UNIVERSIDADE FEDERAL RURAL DO SEMI-ÁRIDO
PRÓ-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO
PROGRAMA DE PÓS-GRADUAÇÃO FITOTECNIA
DOUTORADO EM FITOTECNIA

KAROL ALVES BARROSO

MANAGING MELON ROOT ROT DISEASES VIA SOIL INCORPORATION OF PLANT RESIDUE AND BIOACTIVE INDUCERS AND CONTROLLING PLANT VIRUSES VIA RNA INTERFERENCE

MOSSORÓ

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Tese apresentada ao Doutorado em Fitotecnia do Programa de Pós-Graduação em Fitotecnia da Universidade Federal Rural do Semi-Árido para obtenção do título de Doutor em Fitotecnia.

Linha de Pesquisa: Proteção de Plantas

Orientador: Márcia Michelle de Queiroz Ambrósio, Prof.ª Dra.

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2023

Bm Barroso, Karol Alves.
MANAGING MELON ROOT ROT DISEASES VIA SOIL INCORPORATION OF PLANT RESIDUE AND BIOACTIVE INDUCERS AND CONTROLLING PLANT VIRUSES VIA RNA INTERFERENCE / Karol Alves Barroso. - 2023.
78 f. : il.

Orientadora: Márcia Michelle de Queiroz Ambrósio.
Coorientador: Washingtons Luís da Silva.
Tese (Doutorado) - Universidade Federal Rural do Semi-árido, Programa de Pós-graduação em Fitotecnia, 2023.

1. Cucumis melo. 2. soil-borne pathogens. 3. potato virus Y. 4. RNA interference. I. de Queiroz Ambrósio, Márcia Michelle , orient. II. da Silva, Washingtons Luís, co-orient. III. Título.

Ficha catalográfica elaborada por sistema gerador automáto em conformidade com AACR2 e os dados fornecidos pelo) autor(a).
Biblioteca Campus Mossoró / Setor de Informação e Referência
Bibliotecária: Keina Cristina Santos Sousa e Silva
CRB: 15/120

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AGRADECIMENTOS

É muito difícil encontrar palavras que traduzam o sentimento de gratidão que sinto no momento em que finalizo esta tese. Foram muitas pessoas, momentos e lugares que me levaram ao que sou hoje e a tudo que conquistei nestes anos. Quando falo de conquista, falo de conhecimento, amizades, sabedoria e paciência.

Se eu puder enumerar, gostaria de agradecer primeiramente a Deus, por me tornar uma pessoa forte e corajosa para vencer todos os obstáculos que surgiram no meu caminho. Obrigada minha família, Vera, Ramos, Priscila e Gilvan Júnior. Vocês são porto seguro e as primeiras pessoas em que penso em momentos como este. Este trabalho dedico a vocês.

Agradeço também minha orientadora Márcia Michelle, por todo o ensinamento e compreensão. Não canso de dizer que você é inspiração a todos a sua volta e um exemplo de ética, profissionalismo e humanidade. Gratidão também ao meu coorientador, Washington da Silva, por todas as oportunidades e parceria. As oportunidades que você me deu e os ensinamentos que você compartilhou foram um divisor de águas na minha vida. Obrigada por me fazerem crescer e me tornar a profissional que eu sou hoje.

Obrigada as instituições nas quais desenvolvi o meu trabalho, UFERSA e CAES, por toda a estrutura e apoio. Também agradeço aos amigos que foram fundamentais nesta jornada. Não ouso nomear a todos, pois a lista é imensa, do mesmo tamanho da gratidão que sinto por vocês.

Obrigada a todos.

ABSTRACT

The first part of the research was carried out at the Universidade Federal Rural do Semi-Árido (UFERSA). The aim was to develop novel management strategies for melon root rot and to evaluate the effects of these management strategies on the soil microbiota. Experiments were first performed in a greenhouse and the treatments consisted of incorporating plant material, crambe (*Crambe abyssinica*) and Jack bean (*Canavalia ensiformis*), and using the bioactive inducers, Compost Aid® and Copper Crop®, compared to treatments used by melon-growers as a control. The use of these different approaches in combination was carried out in field experiments in two melon-producing areas with history of root rot disease in melon plants. Overall, the treatments containing jack bean materials were superior in controlling the disease and increasing melon productivity compared to the control treatment. The treatment jack bean incorporation combined with the use of Compost Aid® reduced the incidence of the disease by 61.29% and 40.62% in production areas 1 and 2, respectively, and reduced the severity of the disease by 59.48% and 54.21%, compared to the control. It also increased productivity by 103.46% (Exp. 1) and 84.28% (Exp. 2). The same treatment provided the highest averages for basal soil respiration, microbial biomass carbon, numbers of total bacteria, sporulating bacteria, total actinomycetes, and total fungi colonies forming units compared to the control treatment. The second part of this research was carried out at The Connecticut Agriculture Experimental Station (CAES). The main goal was to test the exogenous application of different dsRNA molecules to control potato virus Y (PVY) via RNA interference (RNAi). Regions with a high concentration of 21nt siRNAs were selected to synthesize dsRNA from each cistron in suppressing PVY infection. The dsRNAs synthesized from CP, HC-PRO, and NIb cistrons were applied and two weeks after viral inoculation, PVY symptoms were assessed. The translocation of the applied dsRNA molecules was also evaluated for 14 days, and in this step no virus inoculation was performed. The dsRNAs applied protected treated plants against PVY infection, and HC-PRO-dsRNA induced greater protection, entered, and moved fast compared to CP and NIb-dsRNAs, The synthesis and application of exogenous dsRNAs targeting the HC-PRO genomic region of PVY proved to be a promising technique for controlling this disease.

