Silencing (SIGS). The mechanism of RNA interference leads to post-transcriptional gene silencing (PTGS), wherein the long double-stranded RNA given to the pathogen triggers the system to prevent the expression of specific genes.[38] The double-stranded RNA is cut by an RNase III-like enzyme called Dicer or Dicer-like into small interfering RNAs, which are then uncoiled into single strands and further incorporated into an RNA-induced silencing complex (RISC). The antisense strands pick out the complementary mRNA, and argonaute proteins in the complex cut the target mRNA, resulting in degeneracy.[3]

In spray-induced gene silencing, which is a non-GMO method, the double-stranded RNA focusing on the virulence gene is sprayed onto the surfaces of plants or crops. The fungal infectious agent takes up the double-stranded RNA and induces fungal RNA interference machinery that silences the virulence gene without introducing modifications that can be heritable(Table 1).[31]

4.2. SIGS in Various Types of Plant Pathogen Prevention. *4.2.i. Rice (Oryza sativa).* A few famous pathogens cause rust, Fusarium seedling blight, Fusarium head blight, powdery mildew) that target cereal crops and as a result, a striking downfall is visible in the economic as well as agricultural grain production industry.[39] Spray-induced gene silencing application of RNAi on specific genes of rice crops was performed by several research groups across the globe.[40] Fungal pathogens known as *Magnaporthe oryzae*, *R. solani*, and *Ustilaginoidea virens* typically infect rice plants, and therefore, the SIGS approach can be initiated to target *AP1*, *SSADH*, *MAC1*, *PMK1*, *tps2*, *Chs2*, etc. genes related to transcription and pathogenetic factors to increase disease resistance.[41−43]

The brown plant hopper (*Nilaparvata lugens*), an insect pest, and *Xanthomonas oryzae*, a bacterial pathogen causing bacterial blight in rice crops, can also be targeted by spraying dsRNA directly over the plant leaves and other wounded areas.[44] Sarkar and his colleagues reported that spraying artificial siRNAs straight over rice leaves targeting the MoAP1 gene can control the disease progression by *M. oryzae*. They also conveyed that spraying the dsRNA was more successfully withdrawn by the fungus, and eventually the *MoDES1* gene took part in diminished pathogenicity.[45] Carbon quantum dots and silica-based and chitosan-made nanoparticle mediated RNAi delivery can stunt the infection caused by the rice striped stem borer (*Chilo suppressalis*).[46]

4.2.ii. Potato (Solanum tuberosum). One of the most common potato diseases, "late blight," is caused by the oomycete *Phytophthora infestans*, which forms into zoosporangium and affects the epidermis and cuticle by making a way inside the leaf tissues.[47] Thus, it expands throughout the plant body, and the lesion looks black after a few days. By the incorporation of RNAi-mediated post-transcriptional gene silencing, *StDND1*, *StDMR1*, and *StDMR6* genes can be downregulated to initiate resistance against such pathogens.[48]

Leaf malformations, stunted plant growth, mosaics on leaves of potatoes, and a lot more are visible when any viral pathogen (potato virus Y, potato virus S, potato leaf roll virus, potato mop-top virus) attacks a potato plant.[49] A scientific study in 2016 suggested that short-hairpin RNA and its corresponding RNAi technology can target the potato virus Y capsid protein gene and can increase viral resistance.[50] Moreover, RNAi gene silencing works as an effective tool against potato spindle tuber viroid by silencing the *StTCP23* gene to provide resistance.[51] The SIGS mode of RNA-based regulation can also lessen the pathogenicity of *P. infestans* causing late blight.

