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TÁLISON EUGÊNIO DA COSTA

USE OF MOLECULAR TECHNIQUES FOR REPORTING A VIRAL DISEASE IN VINEYARDS IN NEW ENGLAND (FIRST REPORT) AND RNAI FOR CONTROLLING A FUNGAL DISEASE IN MELON IN THE BRAZILIAN SEMIARID REGION

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Thesis presented to the Crop Science Graduate Program of the Universidade Federal Rural do Semi-Árido to fulfill the requirements for obtaining the degree of Doctor in Crop Science.

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Principal Advisor: Professor Dr. Ioná Santos Araújo Holanda

Co-advisor: Professor Dr. Rui Sales Júnior

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ABSTRACT

The occurrence of plant diseases is one of the main factors affecting food production worldwide. In this study, we address the application of molecular techniques in detecting viral pathogens and developing new methods for managing fungal diseases in melon. In the first chapter, we describe the initial report of the detection of an important viral pathogen in grapevines. Grapevine red blotch virus (GRBV) is an emerging pathogen that significantly impacts grape production. This pathogen causes Grapevine Red Blotch Disease (GRBD). The virus has been spreading across several states in the United States, but until now, there had been no information about its occurrence in the New England (NE) region. The objective of this study was to investigate the presence of GRBV in NE vineyards and assess the degree of genetic variability among isolates. To detect GRBV, twenty-eight samples from five vineyards in Connecticut, Rhode Island, and Massachusetts were collected between 2018 and 2020. These samples underwent DNA extraction, rolling cycle amplification (RCA), and PCR using GRBV-specific primers. Samples that produced the PCR product of the expected size were considered positive. These samples had partial and complete genomes sequenced using the Sanger method. Phylogenetic analyses revealed that the samples fell into two separate clades, predominantly with isolates in clade 1, which shows a higher degree of genetic variability, possibly explained by genetic recombination events. This is the first report of the presence of GRBV in the evaluated New England states. Correct diagnosis is the first step toward implementing control measures and reducing virus transmission. In the second chapter, we describe the exploration of an innovative method for disease control in melon. The occurrence of diseases caused by soil-dwelling fungi is one of the primary factors that reduce melon productivity (*Cucumis melo* L.). *Rhizoctonia solani* is one of the species affecting the productivity and quality of melon, causing damage at all stages of development. In this study, we aim to explore the potential of RNAi technology based on SIGS (Spray-induced gene silencing) for controlling *R. solani* in melon. The genes responsible for important enzymes related to the pathogenicity of *R. solani*, polygalacturonase (PG1) and xylanase (Xly), were used as silencing targets. The coding regions of PG1 and Xly were amplified by PCR, sequenced, and used for dsRNA synthesis. For application in detached leaf assays and in melon plants, dsRNA molecules were encapsulated in chitosan nanoparticles. The potential for *R. solani* control via RNAi-SIGS was evaluated in *in vitro* experiments (culture media) containing only dsRNA-PG1 and

dsRNA-Xly solutions. In the detached leaf experiments, solutions containing dsRNA and encapsulated dsRNA (Cs-PG1 and Cs-Xly) were applied to the leaves, which were subsequently inoculated with a medium disk containing the pure culture of the pathogen. In the greenhouse experiments, the same solutions were applied to melon seedlings that were inoculated using the infested toothpick method. In the *in vitro* assay, a reduction in mycelial growth was observed in the treatment with dsRNA-PG1 in pectin-based medium after seven days. A decrease in lesion size was observed in detached leaves treated with dsRNA and encapsulated dsRNA after five days of inoculation. In the greenhouse experiments, a reduction in disease severity and an increase in plant growth parameters were noted in the groups treated with dsRNA, with or without the presence of chitosan nanoparticles. The control of *R. solani* via RNAi-SIGS shows excellent potential for use under field conditions. This study presents an innovative technology for the future management of soil-borne fungi.

Key-words: *Vitis vinifera*, virus survey, RNAi, nanotechnology, chitosan, *Cucumis melo*, dsRNA

RESUMO

A ocorrência de doenças em plantas é um dos principais fatores que afetam a produção de alimentos no mundo. Neste trabalho, abordamos a aplicação de técnicas moleculares na detecção de patógenos virais e o desenvolvimento de novos métodos para o manejo de doenças fúngicas em meloeiro. No primeiro capítulo, descrevemos o primeiro relato da detecção de um importante patógeno viral na cultura da videira. Grapevine red blotch virus (GRBV) é um patógeno emergente que afeta de forma significativa a produção de uvas. Esse patógeno causa a Grapevine Red Blotch Disease (GRBD), ou doença da mancha vermelha da videira. O vírus vem se espalhando por diversos estados dos Estados Unidos, porém, até então, não havia informações sobre sua ocorrência nos estados da região de New England (NE). O objetivo deste estudo foi investigar a presença de GRBV em vinhedos de NE e verificar o grau de variabilidade genética entre os isolados. Para realizar a detecção de GRBV, vinte e oito amostras de cinco vinhedos dos estados de Connecticut, Rhode Island e Massachusetts foram coletadas entre 2018 e 2020. Essas amostras foram submetidas à extração de DNA, amplificação por ciclo rolante (RCA) e PCR com *primers* específicos para GRBV. Amostras que apresentaram o produto de PCR com o tamanho esperado foram consideradas positivas. Essas amostras tiveram seus genomas parcial e completo sequenciados pelo método de Sanger. Análises filogenéticas mostraram que as amostras foram classificadas em dois clados distintos, com a grande maioria dos isolados pertencendo ao clado 1, que apresenta maior variabilidade genética. Uma alta variabilidade genética foi observada entre as amostras, possivelmente explicada por eventos de recombinação genética. Este é o primeiro relato da presença de GRBV nos estados de New England avaliados. O diagnóstico correto é a primeira etapa para a implementação de medidas de controle e redução da transmissão do vírus. No segundo capítulo, descrevemos a exploração de um método inovador para o controle de doenças no meloeiro. A ocorrência de doenças causadas por fungos habitantes do solo é um dos principais fatores que reduzem a produtividade do meloeiro (*Cucumis melo* L.). *Rhizoctonia solani* é uma das espécies que afetam a produtividade e a qualidade do meloeiro, causando danos em todos os estágios de desenvolvimento. Neste estudo, temos como objetivo explorar o potencial da tecnologia de RNAi (RNA de interferência) baseada no SIGS (*Spray-induced gene silencing*) no controle de *R. solani* em meloeiro. Os genes responsáveis por importantes enzimas relacionadas à patogenicidade de *R. solani*, poligalacturonase (PG1) e xilanase (Xly), foram utilizados como alvos de silenciamento. As regiões codificadoras de PG1 e

Xly foram amplificadas por PCR, sequenciadas e utilizadas para a síntese de dsRNA. Para aplicação em ensaios com folha destacada e em plantas de melão, as moléculas de dsRNA foram encapsuladas em nanopartículas de quitosana. O potencial de controle de *R. solani* por RNAi-SIGS foi avaliado em experimentos *in vitro* (meios de cultura) com soluções contendo apenas dsRNA-PG1 e dsRNA-Xly. Nos experimentos com folhas destacadas, as soluções contendo dsRNA e dsRNA encapsulado (Cs-PG1 e Cs-Xly) foram aplicadas sobre as folhas, que foram posteriormente inoculadas com um disco de meio contendo a cultura pura do patógeno. Nos experimentos conduzidos em casa de vegetação, as mesmas soluções foram aplicadas nas plântulas de melão, que foram inoculadas utilizando o método do palito infestado. No ensaio *in vitro*, foi observada uma redução no crescimento micelial no tratamento com dsRNA-PG1 em meio à base de pectina após sete dias. Uma redução no tamanho das lesões nas folhas destacadas foi observada nos tratamentos com dsRNA e dsRNA encapsulado após cinco dias de inoculação. Nos experimentos em casa de vegetação, observou-se uma redução na severidade da doença e um aumento nos parâmetros de crescimento das plantas nos grupos tratados com dsRNA, com ou sem a presença de nanopartículas de quitosana. O controle de *R. solani* por RNAi-SIGS apresenta grande potencial para utilização em condições de campo. Este estudo apresenta uma tecnologia inovadora para o controle futuro de fungos habitantes do solo.

Palavras-chave: *Vitis vinifera,* virologia vegetal, RNAi, nanotecnologia, quitosana, *Cucumis melo*, dsRNA

FIGURE LIST

GENERAL INTRODUCTION

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CHAPTER 2

Figure 4. Control experiment of *R. solani* on melon plants in a greenhouse. (a) Photos of plants 30 days after treatment with dsRNA and chitosan nanoformulations. (b) Severity was ranked using Ambrosio et al.'s scale (2015) and analyzed using the Kruskal-Wallis test at a 5% significance level, followed by Dunn's test with a Bonferroni adjustment. Treatments sharing letters showed no significant difference ($p > 0.05$). (c) Size of aerial parts (above) and root area (below) across different treatments, with bars indicating standard deviation. Different letters represent significant differences per Tukey test ($p > 0.05$).....55

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SUMMARY

GENERAL INTRODUCTION

The global population may approach ten billion by 2050, substantially raising food demand by seventy times (ONU, 2013). Consequently, there is an increasing need for technologies that can enhance agricultural productivity sustainably and ensure economic viability (GAMAGE et al., 2024). A key challenge to crop yield is the prevalence of diseases caused by a range of pathogens, including viruses, bacteria, nematodes, and fungi. These diseases can lead to a decrease in productivity of up to 40% (RANI et al., 2023).

In this challenging scenario, two pathosystems of great economic importance have attracted the attention of producers and researchers due to their significant impact on agricultural productivity: Grapevine red blotch virus (GRBV), associated with grapevines, and melon root rot caused by the fungus *Rhizoctonia solani*. Although they affect different crops, both pathogens pose a serious threat to the sustainability of production systems, requiring the development and implementation of innovative and sustainable technologies for their diagnosis and effective management.

The management of viral diseases such as GRBV primarily focuses on reducing transmission. For this reason, safe and reliable diagnostic methods are essential for producers to make informed management decisions (KRENZ; FUCHS; THOMPSON, 2023). Regarding the management of diseases caused by soil-dwelling fungi, due to their challenging control, it is necessary to explore sustainable and innovative methods to maintain crop sustainability in the future (RANI et al., 2023).

The research presented aims to use innovative molecular tools for managing plant diseases. The first chapter reports on the Grapevine red blotch virus, responsible for Grapevine Red Blotch Disease, in New England vineyards. This virus was identified through genome sequencing, and the study examines its genetic diversity and the recombination events that contribute to this variation. The second chapter investigates the potential of RNAi-SIGS technology to control root rot caused by *R. solani*. This was explored through a series of *in vitro* experiments, detached leaf assays, and experiments with melon plants grown under greenhouse conditions. Additionally, chitosan nanoparticles were utilized to deliver dsRNA molecules, thereby enhancing the effectiveness of RNAi-SIGS technology.