Keywords: *Cucumis melo*; soil-borne pathogens; potato virus Y; RNA interference.

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SUMMARY

GENERAL INTRODUCTION

With the growing world population, there is an increased demand for technologies aimed at increasing crop production and yield. Those technologies include using machinery, fertilizers, chemicals, or any technique that increases the quantity and quality of harvested food products. However, with the continued increase in demand for food production and the expansion of arable areas, the susceptibility of plants to disease attacks has also increased, which can compromise yield and quality of agricultural products (VISHNOI et al., 2021).

Plant diseases can be abiotic in origin, such as lack of nutrients or light, or biotic, caused by infectious organisms, including fungi, viruses, bacteria, and nematodes. It is estimated that in the presence of a susceptible host and favorable climatic conditions, the disease caused by a virulent pathogen affects 40% of food crops, according to the United Nations (FAO, 2021). However, in worse case scenarios, these values can reach up to 98%, causing an increase in food insecurity and leaving many arable areas vulnerable (NAZAROV et al., 2020). These losses have a devastating effect on modern society and combating this challenge is not an easy task. Therefore, disrupting novel technologies are needed to manage these diseases at all levels, either during crop production or after harvesting.

Chemical control of plant diseases has played a crucial role in preventing agricultural losses. Over the years, these products have become more selective and explored different mechanisms of action (NAZAROV et al., 2020). In addition, they are considered easy to apply by farmers and promote immediate results to control plant diseases (PANTH et al., 2020). On the other hand, there are serious concerns related to the wide use of chemicals in agriculture settings. The first is that there are no registered products for many pathogens, which can lead to the indiscriminate use of non-specific products to control diseases in the field. Even for registered products, the continuous use of these synthetic formulations can promote the selection of resistant phytopathogens and represent a risk to the biodiversity and the health of humans and animals by contaminating soils, groundwater, and consumer products (GHINI, KIMATI, 2002; SEEPE, 2021). These negative effects of using chemical control further increase the demand for alternative and safe management of plant diseases. Examples of systems where there is no efficient control are the case of root diseases in melon crops in Northeast Brazil and

the incidence of viruses in potato crops and other members of the Solanaceae family in North America, diseases that significantly affect agriculture in global level.

Melon (*Cucumis melo* L.) production is the main economic activity in the state of Rio Grande do Norte, Brazil. The cities of Mossoró and Apodi are regarded as the largest melon-producing areas in the state (IBGE, 2021). However, intensive monoculture and successive planting, associated with favorable conditions of humidity and temperature in these producing areas, can promote changes in soil microbiota and may impact long-term crop productivity mainly due to the surge of diseases caused by soil-borne pathogens (SILVA et al., 2007; SILVA et al., 2011; GOMES, 2018). Current intensive production techniques make it difficult to maintain a healthy and sustainable production system.

The presence of soil-borne microorganisms, either phytopathogenic or not, can affect plant productivity (MARON et al., 2011). Regarding the organisms that cause plant diseases in melon plants, the fungi belonging to the genus *Fusarium* deserve special attention, they cause vascular wilting known as fusarium wilt and rot root, especially at the end of the melon cycle (SILVA et al. 2023), and they can also cause rot on the fruits (ARAÚJO et al., 2021). *Rhizoctonia solani*, which causes seed rot and pre and post-emergence damping-off, and *Macrophomina phaseolina* (Tassi) Goidanich, which causes gray rot (MICHEREFF et al., 2005), are also important pathogens of melon that need consideration. An important feature of these pathogens is their ability to form resistance structures in adverse conditions, such as chlamydospores produced by *Fusarium* spp. and microsclerotia produced by *M. phaseolina*, those structures facilitate the survival of these pathogens in the soil for several years, making their control extremely difficult (ANDRADE et al., 2017).

For years seed treatment and soil fumigation, mainly with methyl bromide, were the most efficient way to manage these pathogens in the field. However, when using these strategies, the soil ran into risks of becoming a biological vacuum since these products were not selective and could affect both malefic and beneficial organisms, causing a serious biological imbalance in the system (TAMIETTI & VALENTINO, 2005; LIMA & MORENO, 2007). Currently, there is no chemical control registered at the field level for the control of fungal root rot in the melon crop (AGROFIT, 2023). However, there are cases of indiscriminate use of non-specific products in the crop, which can lead to the emergence of new races of pathogens or the appearance of others that remained in balance (SALES JR. et al., 2005). Melon fruits produced for export, especially to Europe and China are subject to a set of restrictions and inspections for residues of fungicides and

other products, which must meet the maximum residue limits (MRL) set by the Brazilian legislation and by the importing countries. Therefore, there is a need for the implementation of techniques and strategies to sustainably manage diseases, without increasing production costs, to provide healthy and environmentally friend food products.