Green fluorescent protein (GFP) linked to *P. infestans* showcased such an ability to engulf dsRNA homologous to the GFP from the sprinkles sprayed over the leaves.[52] However, specific genes (*SDH*, *EF-1α*, *GP1-HAM344*, *PLD-3*, *HSP-90*) can get targeted by dsRNA nanoclay sprayed on the lesion area and thus, late blight of potatoes can be recovered.[53]

4.2.iii. Barley (Hordeum vulgare). Using the spray induced gene silencing (SIGS) method for plant disease prevention and protection, it was observed that when siRNAs or dsRNA was drizzled over barley crops, a particular fungal pathogen known as *Fusarium graminearum* quickly took the foliar spray, and therefore, the genes *CYP51A*, *CYP51B*, and *CYP51C* were targeted. The growth of *F. graminearum* halted as only *CYP51A* got silenced.[54] Microbes like *M. oryzae* and *F. graminearum* experienced drastic inhibition by the administration of foliar

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dsRNA spray over *H. vulgare* crops.[6,55] DICER and ARGONAUTE genes of *F. graminearum* were chosen to interact with RNAi-based SIGS biopesticides concerning barley. Likewise, the *AGO* and *DCL* genes got triggered to downregulate by the dsRNA machinery and protect from Fg infection.[56,57] Besides this, the *β2Tub* gene of the *F. asiaticum* fungus can also encounter antifungal properties by RNAi spray on barley crops.[58] Another fungal pathogen, *Sclerotinia sclerotiorum* (white mold), also affects barley crops, which can be prevented using SIGS.[59]

4.2.iv. Thale Cress (Arabidopsis thaliana). The RNAi/dsRNA mediated spray induced post-transcriptional procedure points out that the *DCL-1* and *DCL-2* genes of the *Botrytis cinerea* fungus infecting *A. thaliana* plants showcase the suppression of its disease invasion.[60] Furthermore, the foliar spray technique of incorporating siRNA or dsRNA can soon diminish the quick magnification of gray mold disease via *B. cinerea*.[61] Talking about nanomaterial-based delivery of dsRNA, the LDH nanosheet is one of the most powerful and efficient processes to spray RNAi on *A. thaliana* leaves. CMV2b-dsRNA-Cy3 got erased by sudden rain and irrigation, whereas CMV2b-dsRNA-Cys3-LDH was still attached to its leaves, providing better crop protection against the pathogens.[62] *Sclerotinia sclerotiorum* is another pathogen that can be reduced by SIGS application infecting thale cress and oil rape seed plants.

Additionally, the downy mildew pathogen (*Hyaloperonospora arabidopsidis*) attacking *A. thaliana* can also be killed using this very RNAi approach by suppressing the cellulose synthase corresponding gene.[59,63] A few more examples of pathogens that infect thale cress plants are *Macrophomina phaseolina*, *F. graminearum*, and *Verticillium dhaliae*.[41] Rapeseed (*Brassica napus*) is an example of a plant falling under the Brassicaceae family, which also gets infected by *B. cinerea* and *S. sclerotiorum*, respectively, that can be regulated by SIGS.[36]

5. FUTURE PERSPECTIVES OF SIGS AS A TOOL

Nanotechnology is viewed to be a potential tool in the case of agriculture as it focuses on the smart delivery of RNAi molecules for pest management. For improving the strength and period of the SIGS approach, nanoparticles are being increasingly used because these double-stranded RNAs are not toxic to the environment, unlike HIGS. With the increasing progress in cost-effective production, it can be foreseen that SIGS will be in major use in agriculture. However, much exploration is needed in terms of the mechanism of action with the cross-kingdom small RNAs holding a lot of potential for future disease and pest management. Much interest has also been shown in the science community to produce multiple knockdowns which protect in a patterned manner against a wide variety of diseases under the same application.

6. CONCLUSION

The widely increasing population is posing a demand in the food supply chain which is threatened by pests and pathogens. SIGS is a prospective technique gaining popularity as it represents an attractive advancement owing to a non-GMO method as well as potential advantages for the environment and human health. A much deeper understanding of the mechanisms for interaction in a complex system is desired, and therefore it is suggested that biologists and material scientists work closely. To keep up with the production costs, biotechnology is seen as one of the helpers to improve profit on an industrial scale; though rigorous field studies with the SIGS approach added to the pest management strategy to increase crop yield should be conducted in the coming years to increase the chance of SIGS being used for precision farming.

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Notes

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