1. THEORETICAL FRAMEWORK

1.1. Crops of economic importance

1.1.1. Global and regional viticulture

Grapevines, one of the crops most affected by pathogens, are also among the most widely grown globally (HUSSAIN *et al.*, 2021). Grapes can be consumed as fresh berries or processed into products like wines, juices, and jellies (YEPES *et al.*, 2018). Taxonomically, the cultivated grapevine is classified within the *Vitaceae* family and *Vitis* genus, with *V. vinifera* being the most significant cultivated species, followed by *V. labrusca* and *V. borquiniana* (HUSSAIN *et al.*, 2021).

Grapevines are cultivated globally and hold significant socio-economic value. They comprise 8% of the world's fruit production, totaling 72 million tons in 2023 (FAO, 2023). The top producers are China and Italy, followed by the United States, which ranks fifth with an annual output of 5.61 million tons (USDA-NASS, 2025).

Brazil covers a production area of 83,000 hectares with an estimated output of 1.8 million tons (IBGE, 2025; OIV, 2025). Production is mainly located in the South and Southeast regions, but the Northeast, especially the São Francisco Valley, is becoming increasingly important due to its high production (LEÃO; CARVALHO, 2024).

In the U.S., California and Washington lead in production. The Southeastern New England American Viticultural Area covers Massachusetts, Connecticut, and Rhode Island (APPELLATION AMERICA, 2021; DUMAS *et al.*, 2023). Although its size is relatively small at 0,427 hectares, the production of wine grapes in this region is growing rapidly and is economically vital (USDA-NASS, 2023; BORGES *et al.*, 2020).

1.1.2 Melon cultivation and its socioeconomic significance

The melon (*Cucumis melo* L.) belongs to the *Cucurbitaceae* family and includes various types that offer substantial socio-economic value because of their high production volumes and global popularity. In terms of taxonomy, the melon is classified under the genus *Cucumis*, subtribe *Cucumerinae*, tribe *Melothrieae*, subfamily *Cucurbitoideae*, family *Cucurbitaceae*, and species *Cucumis melo* L. Melons can also be divided into two types: *C. melo* var. *inodorus* (which includes Yellow, Piel de Sapo, and Honeydew) and *C. melo* var. *cantaloupensis* (which includes Cantaloupe, Galia, and Charentais)

(CRISÓSTOMO; ARAGÃO, 2013). The fruit is consumed fresh, as an ingredient in salads, whether fruit or vegetable, and in juice form. It is rich in mineral elements like potassium, sodium, and phosphorus, but also low in calories (MAIA; LIMA; LIMA, 2013).

Melon is one of the most produced fruits in the world. Brazil ranks 12th in the world in melon production, with 862.000 tons of fruit produced in 30.500 hectares (IBGE, 2025a). The Northeast Region is responsible for 71% of the national output, with the states of Ceará and Rio Grande do Norte standing out (IBGE, 2025a). A large part of this production is destined for the international market, where 243.000 tons are worth around 185 million dollars (ABRAFRUTAS, 2025).

The melon tree thrives exceptionally well in the Brazilian northeastern soil and climate, which are characterized by high temperatures, abundant sunshine throughout the year, and fertile ground (MAIA; LIMA; LIMA, 2013). The success of melon cultivation in this region is not only due to advanced agronomic practices but also to the adoption of improved seed varieties and the application of high technology throughout the production process (GUIMARÃES, 2016).

1.2 Pathosystems and their impacts

1.1.2 Viral diseases in grapevines and the Grapevine Red Blotch Virus (GRBV)

Grapevines can be affected by over 80 different virus species. Vine propagation typically occurs through grafting or the use of vegetative material, which makes the vine highly vulnerable to viral pathogens (PEDRO *et al.*, 2017; THAGUNNA, 2023). Viral diseases lead to various symptoms, often resulting in general plant degeneration (e.g., *Grapevine fanleaf virus*-GFLV), vein clearing (e.g., *Grapevine vein clearing virus*-GVCV), deformities (e.g., *Grapevine Pinot gris virus*-GPGV), leaf curling (e.g., *Grapevine leafroll-associated virus*-GLRaV), and red spots on the leaves (*Grapevine red blotch virus*-GRBV) (FUCHS, 2020; MARTELLI, 2017).

Grapevine red blotch disease (GRBD) was first documented in vineyards in California's Napa Valley in 2008 (CALVI, 2011). At the time, researchers were intrigued by plants showing symptoms similar to grapevine leafroll disease, but they tested negative for all GLRaV strains (KRENZ; FUCHS; THOMPSON, 2023). The causative agent of GRBD was identified in 2012 through deep sequencing of dsRNA fractions extracted from

symptomatic vines, followed by RCA (rolling cycle amplification) on total nucleic acid extracts (AL RWAHNIH *et al.*, 2013). The virus was named *Grapevine red blotch virus* (GRBV) and was confirmed as a GRBD agent with Koch's postulates (YEPES *et al.*, 2018).

GRBV is part of the *Geminiviridae* family, specifically the *Grablovirus* genus. It features a circular single-stranded DNA (ssDNA) genome measuring 3206 nucleotides organized into bidirectional open reading frames (ORFs) (AL RWAHNIH *et al.*, 2013; KRENZ *et al.*, 2012). On the complementary strand, there are three overlapping ORFs (C1, C2, and C3), while the virion strand also contains three (V1, V2, and V3). These ORFs code for proteins responsible for replication (RepA and Rep), the coat protein (CP), and potential movement protein proteins (RUMBAUGH; SUDARSHANA; OBERHOLSTER, 2021; WELIGODAGE *et al.*, 2023).

Symptoms of GRBD differ by grapevine variety. In red berry varieties, they manifest as small spots at the edges, which expand along the leaf blade to the vein. In white berry varieties, symptoms appear as chlorotic spots that progress to necrosis on the leaves (CIENIEWICZ *et al.*, 2019; RUMBAUGH; SUDARSHANA; OBERHOLSTER, 2021). Both types suffer from early leaf drop, irregular ripening, and a decrease of up to 20% in the Brix berries (ACHALA *et al.*, 2022; MARTÍNEZ-LÜSCHER *et al.*, 2019)

The disease significantly impacts wine production. Wines from infected grapes display decreased levels of anthocyanins and pigments, decreased volatile compounds, reduced alcohol content, and elevated tannin levels (KRENZ; FUCHS; THOMPSON, 2023; BLANCO-ULATE *et al.*, 2017). Consequently, the disease can lead to economic losses estimated between $2,200 and $68,500 (USD) per hectare throughout its lifespan vineyard (RICKETTS *et al.*, 2017).

1.2.2 Fungal diseases in melon and *Rhizoctonia solani*

Due to the intense cultivation of melon, often without an off-season and proper disease management, the productivity of the melon crop is affected by phytosanitary problems (CUNHA *et al.*, 2019; MEDEIROS *et al.*, 2015). Among these problems, the incidence and severity of diseases caused by soil-borne fungi have become an emerging problem, especially in hot climate regions (PORTO *et al.*, 2019; SALES *et al.*, 2019).

Root rot and vine decline are significant threats to melon quality and productivity. Several fungal species can cause these diseases, with the most common being complexes

of *Macrophomina* species, *Fusarium* species, *Monosporascus cannonballs*, and *Rhizoctonia solani* Kühn (PORTO *et al.*, 2019).

The *R. solani* fungus, *Thanatephorus cucumeris* (Frank) Donk teleomorph, belongs to the *Ceratobasidiaceae* family and is characterized by its great genetic diversity (YANG *et al.*, 2012). Based on its morphology, hosts, and aggressiveness, it is classified into 14 anastomosis groups (AJAYI-OYETUNDE; BRADLEY, 2018). The species is cosmopolitan and polyphagous, with a wide range of hosts, and it has been reported to cause disease in more than 500 crops in more than 80 countries (FARR; ROSSMAN, 2019).

On melon plants, *R. solani* can cause damage at all plant development stages. The pathogen infects the plant through the roots, which, through the action of enzymes and the release of toxins (LI *et al.*, 2019; CHEN *et al.*, 2017), colonizes the plant's entire root system and, in high humidity, the stem up to the plant's leaves (GOULART, 2018). As a result, depending on the degree of severity of the pathogen and the host's susceptibility, the fungus can cause root rot and damping-off in young plants. In adult plants, as the root system is compromised, the branches can collapse, leading to wilting and death of the plant (MEDEIROS *et al.*, 2015; SALES JÚNIOR *et al.*, 2015; SALARI *et al.*, 2012).

1.3 Technologies in plant disease management and their challenges

1.1.2 Control of GRBV in grapevines

Management of viral diseases in a vineyard primarily focuses on preventing virus introduction (KRENZ; FUCHS; THOMPSON, 2023). The primary control strategy relies on using certified virus-free materials, which should serve as the foundation for future generations (RICKETTS *et al.*, 2017). Utilizing virus-free materials significantly reduces the incidence of viral diseases in the vineyard. Secondary control involves monitoring symptomatic plants and identifying potential viral vectors (FUCHS, 2020). Although random sampling and testing of symptomatic plants can be costly, this practice may effectively decrease the prevalence of these diseases in the vineyard (KRENZ; FUCHS; THOMPSON, 2023).

In this regard, the initial step to manage GRBV involves testing symptomatic plants. GRBV detection can be achieved through field technologies, such as Loop-Mediated Isothermal Amplification (LAMP), as well as laboratory techniques, including

PCR (Polymerase Chain Reaction) and qPCR . These methods are crucial for the effective confirmation of viral presence. Genetic sequencing and phylogenetic analyses can provide insights into the genetic diversity and possible origins of the isolates.

Phylogenetic studies revealed that GRBV can be grouped into two clades based on genetic variability (FLASCO *et al.*, 2024). Clade 1 exhibits greater diversity, with genetic differences between isolates reaching up to 8.5%. In contrast, clade 2 is more uniform, showing an average genetic similarity of 98.8% among isolates (POOJARI et al., 2017). This variability is detectable only through nucleotide similarity analysis (POOJARI et al., 2020).

Confirming the presence of the virus in the production area is crucial for producers. Implementing transmission control measures, including the removal of symptomatic plants and managing insect populations, may help limit secondary transmission by flying insect vectors (FUCHS, 2020; KRENZ; FUCHS; THOMPSON, 2023). The three-toed alfalfa hopper, *Spissistilus festinus*, has been identified as a vector for GRBV (POOJARI et al., 2020). This virus has already been found in vineyards throughout most U.S. states, as well as in Canada, Italy, Australia, and India (EPPO, 2025). GRBV has not yet been officially reported in vineyards in Brazil, although similar viruses, such as GLRaV, have already been confirmed (BASSO; FAJARDO; SALDARELLI, 2017; FAJARDO *et al.*, 2017).

1.3.2 Management of *Rhizoctonia solani* in melon

Like other soil-borne fungi, *R. solani* has a high capacity to remain in cultivated areas. It can either actively saprophytically in crop remains and alternative hosts or remain dormant, forming sclerotia resistance structures (AKBER *et al.*, 2023; SALES JÚNIOR *et al.*, 2015). Due to these characteristics, combined with the fact that symptoms may only manifest at the end of the cycle, controlling *R. solani* is challenging for producers.