Among the potential measures for use in the management of soil-borne diseases, the incorporation of organic materials stands out as a viable alternative because it helps to balance soil microfauna, increasing the potential for disease control (CRUZ et al., 2013). Emphasizing this idea, Viana & Souza (2000) state that the release of toxic metabolites from these materials can suppress the population of microorganisms, as well as increase the population density of antagonists present in the soil.

In the state of Rio Grande do Norte, covering the soil with polyethylene plastic in areas with melon cultivation is a consolidated technique. In these areas, the polyethylene mulch is applied to the newly fertilized and irrigated soil with the white side facing upwards and the black side facing the soil and the irrigation hose underneath this structure. Soon afterward, holes are made in the center of the polyethylene mulch following the spacing chosen for the planting of the plant, which in general is 40 cm between plants. The plant remains in these conditions until the end of its cycle, and the polyethylene mulch is generally reused in subsequent plantings after removing the plant, starting a new plant cycle. This technique aims to reduce weeding costs in the areas, reduce evaporation losses, and protect the plant from large thermal amplitudes of the covered soil. Thus, losses can be reduced and an increase in the production and quality of the fruits are achieved (MIRANDA et al., 2003).

Since they already have consolidated management techniques for melon cultivation, any viable proposed alternative must be adapted to this already established system. Our proposed alternative solution consists of incorporating plant materials associated with the use of polyethylene before the transplanting phase of the seedlings for a certain period. The aim was to accelerate the material decomposition process, release toxic substances to pathogens, and favor plant development and soil quality. Nascimento et al. (2018) proposed a similar approach based on the melon management system already adopted by producers, where they compare a conventional tillage system with a no-tillage system. They incorporated sunn hemp plant materials, millet, corn, brachiaria, and spontaneous vegetation, associated with the use of polyethylene mulch in their no-tillage tests. Compared to the conventional management system, the incorporation of pearl millet and spontaneous vegetation reduced the incidence of the main pathogens that cause melon

root rot (*Fusarium solani*, *Macrophomina phaseolina*, *Monosporascus cannonballus*, and *Rhizoctonia solani)* by more than 50%. Furthermore, among the potential plants for controlling soil-borne pathogens, Brassicas stand out as good candidates due to their chemical composition (OLIVEIRA et al., 2011).

Brassicas produce glucosinolates and derivatives that after enzymatic hydrolysis are converted into isothiocyanates, nitriles, thiocyanates, and epinitrils, those compounds are known to have biological activity against nematodes, fungi, and other soil-dwelling pathogens (OLIVEIRA et al., 2011). The crambe (*Crambe abyssinica* Hochst.) is a brassica that has great adaptability, rusticity, precocity, tolerance to water deficit, and low production cost (COLODETTI et al., 2012). Crambe has demonstrated efficiency in controlling nematodes, reducing 49.27% of the galls caused by *Meloidogyne incognita* on tomatoes (COLTRO-RONCATO et al., 2018). In studies of crop succession, the introduction of crambe 90 days before soybean planting reduced the population of *Pratylenchus brachyarus* and promoted less reproduction of *M. javanica* when compared to treatments with black oat, turnip, corn, and bean (TAVARES-SILVA et al., 2017). Crambe has also been used in the form of an extract to control soil-borne pathogens, which, when applied to soil cultivated with soybeans, was able to promote mortality of 81.4% in juveniles of *M. javanica*, and even influence the height and fresh weight of plants (TARINI et al., 2020). The use of crambe is already known for the control of nematodes in the soil (COLTRO-RONCATO et al., 2016; SANTOS et al., 2016), but there are still few studies on this crop aimed at controlling fungal diseases. Thus, this culture is a great candidate to be tested in association with polyethylene mulch in the system we are proposing.

Jack bean (*Canavalia ensiformis* (L.) DC.) is another crop well known for its use as green manure, intercropping or rotation with annual crops, and as soil cover (LOPES, 1998; PADOVAN et al., 2011). According to Oliveira et al. (2002), when using a legume as green manure, there is the advantage of a significant reduction in the application of nitrogen via synthetic soil fertilizers, as plants of this family are capable of fixing nitrogen from the air through symbiosis with bacteria of the genus *Rhizobium*, enriching the soil with this macronutrient. Its deep root system also ensures an increase in its efficiency as green manure and in opening galleries in the soil, which facilitates air circulation (RUSSELL et al., 1981; PADOVAN et al., 2011). Cruz et al. (2013), studying the control of tomato fusariosis, found that the incorporation of legumes, including jack beans, was efficient in controlling the disease, having a control potential of 73.34% of the disease,

compared to the control, using a dose of 60 g of jack bean per kg of soil. Porto et al. (2016), studying the effect of using jack beans in the management of melon root rot, observed that there was a reduction in the incidence of the disease caused by *F. solani*. When using polyethylene mulch in addition to the treatment with jack beans, there was an increase in melon dry matter. This fact is mainly due to the presence of substances such as enzymes, glycoproteins, polypeptides, and compounds from the metabolism of amino acids that can reduce the population of the pathogen. The inhibition of the soil pathogen may also occur due to the isomorphic form of the urease enzyme, which directly inhibits the vegetative growth and germination of some pathogens, such as *R. solani*, *F. solani*, and *F. oxysporum* (SILVA LÓPEZ, 2012).