Due to the lack of resistant melon cultivars and the persistent resistance of international markets to chemical control, searching for alternative methods is crucial for ensuring the sustainability of the crop in the future. Among the methods currently under investigation, control based on gene silencing through interference RNA has been explored to manage insects, viruses, and fungi (WYTINCK *et al.*, 2020; VOGEL *et al.*, 2019).

1.3.3 RNA Interference (RNAI) in the control of fungal diseases

RNA interference (RNAi) is a natural mechanism in most eukaryotic organisms for post-translational genetic control (WANG et al., 2024; VOGEL et al., 2019). This endogenous mechanism can be manipulated to silence specific genes in agriculturally important pathogens by introducing dsRNA or siRNA molecules (HE *et al.*, 2024; MCRAE *et al.*, 2023).

In the dsRNA-induced RNAi mechanism, dsRNA is recognized by class III RNAse enzymes called Dicer (LAX *et al.*, 2020; AGRAWAL *et al.*, 2003). This enzyme cleaves the dsRNA into smaller single-stranded RNAs of 20 to 25 nucleotides, called small interfering RNAs (siRNA). These, in turn, are recognized and associated with a complex of proteins, including the Argonauts, which form the RISC (RNA-induced silencing complex). In this complex, only one of the strands is maintained, and if it encounters complementary messenger RNA (mRNA), it is degraded, thus preventing translation (HE *et al.*, 2024; WYTINCK *et al.*, 2020; ROSA *et al.*, 2018).

Initially, RNAi was used to introduce dsRNA into the host plant through transgenic techniques. Host-induced gene Silencing (HIGS) has proven to be an effective technique for controlling phytopathogenic fungi such as *Fusarium spp.* (RAY *et al.*, 2022), *Botrytis cinerea* (XIONG *et al.*, 2019) and *R. solani* (RAO *et al.*, 2019) by reducing fungal colonization, virulence, and symptoms. While HIGS proves effective, its limitations in transformation, lengthy development cycles, regulatory issues, and consumer resistance to transgenic cultivars restrict its field application (HE *et al.*, 2024; MANN *et al.*, 2023).

Spray-induced gene silencing (SIGS) involves applying dsRNA (double-stranded RNA) or siRNA (small interfering RNA) to plant leaves to activate the RNAi mechanism (MCRAE *et al.*, 2023; KOCH *et al.*, 2016). In this strategy, dsRNA molecules are applied to the leaves. Theoretically, the dsRNA molecules could be translocated from the leaves to other parts of the plant, delivered to the fungus, or processed by the plant's RNAi machinery into siRNA (QIAO *et al.*, 2021; CAI *et al.*, 2018). Both pathways would activate the RNAi gene-silencing machinery in the pathogen (RAY *et al.*, 2022) (Figure 1).

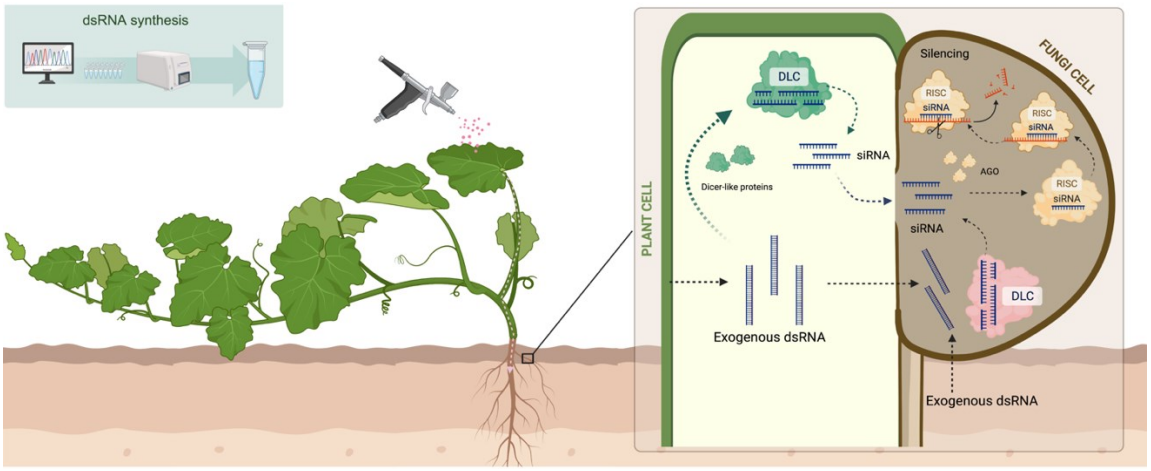

Figure 1. Theoretical approaches and pathways of the RNAi-spray-induced gene silencing (RNAi-SIGS) mechanism (created with BioRender.com).

RNAi-SIGS technology, therefore, has excellent potential for controlling pathogens in agriculture. Several studies have reported the ability of siRNA/dsRNA to protect plants against pathogens such as *Sclerotinia sclerotiorum* (RANA *et al.*, 2022), *Botrytis cinerea* (NIÑO-SÁNCHEZ *et al.*, 2022), *Fusarium spp.*, and *Phakopsora pachyrhizi* (RAY *et al.*, 2022).

A key limitation of SIGS for large-scale applications is the instability of RNA molecules, including siRNA and dsRNA (YANG *et al.*, 2022). These molecules are susceptible to degradation due to unfavorable temperature, pH, and light conditions, as well as by nucleases (HE *et al.*, 2024; DUBELMAN *et al.*, 2014). Consequently, they may degrade in the environment before reaching their intended target. The SIGS approach often involves employing nanoparticles to address these challenges.

Nanoparticles are defined as molecules between 1 to 100 nm in size (SHIDORE *et al.*, 2021). They have been studied in agriculture to increase the efficiency of various active ingredients, such as herbicides, fungicides, fertilizers, and others (SAMPATHKUMAR; TAN; LOO, 2020). In RNAi-SIGS, nanoparticles play a significant role in protecting dsRNA and/or siRNA molecules and increasing their absorption by plant and fungal cells (RAY *et al.*, 2022).

Nanoparticles can be synthesized from organic or inorganic materials. Among the inorganic molecules, nanoparticles made of carbon quantum dots, layered double hydroxides, and nanoparticles synthesized from gold and zinc have increased the efficiency of the RNAi mechanism against pathogenic fungi (WANG *et al.*, 2023; YANG *et al.*, 2022). Among the nanoparticles based on organic molecules, chitosan nanoparticles

have gained significant prominence in the dsRNA delivery system to plants (KHAN; JIN; KIM, 2024; KOLGE *et al.*, 2021).

Chitosan is a polymer obtained through the deacetylation of chitin. Chitin is a structural polymer found in various organisms, such as crustaceans and fungi (MUJTABA *et al.*, 2020). An essential characteristic of chitosan is its ability to complex with negatively charged molecules, such as nucleic acids, through electrostatic interactions (BUSCHMANN *et al.*, 2013). This characteristic also allows these nanoparticles to interact with cell membranes, which are also negatively charged, increasing active membrane uptake (ARJUNAN; THIRUVENGADAM; SUSHIL, 2024) or the activation of clathrin-mediated endocytosis (ZHOU *et al.*, 2023; LICHTENBERG *et al.*, 2019). In addition, nanoparticles are relatively inexpensive in production, non-toxic, and biodegradable, making them an excellent delivery system for dsRNA on a large scale in field conditions (CHEN *et al.*, 2023).

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CHAPTER 1

The format of this chapter follows the rules of the journal Crop Protection, to which the manuscript will be submitted.

First report of Grapevine red blotch virus causing grapevine red blotch disease in New England vineyards

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ABSTRACT

Grapevine red blotch virus (GRBV) is an emerging pathogen that adversely affects grapevine production and quality worldwide. The pathogen has been reported infecting plants in many vineyards in the largest producing areas of the United States (i.e., California, Washington, and Oregon). However, its presence in the Southeastern New England American Viticultural Area (SNE-AVA) states was unknown. Herein, we report the detection of GRBV in Connecticut, Rhode Island, and Massachusetts vineyards surveyed from 2018 to 2020. Symptomatic plant samples from five SNE-AVA vineyards were initially tested for RNA viruses. Samples that tested negative were subjected to Illumina high-throughput sequencing. Subsequently, DNA was extracted and amplified through rolling circle amplification (RCA), and the product was used as a template for PCR with GRBV primers. The PCR product was submitted to Sanger sequencing. GRBV was detected in all five vineyards analyzed. Phylogenetic analysis revealed that samples were clustered in two different clades. The considerable genetic variability observed within and between isolates suggests the occurrence of potential recombination events. These findings underscore the importance of closely monitoring and applying management practices to mitigate the GRBV dispersion in the region and the economic losses that it can cause to New England grape growers.

Keywords: *Geminiviridae*, genetic diversity, virus survey, *Vitis vinifera*, wine grape

1. INTRODUCTION

Grapevines (*Vitis vinifera* L.) are susceptible to more than eighty-six species of viruses (Fuchs, 2020). One of these viruses is the Grapevine red blotch virus (GRBV), which causes symptoms on leaves and fruits. In red-berry cultivars, leaves from infected plants display a red blotch that spreads to the entire leaf throughout the season. In white-berry cultivars, symptoms consist of chlorotic lesions that later become necrotic (Cieniewicz et al., 2017; Krenz et al., 2023). In fruits, the disease reduces ripeness, total soluble sugars, and pigments, which drastically affects the quality of the wine produced from GRBV-infected fruit juice (Rumbaugh et al., 2021).

Grapevine red blotch virus belongs to the genus *Grabovirus* in the family *Geminiviridae* (Varsani et al., 2017). It bears a single circular single-stranded DNA (ssDNA) genome that is about 3,200 kb and encodes seven bidirectional overlapping open reading frames (ORFs) in a complementary (C1-C3) and viral sense (V0-V3). These ORFs are translated into proteins responsible for the virion capsid (CP), replication (Rep), and viral movement (Cieniewicz et al., 2017). Based on their genetic variability, GRBV isolates are divided into two clades. Clade 1 has up to 94.8% nucleotide sequence similarity, while clade 2 has 98.8% similarity (Krenz et al., 2014).

The primary method for GRBV spread among vineyards is through infected propagating material and grafting (Krenz et al., 2023). In vineyards, secondary transmission is associated with the vector three-cornered alfalfa hopper, *Spissistilus festinus* (Hemiptera: *Membracidae*) (Hoyle et al., 2022). GRBV was detected in most producing areas of the United States, Canada, Mexico, Argentina, Australia, and Europe (EPPO, 2024). Despite the detection of GRBV in neighboring states (New York) (Cieniewicz et al., 2019), there have been no reported cases in New England (NE)

vineyards. In this study, we describe the first report of GRBV in SNE-AVA vineyards, based on genome sequencing and phylogenetic analysis.

2. MATERIAL AND METHODS

During an extensive survey conducted from 2018 to 2020 of several grapevine RNA viruses in New England vineyards, we collected a total of 28 leaf samples from five vineyards (Table 1). These samples came from plants displaying symptoms similar to leafroll disease (redness, yellowing, leaf distortion, and chlorotic mottling) that tested negative for all viruses included in our survey. The samples were stored at -80°C for downstream analyses. The total RNA was extracted (PureLink RNA Mini Kit, Invitrogen) from five samples (one leaf sample from each vineyard), and 0.5 μg of each RNA sample was mixed to form a composite sample (Borges et al., 2020; Dumas et al., 2023).

The composite sample was sent to Yale Center for Genome Analysis, Yale University, New Haven, CT, USA, for library preparation using the ribosomal RNA depletion protocol (NEB Waltham, MA) and sequenced on an Illumina NextSeq2000 platform (generating 150 bp paired-end reads). The 66,300,500 clean reads obtained were assembled into contigs using Megahit version 1.1.2 software. Nine contigs shared nucleotide identity ranging from 84% to 97% with the GRBV isolate CF214-1 (KC896623.1); no contigs were mapped to other viral and viroidal sequences in GenBank databases.

Next, total DNA extraction (E.Z.N.A. Plant & Fungal DNA Kit, Omega) was performed on all 28 samples and subjected to amplification by rolling circle amplification (RCA) (Haible et al., 2006). The primers 1402c/1404v and 2287v/604c (Ouro-Djobo et al., 2023) were used to amplify the GRBV genome using the RCA amplicons as the template. These primers can amplify the entire circular genome. The PCR amplicons were

resolved in 1% agarose gel and Sanger sequenced at the Keck Lab at Yale University. The sequences were assembled on SnapGene software (Dotmatics; version 7.2) using the GRBV isolate CF214-1 (KC896623.1) as the reference genome.

Table 1. Isolates of GRBV obtained in this study and from GenBank and used for phylogenetic analyses and recombination studies.

Isolate	Genbank access code	Country - state	Collection year	Reference
WCT01	PQ601718	US - Connecticut	2019	This study
WCT02	PQ601723	US - Connecticut	2019	This study
PRI03	PQ601724	US - Rhode Island	2019	This study
PRI04	PQ601722	US - Rhode Island	2019	This study
PRI05	PQ601720	US - Rhode Island	2019	This study
MA07	PQ601721	US - Massachusetts	2019	This study
FRI09	PQ601707	US - Rhode Island	2020	This study
FRI10	PQ601725	US - Rhode Island	2020	This study
FRI11	PQ601713	US - Rhode Island	2020	This study
FRI12	PQ601719	US - Rhode Island	2020	This study
NCT13	PQ601710	US - Connecticut	2020	This study
NCT14	PQ601708	US - Connecticut	2020	This study
NCT17	PQ601714	US - Connecticut	2020	This study
NCT18	PQ601709	US - Connecticut	2020	This study
NCT22	PQ601715	US - Connecticut	2020	This study
NCT23	PQ601716	US - Connecticut	2020	This study
NCT25	PQ601711	US - Connecticut	2020	This study
NCT28	PQ601717	US - Connecticut	2020	This study
GUADALUPE-JGB	MH557096.1	Mexico	2017	Gasperin-Bulbarela et al. (2018)
LNSA1	MF696145.1	US - Washington	2016	Adiputra and Naidu (2017)

NY147	KF751708.1	US - New York	2012	Krenz et al. (2014)
COCF93	MF795169.1	US - Washington	2016	Adiputra and Naidu (2017)
AZMVTN	OM135587.1	US - Arizona	2021	Jiahuai Hu (2022)
MO-CC7	MH732736.1	US - Missouri	2017	Schoelz et al. (2018)
NY701	KU564249	US - California	2012	Perry et al. (2016)
OH15-44	KY498151.1	US - Ohio	2015	Yao et al. (2021)
CS337-1	KC896624.1	US - California	2010	Rwahnih et al. (2013)
ONRB7	KY316025	Canada	2015	Adiputra and Naidu (2017)
NIAGARA_115	OR478449.1	Canada	2016	Xiao and Meng (2023)
SW6	KU821056.1	South Korea	2014	Lim et al. (2016)
COCF93	MF795169.1	US - Washington	2016	Adiputra and Naidu (2017)
MWC#166	MN186404.1	US - North Carolina	2018	Hoffmann et al. (2020)
GIGV-WA-MR	KC427995.1	US - Washington	2011	Poojari et al. (2013)
GRBV_ID14_SYRAH_2014	MK928383.1	US - Ohio	2014	Thompson et al. (2019)
NY147	KF751708.1	US - New York	2012	Krenz et al. (2014)
NSRB3	MH476296.1	Canada	2016	Poojari et al. (2013)
NPPS7	MF795160	US - Washington	2014	Adiputra and Naidu (2017)
OLSY4	MF795162.1	US - Washington	2015	Adiputra and Naidu (2017)
IDGN596	MF795171.1	US - Washington	2015	Adiputra and Naidu (2017)
TUMR5	MF795164.1	US - Washington	2015	Adiputra and Naidu (2017)

Searches for genetic similarity were conducted using NCBI-BLAST. Multiple sequence alignment was conducted using ClustalW, and phylogenetic trees were constructed by using the Maximum likelihood method based on the Tamura–Nei model on MEGA-11 Software (Version 11.0.3) (Tamura et al., 2021). Nucleotide sequences were

obtained from NCBI to create the phylogenetic tree, determining the clade to which SNE-AVA (The Southeastern New England American Viticultural Area) belongs. To generate the pairwise nucleotide identity scores, aligned sequences were submitted to the Sequence Demarcation Tool (SDT) software (version 1.2) (Muhire et al., 2014). The recombinant analysis was performed using the RPD4 software (version 4.72) (Martin et al., 2015), where seven different recombination detection algorithms (RDP method, GENECONV, BOOTSCAN, MAXCHI, Chimaera, 3SEQ, and SISCAN) were used to detect potential recombination breakpoints, using default settings for each analysis method with 1000 bootstraps and a Bonferroni-corrected P-value of 0.05. Only recombination events detected by at least four methods were considered reliable.

3. RESULTS AND DISCUSSION

Eighteen of the 28 samples analyzed tested positive for GRBV by PCR and were confirmed by blasting analysis results using the Sanger sequences acquired. The whole genome sequence was acquired for seven isolates and partial genome sequences for 11. All sequences were deposited in GenBank (Table 1). All samples tested herein had already been tested for other grapevine viruses as part of a large grapevine virus survey in the Southeastern New England American Viticultural Area (SNE-AVA) (Borges et al., 2020; Dumas et al., 2023), which includes parts of three southern New England states: Massachusetts, Connecticut, and Rhode Island (America-Appellation, 2021). The fact that 10 of the symptomatic plants test negative for all viruses tested; corroborates the hypothesis that other viruses may be infecting those samples, or those samples are suffering from physiological disorder or nutrient deficiency. These samples need to be tested for the presence of other viruses to investigate this hypothesis.

Phylogenetic analyses of the sequences acquired in this study separated the SNE-AVA isolates into two distinct phylogenetic clades (Fig 1). Sixteen isolates grouped in clade 1, while only two isolates, WTC02 and PRI04 from CT and RI, respectively, were grouped in clade 2 (Fig 1). The phylogenetic analyses indicate that the GRBV isolates from SNE-AVA may have different origins. In fact, the information provided by growers reveals that all WTC, FRI, and PRI isolates came from plant material purchased from California nurseries, while all NCT isolates came from plant material purchased from New York nurseries. There was no information available on the origin of the plant material of sample MA07, collected from a Massachusetts vineyard.

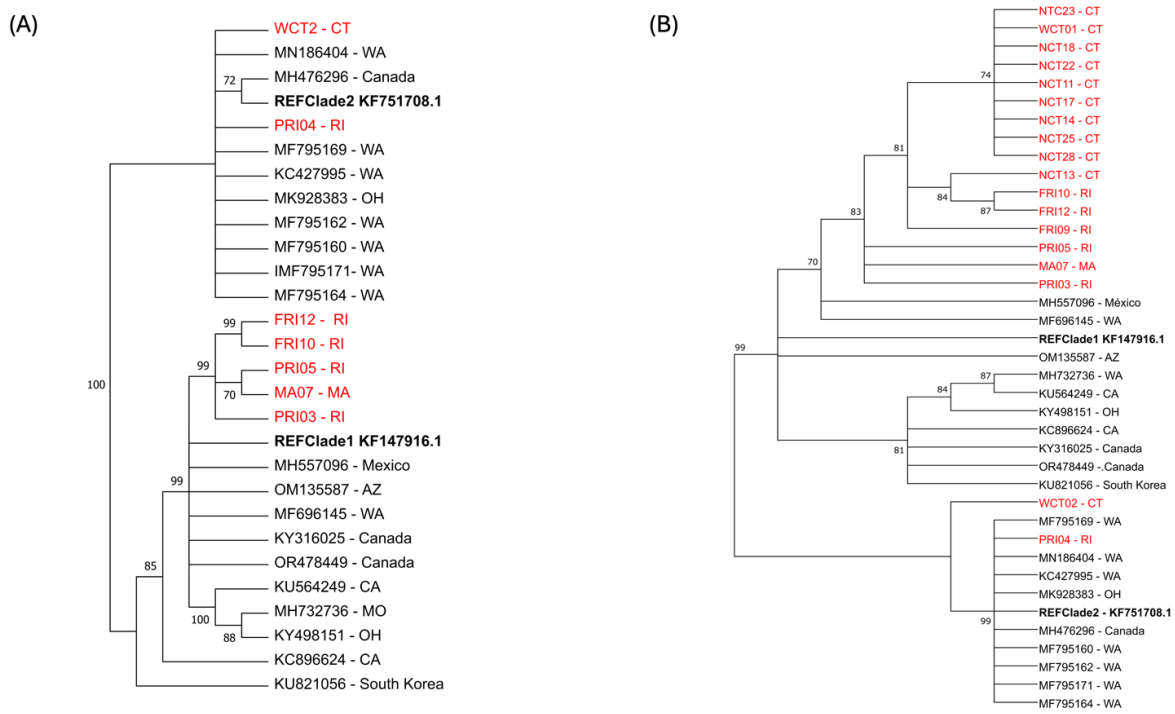

Figure 1 Phylogenetic analysis of GRBV SNE-AVA isolates. An unrooted Maximum Likelihood Phylogenetic tree, created using complete (A) and partial (B) genomes, shows that SNE-AVA isolates (in red) are grouped into two separate clades. The isolates in black originate from NCBI GenBank, while the bold ones denote reference isolates from GRBV clades. The numbers at the nodes indicate the bootstrap reliability level based on 1000 replicates.

The complete genome pairwise nucleotide sequences identified between the SNE-AVA GRBV isolates ranged from 91.9% to 100% nucleotide identity (Fig. 2). PRI04 (RI) was the most divergent isolate, with an average of 92.2% similarity compared with all isolates. There was a comparable observation between clades (91.4% - 100%), with the most divergent isolates being PRI05 (Clade 1) and TUMR5 (Clade 2) from RI (Fig 2). These results indicate significant genetic variability among the SNE-GRBV isolates.