Another factor that must be considered in this management system is the use of bioactive inducers in agriculture. The use of products that act as bioactive inducers is on the rise in agriculture and can provide micronutrients for plants and beneficial microorganisms to the soil, increasing the tolerance of plants to damage caused by diseases and may increase the efficiency of crop productivity, which can act directly or indirectly on the health of the soil and consequently of the plant (PEIRIS et al., 2021). A range of these products are available in the market and are being used by melon growers, that can act as a biostimulant, biopesticide, and biofertilizer (CHOJNACKA, 2015). These characteristics can be found separately or in a single product, depending on its components and depending on the grower's needs. The interactions of these characteristics can affect the health of plants in several ways, ranging from acting in the management of plant diseases, such as bacteria, nematodes, and fungi of the genera *Fusarium*, *Rhizoctonia*, *Alternaria*, and *Aspergillus*, in the control of weeds, and the increment of beneficial organisms in the soil (CHOJNACKA, 2015; SINGH and YADAV, 2020).

An example of these products is Compost Aid®, which has enzymes and microorganisms in its composition, such as *Lactobacillus plantarum* (1.25×10^8 CFU g^{-1}), *Bacillus subtilis* (1.25×10^8 CFU g^{-1}), and *Enterococcus faecium* (1.25×10^8 CFU g^{-1}). Another example of a product that acts as a bioactive inducer is Copper Crop®, which has in its composition bioactive copper (132 g/L), nitrogen (66 g/L), and organic carbon (1.32 g/cm³). According to the manufacturer, this composition guarantees better use of copper by the plant, making it more effective in nutrition and in the involvement of these nutrients in physiological processes such as photosynthesis and respiration. Despite its wide use in melon-producing areas in the Brazilian Northeast, there is still a lack of

studies on the effects of these products in the areas to validate their efficiency. One of the few research testing those products was carried out by Borges et al. (2023). They incorporated *Pennisetum glaucum* associated with the use of commercial products that act as bioactive inducers in melon management. They saw a significant increase in the total population of fungi by 183%, total bacteria by 55%, sporulating bacteria by 21%, and actinomycetes by 146 % when compared to the control treatment. These findings opened the door for future work targeted to test these bioactive inducers as soil conditioner to elevate the benefits of a diverse soil microfauna on root rot disease suppression.

Potato virus Y (PVY) is considered one of the most important virus pathogens globally. PVY is a member of the genus *Potyvirus* in the family *Potyviridae*. Several strains of PVY have been described, but the recombinant strains, PVY^{N-Wi} and PVY^{NTN}, are becoming the predominant strains in potato production areas around the world (KARASEV and GRAY, 2013). In addition to potatoes, PVY can infect other members of the Solanaceae family, such as tobacco, pepper, and tomato. In general, the symptoms on foliage of PVY-infected plants include mosaic, chlorosis, necrosis, and leaf distortion, normally associated with growth retardation and plant stunting. In potato tubers, the symptoms include the potato tuber necrotic ringspot disease (PTNRD), this disease starts slowly usually after tubers are harvested, but with time it develops into sunken necrotic rings, which makes the tuber unmarketable (KARASEV and GRAY, 2013).

Data on the precise impact of PVY on yield and production of potatoes are difficult to quantify, but it is estimated that in areas with high incidence of PVY, there is a reduction in productivity by 40% to 70% (NOLTE et al., 2004). One of the factors that help to explain these high rates of loss is the virus mode of transmission, which occurs in a non-persistent and non-circulating way through many aphid species, such as *Myzus persicae*, found to efficiently transmite different strains of PVY in potato-producing areas around the world (ROBERT and BOURDIN 2001; SRINIVASAN et al. 2012).

The development of effective techniques for controlling viral diseases is essential to reduce losses in agriculture. When it comes to the management of viral infections in agriculture systems, some strategies are widely used to reduce the impact on crop production and quality, such as the treatment of propagating material, the use of insecticide products to control viral vectors, and the use of resistant or tolerant plant cultivars. These techniques are essential to reduce the risk of emergence and dissemination of viral diseases (RAGSDALE et al., 2001; PETROV, 2015; PETROV et

al., 2022), but they are not always efficient, and strategies aimed to directly control the pathogen are much needed. A promising such technique is the induction of post-transcriptional RNA gene silencing (PTGS), also known as RNA interference (RNAi) in plants aimed to block viral replication and by extension, infection suppression.

To understand how the RNAi mechanism works for the control of viral diseases, it is important to know the genomic structure of the virus. PVY is a single-stranded, positive sense, and linear RNA genome, which encodes a large polyprotein, which is essential for the infectious process (Figure 1) (GREEN et al., 2018). The polyprotein when matured transcribed is processed into ten proteins: the viral protein genome-linked (VPg), N-terminal protein (P1), the helper component proteinase (HC-Pro), the P3 protein, the cytoplasmic inclusion protein (CI), two nuclear inclusion proteins (NIa and NIb), two small proteins (6K1 and 6K2), and the capsid protein (CP) (URCUQUI-INCHIMA et al., 2001; ADAMS et al., 2005; DA SILVA, 2008; ESKELIN et al., 2011). These proteins are multifunctional and vital in more than one process during viral infection. For example, the CP protein is involved in the processes of RNA encapsulation, aphid transmission, virus cell-to-cell movement, and viral symptoms expression in the plant. HC-Pro acts in the process of viral transmission via aphids, virus systemic movement, viral symptoms expression, and gene silencing mechanism suppression. The NIb protein is responsible for the viral recognition of viral RNA regulatory sequences and viral host interaction factors to facilitate the infection process (SHUKLA et al., 1994; URCUQUI-INCHIMA et al., 2001; BAO et al., 2020).