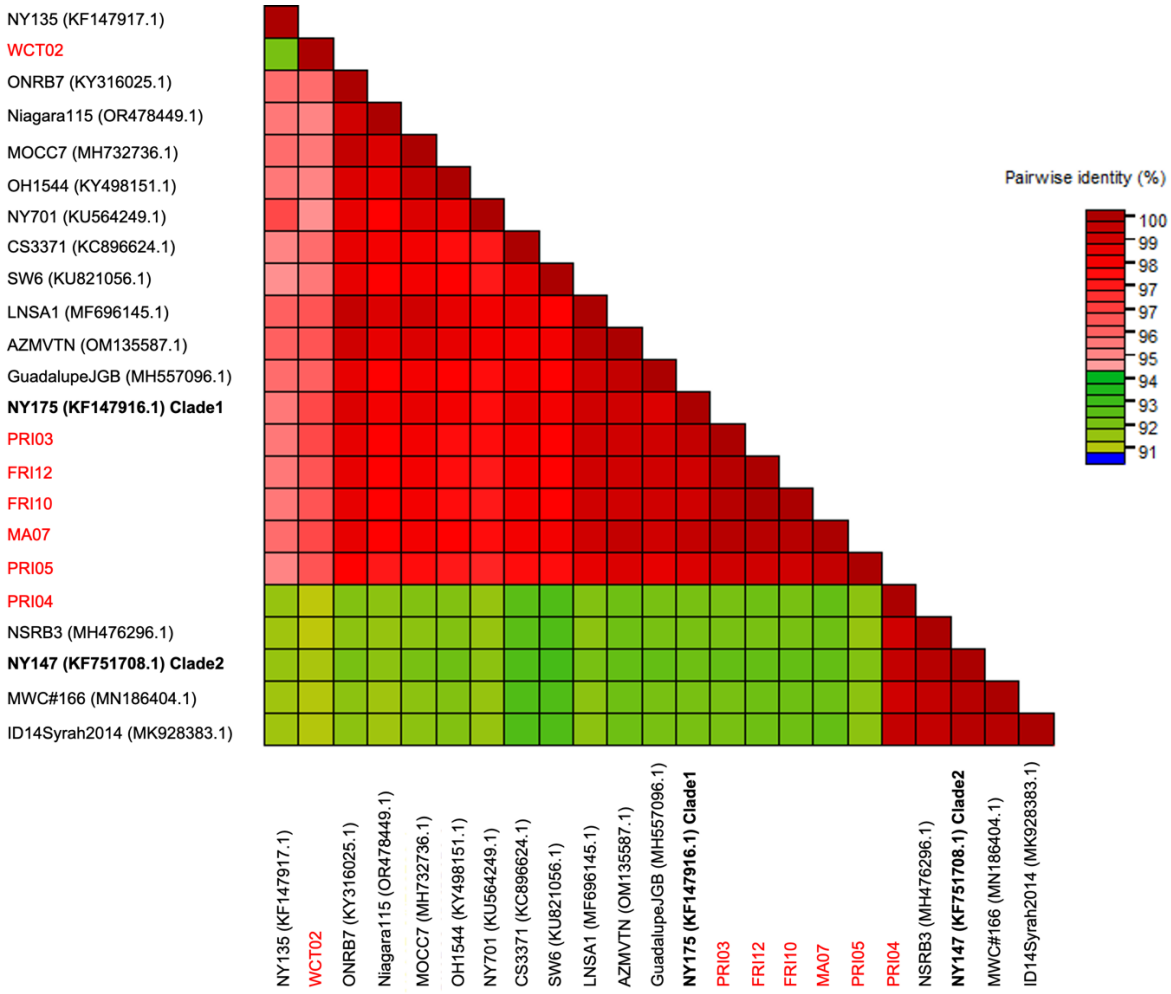

Figure 2. A sequence identity matrix (pairwise identity) generated using the SDT software (species demarcation tool) reveals the percentage identity among NCBI (in black) and SNE-AVA GRBV (in red) isolates.

The high intraspecific variability of viruses allows better adaptation. In *Geminiviridae* viruses, variants accumulate rapidly during the infection process. This rapid

variation enables the virus to evade the plants innate defense mechanisms and adapt to new hosts and environments (Juárez et al., 2019; Sánchez-Campos et al., 2018). In California vineyards, GRBV was found infecting wild grapevines, exhibiting considerable variability among the isolates sampled from cultivated grapevines (Perry et al., 2016).

The sources of genetic variation in GRBV genomes have yet to be fully understood. The increased fitness found in variants of *Geminiviridae* is determined by the level of mixing among viral lineages. To explore this further, a recombination analysis was conducted on NE and GenBank reference isolates to investigate the sources of genetic variability. Eleven recombination events were detected (Table S1), but only eight statistically significant events were considered in at least four analysis methods (Figure 3). The sample PRI03 was indicated to be recombinant in one phylogenetic method (Boostcan) and three substitution methods (Maxchi, Chimarea, and GENECONV). The same event was found in clade-1 samples: KF147916.1, FRI10, FRI12, MA07, and PRI05. The major parent indicated by the analysis is the NY701 (KU564249.1) isolate from Napa Valley (CA). The breakpoint of the recombination event is localized between nucleotides 709 and 832 on the GRBV genome, part of the coat protein coding sequence (CP).

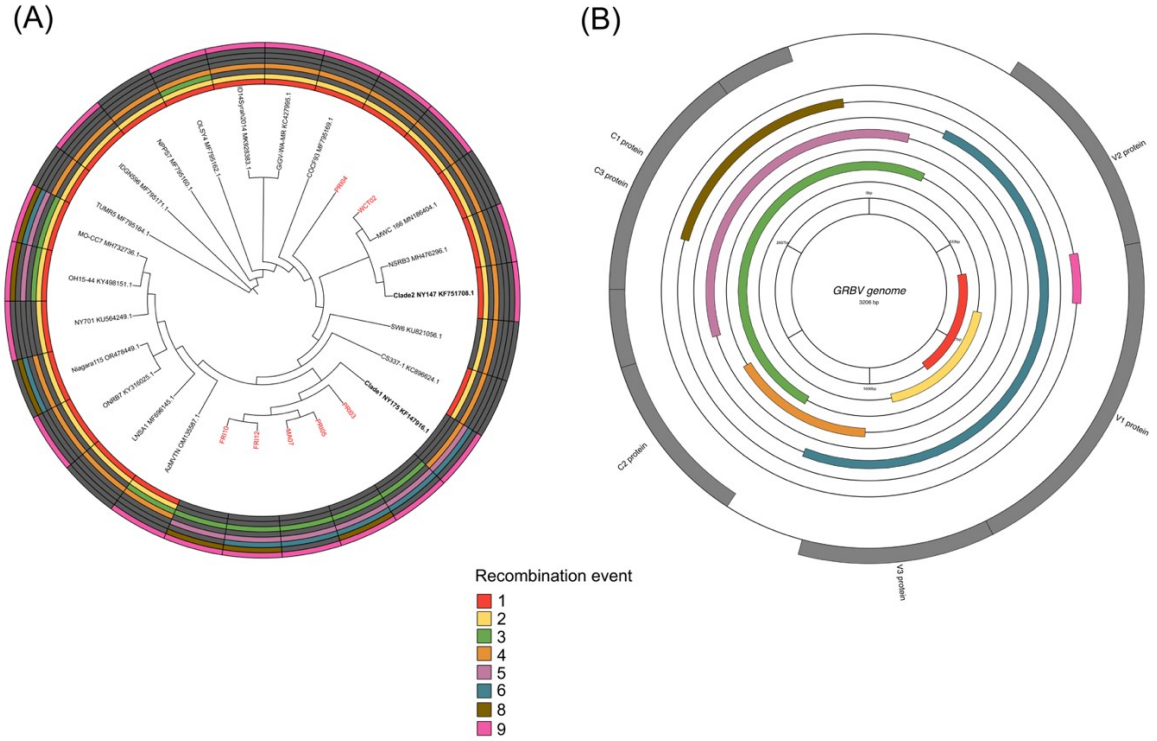

Figure 3. Analysis of recombination events generated by RDP4 software. The heat-map phylogenetic tree (A) shows where in which isolates the recombination event was detected and the location of the recombination event breakpoint in the GRBV genome (B).

The high similarity among isolates from SNE-AVA and other U.S. states, as well as none of the recombination events being intra-isolate, suggests the virus distribution may originate from several inoculum sources. The exponential increase in grape production in NE states may result in producers exchanging materials and using non-certified virus-free plants. There is concern about whether these materials may come from California, where GRBV was detected in germplasm from cultivated vineyards as early as the 1940s in California (Al Rwahnih et al., 2015; Krenz et al., 2023).

This study presents the first report of GRBV in SNE-AVA vineyards. Although the vector of GRBV, the three-cornered alfalfa hopper (*S. festinus*) (Flasco et al., 2021, 2023), has not been reported in the region, a pest management program combined with the use of certified virus-free material is recommended for GRBV management in the region.

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CHAPTER 2

SPRAY-INDUCED GENE SILENCING TO CONTROL ROOT ROT CAUSED BY *Rhizoctonia solani*

ABSTRACT

Soil-borne fungi have a significant impact on crop production worldwide. The cultivation of melons (*Cucumis melo* L.), which are of considerable socioeconomic importance, is profoundly influenced by fungal pathogens. Controlling root rot caused by *Rhizoctonia solani* presents a complex challenge due to its ability to survive in the soil for extended periods and the many potential hosts. RNA interference Spray-Induced Gene Silencing (RNAi-SIGS) technology has been explored for fungal control. In this study, we investigated the application of RNAi-SIGS targeting the genetic silencing of enzymes essential for the pathogenicity of *R. solani* using *in vitro* assays, detached leaves, and melon plants. To enhance the effectiveness of RNAi-SIGS, dsRNA was encapsulated in chitosan nanoparticles. In both *in vitro* and detached leaf assays, we observed reduced mycelial growth on the pectin-based medium and colonization on plant tissues. Greenhouse experiments showed a decrease in the harmful effects of *R. solani* infection in treated plants after thirty days. The increased efficiency of RNAi with chitosan nanoparticles confirms the advantages of using nanoparticles for this purpose. These findings indicate that this technology has considerable potential for large-scale management of soil-borne fungi.

Keywords: RNA interference, *Cucumis melo* L, nanotechnology, chitosan.

1. INTRODUCTION

The presence of soil-borne fungi significantly reduces the productivity of cucurbits. *Rhizoctonia solani* Kuhn (telemorph *Thanatephorus caumeris* (Frank) Donk) is a pathogen with a broad host range (XUE *et al.*, 2018). In melons (*Cucumis melo* L.), productivity damage can reach 50% (PORTO *et al.*, 2019), significantly harming crop quality and productivity. The fungus can cause diseases at all stages of plant development,

including seed rot, early seedling death (MICHEREFF; ANDRADE; SALES JÚNIOR, 2008), root rot, and, most commonly, vine rot collapse (SALES JÚNIOR *et al.*, 2015).