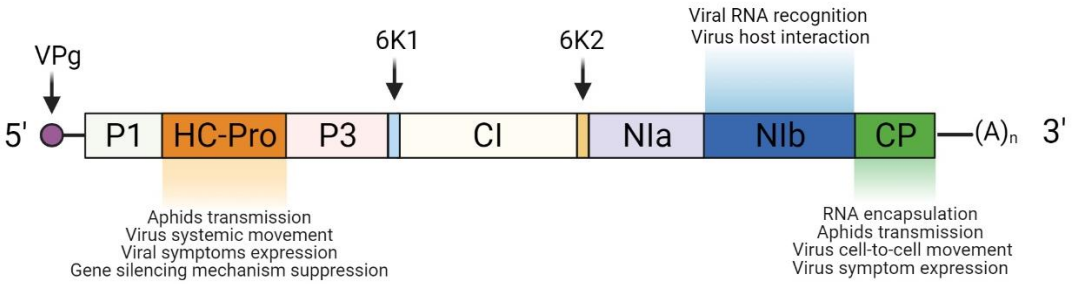

Figure 1. Representation of the potato virus Y (PVY) genome, highlighting the CP, HC-Pro, and NIb cistrons and their respective functions.

Before the PVY polyprotein is translated, the RNAi mechanism can be explored to silence the viral messenger RNA (mRNA) to disrupt the protein translation. RNAi is a gene expression control mechanism that occurs naturally in cells of eukaryotic organisms

and works according to the organism's need to “turn on” or “turn off” certain functions. The mechanism is activated by the presence of double-stranded RNA (dsRNA) molecules, which can be endogenously expressed or exogenously introduced into the cell. Once present in the cytoplasm, dsRNAs are cleaved into small interfering RNAs (siRNA) of 21-25 nt in length by ribonucleases called DICERs. The siRNAs are recruited by argonaut proteins (AGO) and he positive strands of these siRNAs are removed, and the negative strands are incorporated into a complex called RNA Induced Silencing Complex (RISC), which acts in the identification and degradation of target RNA that has a sequence complementar to the siRNAs. This sequence complementarity characteristic guarantees the high specificity of the siRNA mechanism (DEVERS et al., 2020; HUNG et al., 2021). Having a good understanding of this whole process, as well as the viral genome and the function of the viral proteins, help to design dsRNA to precisely target viral genomic regions to control the desirable viral diseases, making the mechanism work in our favor and without affecting non-target species.

The RNAi mechanism was first discovered in nematodes (*Caenorhabditis elegans)* (FIRE et al., 1998), and this strategy has been used, experimentally, mainly aimed to control pests such as aphids (YAN et al., 2021; ZHANG et al., 2022), bedbugs (*Euschistus heros*) (FISHILEVIH et al., 2016), and locusts (*Schistocerca gregaria)* (SUGAHARA et al., 2015; SUGAHARA and TANAKA, 2018). So far, there have been some success stories in using RNAi to suppress fungal diseases, for example, in the control of *Fusarium oxysporum* f. sp. *lycopersici* in tomato (OUYANG et al., 2023) by the exogenous application of dsRNA synthesized from the FolRDR1 gene. This gene is required for vegetative growth, asexual reproduction, and pathogenicity of the fungus, the treatment with FolRDR1-dsRNA resulted in significant relief of tomato wilt symptoms and pathogen development. For plant viruses, this method of disease suppression is still on the making for applied purposes. DsRNAs synthesized from the HC-Pro and NIb cistrons of the pepper mottle virus (PepMoV) genome demonstrated positive results in disease suppression in *Nicotiana benthamiana* (KWEON et al., 2022). Likewise, dsRNA synthesized from p126 and CP cistrons induced resistance against tobacco mosaic virus (TMV) infection in 65 and 50% of dsRNA-treated tobacco plants (*Nicotiana tabacum)*, respectively (KONAKALLA et al., 2016).

A successful management program of plant pathogens must be based on the combination of different techniques and approaches, and the nature of the pathogen and the needs of each producing region must be considered. Therefore, in this research two

distinct themes were addressed, focused on different pathosystems. The first is the management of root rot in melon, a significant problem for agriculture in northeastern Brazil. The second part of the research, focused on the management of PVY, which is a major obstacle in potato and tobacco production worldwide.

Herein, the first part of the research was carried out at the Universidade Federal Rural do Semi-Árido (UFERSA), Mossoró, RN, Brazil. The aim was to develop novel management strategies for melon root rot and to evaluate the effects of these management strategies on the soil microbiota.

The second part of this research was carried out at The Connecticut Agriculture Experimental Station (CAES), New Haven, CT, USA. The main goal was to test the exogenous application of different dsRNA molecules to control potato virus Y (PVY) via RNA interference (RNAi).

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CHAPTER 1

The incorporation of jack bean plant green material and the use of bioactive inducers in melon growing areas reduced root rot disease incidence and increased soil microbial communities

ABSTRACT

Brazil is one of the largest melon (*Cucumis melo*) producers in the world, but the country's melon production is being jeopardized by the surge of soil-borne pathogens. Current management strategies are inefficient in controlling root rot disease; therefore, innovative ways to manage this disease complex are much needed. This research aimed to evaluate the effect of combining strategies in managing root rot in melon plants and the effects on the soil's biological attributes as compared to traditional management in melon-producing areas. Experiments were first performed in a greenhouse and the treatments consisted of incorporating plant material, crambe (*Crambe abyssinica*) and Jack bean (*Canavalia ensiformis*), and using the bioactive inducers, Compost Aid® and Copper Crop®, compared to treatments used by melon-growers as a control. The treatments that showed the best results in the greenhouse were chosen to be used in the field. Field experiments were performed in two different melon-producing areas, named as experiment 1 and 2, respectively, with histories of root rot disease in melon plants. Overall, treatments containing jack bean materials were superior for variables related to crop productivity and disease control compared to the control treatment. The treatment jack bean incorporation combined with the use of Compost Aid® reduced the incidence of the disease by 61.29% (Exp. 1) and 40.62% (Exp. 2) and reduced the severity of the disease by 59.48% (Exp. 1) and 54.21% (Exp. 2), compared to the control. It also increased productivity by 103.46% (Exp. 1) and 84.28% (Exp. 2). The same treatment provided the highest averages for basal soil respiration, microbial biomass carbon, numbers of total bacteria, sporulating bacteria, total actinomycetes, and total fungi colonies forming units compared to the control treatment. In these treatments, microbial biomass was more efficient in using organic compounds, making them more readily available to melon plants. This was the most suitable treatment to suppress root rot disease in melon in our trials, and it is being integrated by local growers as an efficient strategy to manage root rot disease and to ensure a soil microbiological balance in melon farms in the Brazilian Semiarid region.

Keywords: *Cucumis melo*, Crambe, soil-borne pathogens, soil microbiota

1 INTRODUCTION

Melon (*Cucumis melo* L.) is one of the most important fruit crops in Brazil. In 2021 alone, 607,000 tons of melon were produced in the country (IBGE, 2021), representing the main economic activities in the state of Rio Grande do Norte, located in northeastern Brazil, which is responsible for 59.47% of the country's total melon production (IBGE, 2021), generating over 22,000 direct jobs in this region (ABRAFRUTAS, 2022). Many fruits produced in this region are destined for external markets, especially Europe and China. The favorable climatic conditions for melon growth, *i.e.*, high temperature and low humidity, are determining factors for the increased melon production rate in these regions attained in the last decades. However, these climatic conditions, coupled with intense monoculture, also increased the occurrence of diseases caused by soil-borne pathogens (GOMES, 2018).

Members of the *Fusarium solani* (Mart.) complex, *Macrophomina phaseolina* Tassi (Goid.), *Rhizoctonia solani* Kühn, and *Monosporascus cannonballus* Pollack e Uecker are the major agents found to cause melon root rot diseases in this region (NASCIMENTO et al., 2018). These pathogens have been found causing disease alone (single species) or in an association (multiple species) (PORTO et al., 2016). Melon plants can be affected by these pathogens at all stages of development, but more prominently at the end of the crop cycle. The disease usually develops in the roots and the soil line region becomes necrotic, reaching the plant stem and leading to loss of plant vigor, stopping growth, and becoming wilted and drying out (PAVAN et al., 2016). Symptoms can also be observed on the fruits as characteristically water-soaked or dry lesions (ARAÚJO et al. 2021).

The control of root pathogens is a complex task, it requires a combination of different strategies that not only control the disease but also promote plant and soil health. With the global ban on methyl bromide (MARTIN, 2003), there are no registered chemicals to be used in the field for soil fumigation. Plus, there is a lack of resistant cultivars to most melon diseases found in Brazil (AGROFIT, 2023). All these issues combined call for the development of new and alternative ways to control this disease complex, all while promoting soil health, and the preservation of the environment in a climatically fast-changing world. Incorporating green material into the soil can be a viable alternative to the management of soil-borne pathogens and promote the quality of

biological attributes. It can improve soil diversity and enzymatic activities and accelerate the transformation of soil nutrients, all these beneficial factors can result in crop yield improvement (MA et al., 2021; HONORATO et al., 2022). This strategy can also suppress root rot diseases by promoting the development of microbial communities antagonistic to soil-borne pathogens (LARKIN, 2015).

The incorporation of crambe (*Crambe abyssinica* Hochst) and jack beans (*Canavalia ensiformis* (L.) DC.) have the potential to suppress the disease after incorporation into the soil. There are many reports about the successful use of this crop to promote the quality of soil and increase soil fertility (BENTO et al., 2020). Addtionaly, there are no reports of crambe or jack beans being hosts of the primary soil-borne pathogens that causes melon disease. Both crops contain compounds that directly affect soil-borne pathogens, *e.g.*, glucosinolates, enzymes, and polypeptides. Those plants are adapted to high temperatures, have water deficit tolerance, a short growth cycle, and low production cost, making them great candidates for use as soil incorporation green material in northeastern Brazil (RONCATO et al., 2018; PORTO et al., 2016). There is also the advantage of those plant being planted in the same melon planting area during the off-season, making crop management easier.