Studies of pathogen-host interactions have revealed mechanisms of pathogenicity. *R. solani* produces several virulence factors that induce toxicity, inhibit innate immunity, and degrade host plant tissues (LI *et al.*, 2019; CHEN *et al.*, 2017). The expression and activity of cell-wall degrading enzymes (CWDEs) are crucial for initiating the infection process that leads to tissue colonization, nutrient uptake, and disease establishment (CHEN *et al.*, 2018; XUE *et al.*, 2018). The expression of CWDEs like polygalacturonases (PGs) and xylanases (Xly) has been investigated as an essential factor in pathogenicity (ANDERSON *et al.*, 2017; RAO *et al.*, 2019).

PGs (Endo-polygalacturonases, EC 3.2.1.15) are part of the glycoside hydrolase family 28 (GH28) and catalyze the hydrolysis of the α-1,4-glycosidic bond of d-galacturonic acid in pectin (RAO *et al.*, 2019). The production of PGs by *R. solani* is a key virulence factor; when its expression is high, symptoms such as chlorosis, membrane damage, and tissue maceration are observed, while inhibition of its expression leads to decreased severity (TANG *et al.*, 2023; CHEN *et al.*, 2017, 2016). Xylanases (Endo-β-1,4-xylanases, EC 3.2.1.8), which belong to the glycoside hydrolase family 10 (GH10), cleave the internal β-1,4 bonds between xylose units in xylan (DORNEZ *et al.*, 2010). Similar to PGs, this class plays a role in the successful infection of pathogens, and their disruption results in reduced virulence and symptom development (ANDERSON *et al.*, 2017; LU; LIU; ZHANG, 2020).

Understanding these key-pathogenicity mechanisms enables the development of effective control methods. RNA interference (RNAi) is a post-transcriptional regulatory mechanism in eukaryotes that silences specific genes (ROSA *et al.*, 2018). This silencing machinery can be activated using double-stranded RNA (dsRNA) that targets a particular gene. The dsRNA is processed into small interfering RNAs (siRNA) measuring 21-25 nucleotides in length by the enzyme DICER (LAX *et al.*, 2020; AGRAWAL *et al.*, 2003). siRNAs are recognized and associated with a protein complex, which includes argonaute proteins (AGO), to form the RNA-induced silencing complex (RISC). Once incorporated into the RISC, the guide strand of the siRNA recognizes the complementary messenger RNA (mRNA), leading to the degradation of the gene, and the resulting silencing occurs (WYTINCK *et al.*, 2020; ROSA *et al.*, 2018).

Using RNAi based on non-transgenic delivery systems, such as SIGS (Spray-Induced Gene Silencing), facilitates the application of this technology for large-scale

pathogen control (MCRAE *et al.*, 2023; RAY *et al.*, 2022). However, a limiting factor of SIGS is the instability of RNA molecules in the environment. dsRNA and/or siRNA molecules have low stability under adverse abiotic conditions, such as temperature and light, and are susceptible to nucleases (DUBELMAN *et al.*, 2014). The association of RNA molecules with nanoparticles has enhanced their stability and the efficiency of RNAi (MCRAE *et al.*, 2023; RAY *et al.*, 2022).

Nanoparticles are innovative materials that range in size from 1 to 100 nm (SHIDORE *et al.*, 2021). These molecules can comprise various materials, including lipids, peptides, proteins, and polymers (MOAZZAM *et al.*, 2024). One natural polymer gaining prominence in agriculture is chitosan. Chitosan is a polymer derived from the deacetylation of chitin, which is found in the shells of crustaceans and certain fungi (ARJUNAN; THIRUVENGADAM; SUSHIL, 2024). Its unique property of being positively charged allows it to interact with cell membranes, promoting endocytosis and facilitating the internalization of dsRNA into cells, thus reducing the chances of degradation in the environment (LICHTENBERG *et al.*, 2019; SANDAL *et al.*, 2023). Additionally, chitosan is non-toxic, biodegradable, and has a relatively low synthesis cost, making it an attractive option for delivering active molecules (VASQUEZ *et al.*, 2023).

Silencing genes that encode key enzymes involved in *R. solani* pathogenicity offers a promising strategy for sustainable disease management. This study aimed to evaluate the potential of RNAi-SIGS to control *R. solani* through *in vitro* and greenhouse assays. We also assessed the use of chitosan nanoparticles as a delivery system to improve the stability and effectiveness of dsRNA molecules.

2. MATERIAL AND METHODS

2.1 Fungal isolate and plant materials

The Valley Laboratory of the Connecticut Agricultural Experiment Station provided the isolate *R. solani* NT03 (isolated from *Nicotiana tabacum*). It was cultured on a PDA (Potato Dextrose Agar) medium and identified as *R. solani* AG2-1 through the barcoding of genetic regions. Previous tests proved the pathogenicity of the isolate in melon and tobacco (data not shown).

Tobacco leaves served as an experimental model for *in vitro* studies. The Samsun tobacco cultivar was propagated and cultivated in a growth chamber regulated at 27°C with 65% relative humidity, following a 16-hour light and 8-hour dark cycle. Golden

(TopSeed Garden) yellow melon plants were initially sown in seedbeds for the greenhouse experiments. Once germinated, the seedlings were moved to 1.0 L pots containing a 2:1 mix of soil and organic substrate, both autoclaved before use. The melon plants were grown in the greenhouse under natural light and temperature conditions.

2.2 Selection of target genes and dsRNA synthesis

Endo-polygalacturonase 1 (AG1IA_04727) (RAO *et al.*, 2019) and 1,4- Beta-xylanase (RSAG8_07159) (ANDERSON *et al.*, 2017) were selected due to their relation with the pathogenicity of *R. solani* AG1 and AG8. The protein-coding regions were accessed on the EnsemblFungi website (http://fungi.ensembl.org/index.html). To find homologous sequences in *R. solani* AG1-2, similarity searches were performed in NCBI BLAST (National Center for Biotechnology Information). Then, using the software siFi21 (LÜCK *et al.*, 2019) and sidirect2 (https://sidirect2.rnai.jp/), the sequences with the highest probability of generating small RNAs were identified.

Gene fragments were amplified by PCR to synthesize PG1 and Xly dsRNAs, using primers designed on the NCBI PrimerBlast website (listed in Table 1). PCR amplifications were performed using Phusion High–Fidelity DNA Polymerase (Thermo Scientific, USA) following the instructions of the manufacturer. The resulting PCR products were sequenced and purified with the ChargeSwitch PCR Clean-Up Kit (Invitrogen, CA-USA). The dsRNA from each region was synthesized with the T7 RiboMAX Express Large-Scale RNA Production Kit (Promega). The concentration was determined using a Nanodrop.

Table 1. Primers used for PCR amplification, sequencing, and dsRNA synthesis.

Primer ID	**Primer sequence (5´to 3´)**
PG1_F	CGATGCACTGGCACAATCTC
PG1_R	GTTGAGCACCTTGACGTTCG
Xly_F	ATTTCAGCGCCACAACCATC
Xly_R	ACGGAGGGTTCAATCGCATC
PG1dsRNA_F	TAATACGACTCACTATAGGGCGATGCACTGGCACAATCTC
PG1dsRNA_R	TAATACGACTCACTATAGGGGTTGAGCACCTTGACGTTCG
XlydsRNA_F	TAATACGACTCACTATAGGGATTTCAGCGCCACAACCATC
XlydsRNA_R	TAATACGACTCACTATAGGGACGGAGGGTTCAATCGCATC

2.3 Chitosan nanoparticle synthesis, characterization, and dsRNA loading

Chitosan (MW: 27 kDA, degree of deacetylation ≥ 75%) and tripolyphosphate (TPP) were purchased from Sigma-Aldrich (St. Louis, MO, USA). A chitosan stock solution was prepared by dissolving the powder in 0.2% acetic acid while stirring magnetically for 16 hours at pH 4.7. A 0.1% TPP solution was made by dissolving the powder in deionized water. Before synthesis, both solutions were filtered through a 0.45 μm membrane (Millipore) (ZHANG; ZHANG; ZHU, 2010).

To determine the optimal ratio between dsRNA and chitosan, dsRNA was mixed with chitosan at various ratios: dsRNA to chitosan (w/w) (1:0.5, 1:1, 1:1.5, 1:2, 1:4, 1:6, and 1:8). The samples were stirred at 1000 rpm for 30 minutes on a magnetic stirrer. Then, 20% of the 1% TPP solution volume was added (ZHANG; ZHANG; ZHU, 2010). To verify the dsRNA encapsulation in chitosan nanoparticles, an Electrophoretic Mobility Shift Assay (EMSA) was performed by adding the samples to an agarose gel; these samples were subjected to electrophoresis in 1X TE buffer at a constant voltage of 70 V. The gel was stained with ethidium bromide (0.5 $\mu g/ml^{-1}$) and photographed using a UV photodocumenter. The sample with the optimal ratio was characterized using a Zetasizer (Malvern MAN0486-0), where size (nm), polydispersity (Pdi), and charge (Z potentials) were analyzed. Each sample was analyzed in three replicates of 10, and the averages were observed.

2.4 *In vitro* growth inhibition assay

The inhibition capacity of the synthesized dsRNA molecules was tested in two types of culture medium. The commercial culture medium PDA was prepared according to the instructions of the manufacturer. The second medium was Pectinase Screening Agar Medium (PSAM), prepared according to the provided recipe (KHALIL *et al.*, 2020). The mediums were autoclaved at 121°C for 15 minutes and poured into Petri dishes.

An inhibition assay was conducted by pipetting 20 μg of each dsRNA solution into the center of the plate and then adding a disk of a pure culture of *R. solani* NT03. The mock included only sterile water. The plates were sealed with parafilm and transferred to an incubator at 28°C for 5 days. After this period, the plates were photographed.

2.5 Detached leaf assay

Medium-sized (± 12 cm) healthy *N. tabacum* leaves were collected by cutting the base with a sterile scalpel. The leaves were subjected to a surface disinfestation with a 0.7% PPM (Plant Preservative Mixture, Plant Cell Technology, Inc., Washington, DC) solution for 10 minutes and washed three times with sterile deionized water. The leaves were transferred to square Petri plates (100 x 100 mm) with a drop of approximately 3 ml of MS Agar medium in the corner of each plate. The petiole was cut with a sterile scalpel and placed in the drop to prevent drying and supply minerals to the tissue during the assay.

The treatments used in the experiments consisted of naked dsRNA (dsRNAPG1 and dsRNAXly14), dsRNA encapsulated by chitosan nanoparticles (Cs-dsRNA-PG1 and Cs-dsRNA-Xly), and experimental controls (Silwet LT-77 solution, chitosan, and mock). For treatments containing naked dsRNA, 20 ug was added in 0.015% Silwet LT-77 (final volume of 100 µl per treatment). For treatments with dsRNA encapsulated by chitosan nanoparticles, the solutions were centrifuged, the supernatant was collected, and 20 µg of dsRNA/cs-nanoparticles was applied to the leaf surface. Only the Silwet LT-77 solution and chitosan (Cs) nanoparticles were applied as an experimental control. The mock was the sterile water solution. The solutions were applied to the leaves using a pipette and spread with a Drigalski handle. After the solutions had dried on the surface of the leaves (approximately two hours), the leaves were inoculated with a seven-day-old 5 mm PDA pure culture of the *R. solani* NT03 placed on the center of the leaf. The plates were sealed with parafilm and transferred to a growth chamber (27°C, 65% relative humidity, 16h day, eighth night cycle) for 7 days. Each plate was photographed, and the relative infected area was measured using the Leaf Doctor application (PETHYBRIDGE; NELSON, 2015).

2.6 Greenhouse assays

To verify the ability of dsRNA treatments to control melon root rot, we performed plant assays under greenhouse conditions. The experiment consisted of four treatments (naked dsRNA: dsRNAPG1 and dsRNAXly14; dsRNA encapsulated by chitosan nanoparticles: Cs-dsRNA-PG1 and Cs-dsRNA-Xly) and two experimental controls (chitosan and the mock). Ten-days-old melon plants were treated with 100 µg of each dsRNA in a 0.05% Tween 20 solution (final volume of 200 µmL per plant). For the solutions containing chitosan nanoparticles, the prepared solutions were centrifuged at 10,000 rpm for 5 minutes, and the same 100 µg of dsRNA/cs-nanoparticles (final volume

per plant of 200 μL) was applied to young leaves. As experimental controls, chitosan nanoparticles (cs-control), and sterile distilled water (mock) were applied. The treatment was applied manually using a pipette and gently massaging the leaf.

Twenty-four hours after treatment, the pathogen was inoculated using the infested toothpick method (AMBRÓSIO *et al.*, 2015). The toothpicks were inserted into the seedling stem about 5 cm from the substrate. Autoclaved toothpicks served as a mock. The seedlings were kept in a greenhouse, and the severity and incidence were assessed at 15 and 30 days using a severity scale proposed by (AMBRÓSIO *et al.*, 2015), where 0 = symptomless, 1 = less than 3% of shoot tissues infected, 2 = 3–10% of shoot tissues infected, 3 = 11–25% of shoot tissues infected, 4 = 26–50% of shoot tissues infected, and 5 = more than 50% of shoot tissues infected. The size of the aerial and root parts of all the plants was measured with a caliper.

2.7 Statistics and reproducibility

In vitro experiments consisted of five replicates for each treatment and control. Mycelial growth was assessed using a caliper. Treatment means underwent one-way ANOVA, followed by Tukey test at a significance level of 5% ($p < 0.05$). Detached leaf assays were performed with five replicates per treatment and independently repeated at least twice. The relative infected area of the leaves, evaluated with the Leaf Doctor app, was analyzed using the Kruskal-Wallis test. When significant differences were found, the Conover post hoc test with Bonferroni correction was applied. Severity data from two replicated greenhouse experiments (5 replicates per treatment) were analyzed using the Kruskal-Wallis test, followed by Dunn test ($\alpha = 0.05$) for multiple comparisons. Data regarding shoot and root growth were also analyzed using one-way ANOVA followed by Tukey test at a 5% significance level. All statistical analyses were conducted in R Studio with the PMCMRplus, ggplot2, ggpubr, and ggsignif packages.

3. RESULTS

3.1 Synthesis of chitosan nanoparticles

The chitosan-dsRNA nanoparticles were prepared using the ionic gelation method. Several formulations with different mass ratios were created to find the optimal balance between the amount of dsRNA and chitosan. These formulations were tested with a retardation assay in an electrophoresis gel (Figure 1a). Encapsulation is considered

successful when the dsRNA fragment is no longer visible. The tests confirmed that complete encapsulation of the dsRNA occurs starting from a 1:1 ratio.

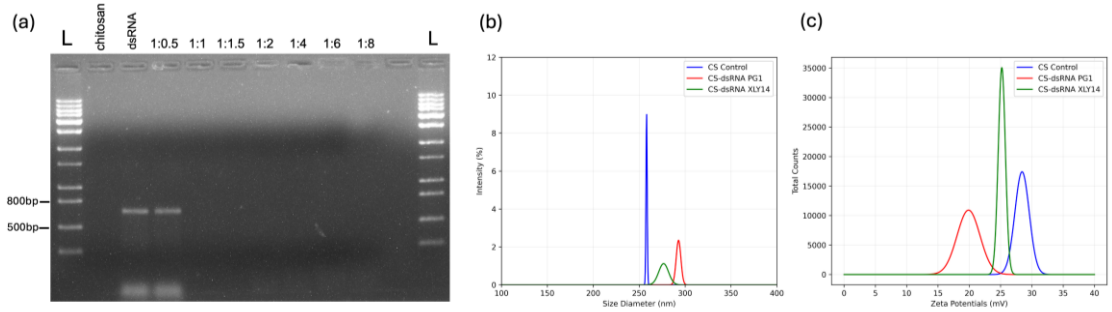

Figure 1. Agarose gel EMSA of dsRNA-chitosan nanoparticle complexes was conducted at various mass ratios to determine the optimal ratio (a). At the 1:1 ratio, no dsRNA is detected in the gel, confirming it as the best dsRNA: chitosan ratio. This ratio was analyzed using Zetasizer equipment, and the size (b) and charge (c) characteristics are presented. The data represent the average of three analyses with ten replicates each.

The encapsulation can also be verified by the differences in size and charge between the complexes, according to data generated by the Zetasizer. The nanoparticles presented an average size of 292.9 nm for PG1-Cs and a charge of 19.9 mV, and 276.6 nm for Xly-Cs and a charge of 25.2 mV (Figures 1b and 1c). The controls (chitosan nanoparticles only) were smaller (258.1 nm) and had a higher positive charge (28.4 mV) (Table 2).

Table 2. Physicochemical analysis of nanoparticle formulations generated by the Zetasizer. Size (Z-average size), polydispersity (PDI), and charge (mV) of chitosan nanoparticles associated with dsRNA and the control. Data represent the average of 3 analyses with 10 replicates each. ± represents the standard deviation.

Nanoparticles	Z-average size (nm)	PDI	Z potentials (mV)
Cs-Control	258.1 ± 1	0.313 ± 0.01	28.4 ± 1.25
PG1-Cs	292.9 ± 3	0.428 ± 0.03	19.9 ± 2
Xly-Cs	276.6 ±7	0.335 ± 0.03	25.2 ± 0.63

3.2 Inhibition of *R. solani* growth *in vitro*

To verify the RNAi silencing capacity of CWDEs, the synthesized dsRNAs were tested *in vitro* in two different culture media. In the culture medium where the sole carbon source was pectin, a reduction in mycelial growth of *R. solani* was observed in the dsRNA_PG1 treatment (Figure 2). In the dsRNA_Xly14 treatment, mycelial growth was similar to the mock treatment. As expected, in the culture medium with simple carbohydrate sources (PDA), no difference was observed among the treatments (Figure 2).

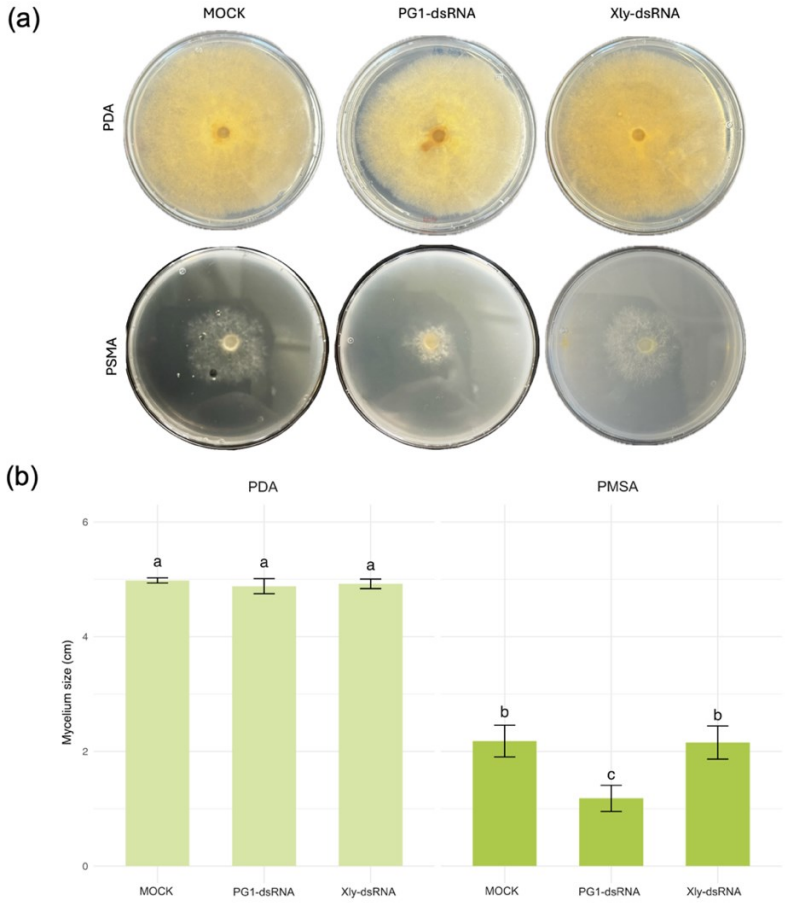

Figure 2. Photographs (a) and average mycelium size (cm) (b) from treatments in the *in vitro* assay of mycelial growth inhibition of *R. solani* after seven days are presented. Two types of culture media were utilized: a commercial PDA and a PMSA medium using pectin as a carbon source. An amount of 20 µg of each dsRNA was applied to the seven-days-old *R. solani* culture disc, while the mock treatment consisted of distilled water. Equal letters above the bars do not differ statistically from each other by Tukey test ($p > 0.05$).

The ability to control *R. solani* development by applying dsRNA was also observed in tests with detached leaves of *N. tabacum*. A significant reduction in pathogen

colonization and development was noted in treatments where dsRNA molecules were applied. The most significant decrease was found in the Xly_Cs treatment, with an average of 4.78% of affected leaf area compared to 34.54% in the control, where sterile water was applied. The PG1 treatment also reduced the affected leaf area, with an average of 5.49% of the affected area. In treatments where dsRNA was encapsulated with chitosan nanoparticles, inhibition was comparable, showing 11.12% and 7.71% for Cs_PG1 and dsRNA_Xly, respectively. A similar reduction was observed in the experimental control, where only chitosan nanoparticles were applied.