To increase the changes of local melon growers to adopted such strategy, it is necessary to adjust the incorporation of these plant materials to existing techniques already in use in their planting system, such as the polyethylene plastic (mulch) as ground cover. This technique is used by growers to decrease evapotranspiration and improve weed control, reducing production costs. Polyethylene plastic covers the entire planted area; holes are made in the center of the plastic to where melon plantlets are transplanted, and the polyethylene plastic is kept throughout the crop cycle. Moreover, incorporating plant material prior to covering the field with the polyethylene mulch without the hole for a certain period of time before plantlets are transplanted, may promote an accelerated decomposition of the plant material, releasing toxic substances and increasing the temperature of the soil, possibly achieving a biofumigation effect. This strategy could be explored further by adding bioactive inductors to the system.

Bioactive inductors are alternative products, such as Compost Aid® and Copper Crop® (Alltech Crop Science), that are known to increase plant nutrition and foster the development of microbes in the soil that stimulate plant growth. Currently, a range of these products are available in the market and are being used by melon growers, that can act as a biostimulant, biopesticide, and biofertilizer (CHOJNACKA, 2015). According to

the manufacturer, Compost Aid® is an additive resulting from enzymes (cellulase, protease, and xylase) and bacteria (*Lactobacillus plantarum*, *Bacillus subtilis*, and *Streptococcus faecium*). This product facilitates and accelerates the decomposition process of material in the soil, fostering the creation of a rich and balanced environment. Copper Crop® provides copper in the form of an organic complex that allows the best use of this element for the plant, increasing its performance. In addition, it has nitrogen and organic carbon in its composition. There are few studies reporting the use of these products alone or associated with other techniques, with high efficiency in controlling diseases caused by soil-borne pathogens (PEIRIS et al., 2021; MOURA et al., 2022). We hypothesize that combining the incorporation of green material (crambe or jack beans) in the soil with polyethylene mulch, and bioactive inductors can be a viable tool to suppress root rot diseases by reducing the population of root pathogens and enhancing soil health in melon fields. In this study, we aimed to evaluate the effect of combining these strategies in managing root rot in melon plants as an effective integrated disease management plan. We also evaluated the effects of these techniques on the soil's biological attributes as compared to traditional management in a melon-producing area.

2 MATERIAL AND METHODS

2.1 Greenhouse experiment

The first stage of the experiment was carried out in a greenhouse located at the Universidade Federal Rural do Semi-Árido (UFERSA) at the Center for Agricultural Sciences (5° 12' 48" S; 37° 18' 44"W).

2.1.1 Experimental design

A completely randomized design with eight treatments and five replicates was evaluated in the greenhouse experiments, including incorporating green material into the soil (crambe or jack bean) and applying bioactive inductors. The treatments were: (Control) - Bare soil; (PM) - Polyethylene mulch; (C) - Crambe incorporation; (C + CA) - Crambe incorporation and Compost Aid® application; (C + CC) - Crambe incorporation and Copper Crop® application; (JB) – Jack bean incorporation; (JB + CA) – Jack bean incorporation and Compost Aid® application; and (JB + CC) - Jack bean incorporation

and Copper Crop® application. With the exception of the control treatment, PM was included in all treatments.

2.1.2 Preparation and application of treatments

Crambe and jack bean were chosen because they can potentially suppress root rot disease after incorporation into the soil. Crambe is a new plant being studied in this region and there is no information on the possibility of this plant being host of root rot pathogens. Therefore, before starting the experiment, a test was carried out to verify if crambe was host of *F. falciforme* (CML3946 - Genbank MH709261) and/or *M. phaseolina* (CMM1531 - Genbank MN136199) isolated from melon plants. We conducted the experiments using two different methodologies (wooden toothpick and soil infestation). No root rot symptoms were observed in crambe when inoculated with the two pathogens, suggesting that it is not a host of the referred pathogens. Therefore, we followed with the use of crambe for incorporation purposes.

The pots used in the greenhouse experiment (14.3 L) were filled with soil collected from one melon-producing area with a history of problems with soil-borne pathogens (Baraúna, RN, Brazil). They were infested in the first layers (up to 15 cm deep) with a substrate mix infested with two pathogens, *F. falciforme* and *M. phaseolina* (9 grams of inoculum from each isolate/L of soil, totaling 18 grams of inoculum/L of soil), isolated from plant roots with root rot symptoms. These isolates grew in a sandy-organic substrate (LEFÈVRE; SOUZA, 1993) for 15 days. After 48 hours, Crambe (40 days after transplanting - DAT), and Jack bean (45 DAT) were collected in full bloom (> 50% of flowering plants) and shredded in forage (leaves and branches) into pieces of around 5 cm, and incorporated into the soil in a depth of 0 - 10 cm in the proportion of 4 $kg.m^{-2}$ of green material (AMBRÓSIO, 2003). With the exception of the control, polyethylene mulch was applied on all treatments before melon planting, with the white side facing up and the black side facing down. The polyethylene mulch remained in the soil for 25 days to simulate solarization. Yellow melon seedlings (11 days after seedling) of the susceptible hybrid Goldex, commonly used in melon-producing areas, were transplanted on the pots and kept until the end of the crop cycle. The treatment that contained alternative products were set up via fertigation following the application recommendations determined by the manufacturer. The application of Compost Aid® was performed at 3, 14, and 21 days after planting seedling (DAP), at 3 kg/ha for the first

application and 2 kg/ha for the last two applications. Copper Crop® (1 L/ha) was applied weekly from 40 days after transplanting (DAT).