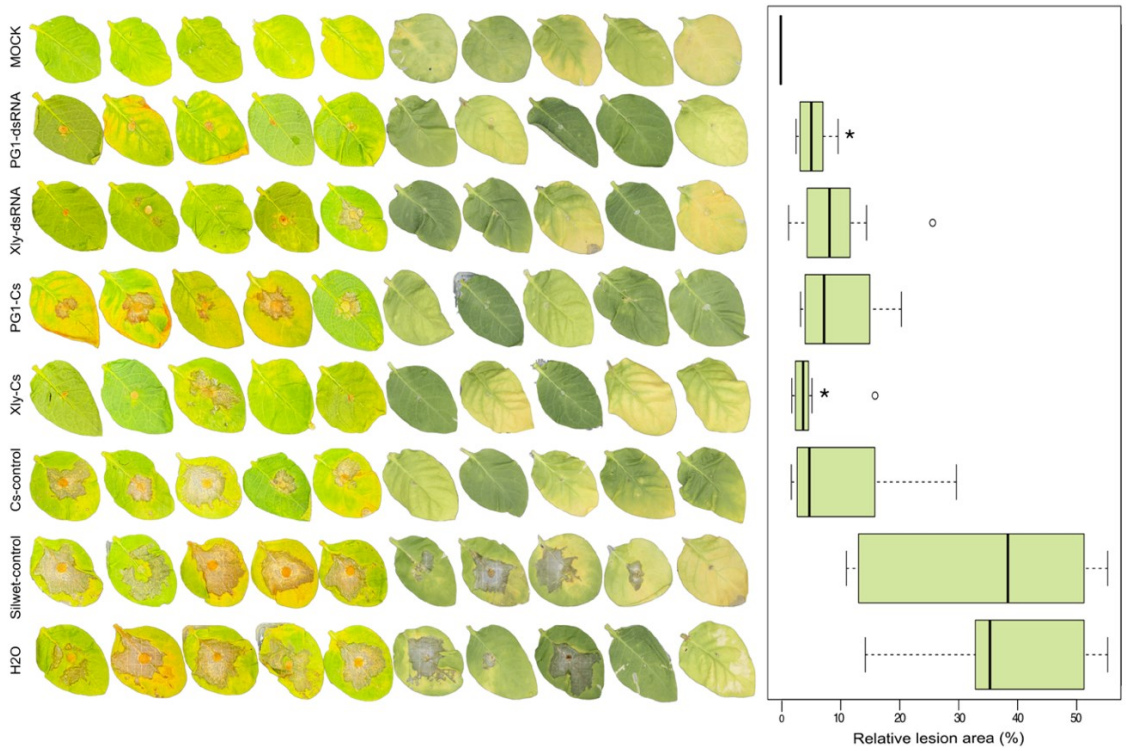

Figure 3. Detached leaf assay with *N. tabacum* leaves. On the right are photos of all replicates of the two detached leaf experiments. Naked dsRNA (PG1-dsRNA and Xly-dsRNA), chitosan-encapsulated dsRNA (PG1-Cs and Xly-Cs), and experimental controls (Cs-control and Silwet-control) were applied. Seven days after inoculation, the relative infected area was analyzed using the Leaf Doctor application and analyzed with the Kruskal-Wallis test at a probability of 5% (on the right). Bars represent the maximum and minimum values. Significance (compared to the H2O group) is defined by the arteritis (*) on the boxplot on the right (*$p < 0.05$; Conover-Bonferroni test).

3.3 Control of melon root rot caused by *R. solani*

The effectiveness of RNAi-SIGS in controlling root rot in melon seedlings was investigated through greenhouse experiments. The assessment of disease severity revealed that all inoculated treatments exhibited symptoms, with average scores ranging from 1.6 to 2.6 (Figure 4b). The control treatment (MOCK) showed no symptoms, which differed significantly from those of the others. Although the Rhz_control treatment had the highest average severity (2.6), this was not statistically significant compared to other inoculated treatments, which had averages between 1.6 and 1.8. This indicates that treatments with PG1, Xly, Cs_PG1, Cs_Xly, and Cs_control had similar susceptibility levels to the pathogen under experimental conditions.

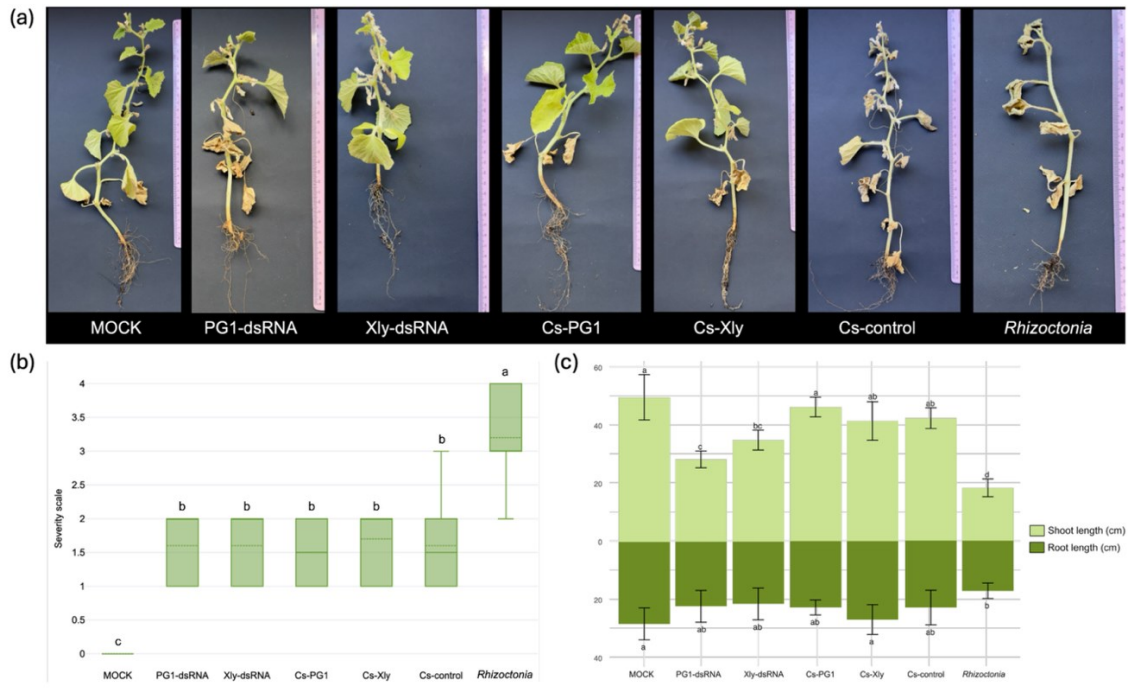

Figure 4. Control experiment of *R. solani* on melon plants in a greenhouse. (a) Photos of plants 30 days after treatment with dsRNA and chitosan nanoformulations. (b) Severity was ranked using Ambrosio et al. scale (2015) and analyzed using the Kruskal-Wallis test at a 5% significance level, followed by Dunn test with a Bonferroni adjustment. Treatments sharing letters showed no significant difference ($p > 0.05$). (c) Size of aerial parts (above) and root area (below) across different treatments, with bars indicating standard deviation. Different letters represent significant differences per Tukey test ($p > 0.05$).

In contrast to disease severity data, vegetative growth parameters showed a positive response to treatments. All treatments with dsRNA or encapsulated dsRNA and the Cs-control exhibited comparable shoot and root growth to the mock group after 30 days.

Plants treated with dsRNA, Cs_PG1, and Cs_Xly nanoformulations with chitosan had positive effects on shoot size, averaging 46.2 cm and 41.4 cm, respectively, similar to the mock (without fungus inoculation). For roots, Cs_Xly, Cs_PG1, dsRNA-PG1, dsRNA-Xly, and Cs_control performed statistically similarly to the control (MOCK). Untreated plants had average shoot and root sizes of 18.2 cm and 17.2 cm, respectively.

4. DISCUSSION

Soil-borne phytopathogenic fungi must find a suitable environment and bypass the innate immune defenses of the plant to establish themselves and infect plants. Successful infection requires them to overcome physical barriers, specifically the cell walls of complex plant molecules (RAO et al., 2019; CHEN et al., 2017; XUE et al., 2018). Therefore, limiting the initial colonization ability of the fungus within plant tissues could be essential for effectively managing these pathogens. This study found that silencing genes linked to CWDEs decreased infection rates and lessened the negative effects of *R. solani* infection.

Gene silencing via RNAi is highly target-specific. This specificity was evident in the reduced mycelial growth of *R. solani* when dsRNA targeting the PG1 enzyme was applied in a medium containing only pectin as the carbon source. In contrast, no reduction was observed with dsRNA molecules targeting the enzyme xylanase. Such selectivity is particularly pronounced *in vitro* under nutrient-limited conditions, where fungi rely on specific molecular pathways for growth (RAO *et al.*, 2019; SONG *et al.*, 2018).

The disruption of genes involved in CWDEs production impacts *R. solani* colonization capacity. That was demonstrated when these genes were knocked out (DE LORENZO; FERRARI, 2002), silenced by RNAi-HIGS (RAO *et al.*, 2019; YANG *et al.*, 2012), or inhibited by host molecules (CHEN et al., 2016). A significant reduction in *R. solani* colonization was observed on leaves treated with exogenous dsRNA in the detached leaves assay. Surprisingly, dsRNA naked treatments showed a slight advantage over chitosan-encapsulated formulations, possibly due to faster uptake under controlled conditions. Nanoparticle encapsulation enables controlled release of dsRNA, which is particularly important in field conditions (NIÑO-SÁNCHEZ *et al.*, 2022).

Plants treated with encapsulated dsRNA exhibited greater shoot and root growth, suggesting a positive effect of the nanoparticle formulation, despite similar severity scores between all treatments. Like other soil-borne fungi, *R. solani* damages the root system and

indirectly affects the shoot (AJAYI-OYETUNDE; BRADLEY, 2018), which becomes more apparent in later stages, when water and nutrient demands increase (MICHEREFF; ANDRADE; SALES JÚNIOR, 2008; GARCÍA-JIMÉNEZ *et al.*, 2000).

The effectiveness of RNAi-SIGS in living plants relies on multiple factors. Exogenous dsRNA uptake by fungi has been demonstrated for *R. solani* and other fungi (QIAO *et al.*, 2021), and its systemic mobility has been observed in crops such as melon (DELGADO-MARTÍN *et al.*, 2022). However, it remains unclear whether control efficacy depends on the dose, formulation, or application frequency (ŠEČIĆ; KOGEL, 2021). It is plausible that, between the application, inoculation, and fungal colonization, the dsRNA lost some activity. Studies in other plant-pathogen systems have shown that dsRNA can remain active on the plant for up to eight days (SONG *et al.*, 2018) and that reapplications can enhance silencing by increasing siRNA production and transcriptional repression (ŠEČIĆ; KOGEL, 2021; MCLOUGHLIN *et al.*, 2018).

Despite advances in RNAi-SIGS-based control technologies, significant gaps remain regarding the practical application to control soil-borne pathogens under field conditions. The present study demonstrates that silencing genes related to CWDEs via exogenous dsRNA application has the potential for *R. solani* control. While the inhibition of CWDEs has been previously reported in other pathosystems, these findings contribute to the development of a scalable control method. Further research is required to optimize key parameters to enable successful application in commercial field settings.

5. CONCLUSION

This study assessed the potential of RNAi-SIGS technology to control *R. solani*. In vitro experiments, including growth on pectin medium and detached leaf tests, showed that dsRNA-based treatments decreased fungal growth and colonization ability. In greenhouse conditions, these treatments also reduced the harmful effects of *R. solani* infection on plant growth. These findings emphasize the potential of RNAi-SIGS as a sustainable disease management approach. However, further research is needed to optimize key factors to improve its effectiveness.

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