2.1.3 Epidemiological components and plant evaluation

Epidemiological components were evaluated at the end of the melon cycle (60 DAT) (Figure 1E). The disease incidence (INC) was measured through the percentage of symptomatic plants within each treatment. The severity of the disease (SEV) was estimated using the rating scale described by Ambrósio et al. (2015), where: 0 = asymptomatic tissue, 1 = less than 3% of infected tissues, 2 = 3-10% of infected tissues, 3 = 11-25% of infected tissues, 4 = 26-50% of infected tissues, 5 = more than 50% of tissues infected. Tissue from plants with root rot symptoms was isolated indirectly in PDA media (Potato-Dextrose-Agar) to confirm the causal agents of rot in the root systems of plants.

For the correct identification of the causal agents, the fungi grown in the PDA culture medium were grown in a single colony and separated into morphotypes according to the characteristics of the colonies. Representatives of each morphotype were chosen for molecular identification. Total fungal DNA was extracted using the plant total DNA extraction kit (Thermo Fisher Scientific, Waltham, MA), following the manufacturer's recommendations. The total DNA obtained was amplified using primers design from fungi conserved genomic region as described in Table 1, and using the Green Taq 2x master mix (Thermo Fisher Scientific, Waltham, MA), following the manufacturer's recommendations. Amplicons generated were cleaned using Charge Switch PCR Clean-Up Kit (Thermo Fisher Scientific, Waltham, MA) and quantified using Nanodrop 2000 (Thermo Fisher Scientific, Waltham, MA). The amplicon sizes were verified in 1.5% agarose gel electrophoresis and then Sanger sequenced at the Yale Center for Genome Analysis, Yale University, New Haven – CT, USA.

The number of leaves (NL), stem diameter (SD), using a digital caliper, and plant height (HE) were measured. Then, the plants were weighed to obtain fresh mass (FM) and fresh root mass (FRM). With a graduated cylinder, the root volume (RV) was estimated. The aerial part of the plant was packed in paper bags and placed in an oven with forced air circulation, at a temperature of 65 °C, until obtaining constant mass and subsequent determination of dry mass (DM).

Table 1. Primers used for amplification of specific regions of the genome of *Fusarium* and *Macrophomina* isolates and subsequent identification.

Target region	Primer	Sequence (5' – 3')	Amplicon size
Nuclear ribosomal internal transcribed spacer	ITS5_F	GGAAGTAAAAGTCGTAACAAGG[1]	500 bp
	ITS4_R	TCCTCCGCTTATTGATATGC	
β-tubulin	BT1_F	AACATGCGTGAGATTGTAAGT[1]	600 bp
	BT22_R	TCTGGATGTTGTTGGGAATCC	
Translation elongation factor 1-alpha	EF1α_F	ATGGGTAAGGARGACAAGAC[2]	650 bp
	EF1α_R	GGARGTACCAGTSATCATGTT	

[1] O'DONNELL and CIGELNIK (1997); [2] O'DONNELL et al. (1998).

2.2 In-field experiments

The field experiment was carried out in two melon-producing areas with history of root rot in melon plants in Mossoró, Rio Grande do Norte, Brazil.

2.2.1 Experimental design and treatments application

The three best treatments in the greenhouse experiment were compared with the management commonly performed by melon growers in the Brazilian semi-arid region (the control treatment). The experiment was performed under a randomized blocks design, with five treatments and four replications, each plot represented by eight melon plants. The treatments were: (Control) – Usual management of melon growers; (PM) - Polyethylene mulch; (JB) – Jack bean incorporation; (JB + CA) – Jack bean incorporation and Compost Aid® application; and (JB + CC) - Jack bean incorporation and Copper Crop® application. In this experiment, PM was included in all treatments.

At the time of opening the furrows for fertilizing the melon foundation, branches and leaves of shredded jack beans (40 days DAT) were added to the rows with the incorporation of green materials at a dose of 4 $kg.m^{-2}$. The soil was mixed, the furrow was covered, and the polyethylene mulch was applied. Except for the control treatment, the soil remained in this solarization simulation state (biofumigation process) for 25 days. After this period, holes were drilled on the polyethylene mulch with 40 cm spacing between holes. Holes were kept open for 48 hours to volatilize gases resulting from the decomposition of jack bean plant material that can be toxic to plants. Then, yellow melon seedlings (11 days after seedling), a susceptible hybrid Goldex, were transplanted. Plants were grown in the field until harvest.

As the experiment carried out in a greenhouse, the treatments that contained alternative products were set up following the application recommendations determined by the manufacturer.

2.2.2 Epidemiological components and plant evaluation

At the end of the melon cycle (60 days), the plants (aerial part and roots) and fruits were collected for analysis. Fresh mass (FM) and dry mass (DM) of aerial parts were measured. The fruits were weighed, and the average fruit weight, the number of fruits in each treatment, the number of fruits per plant, and productivity were estimated. Post-harvest analyses were also carried out by measuring the fruit soluble solids (SS) content and fruit firmness.

The roots collected were cleaned with water, and disease incidence and severity were quantified using the scale developed by Ambrósio et al. (2015). To confirm the nature of the disease, tissue fragments (≅ 1 cm) from plants with root rot symptoms were isolated by the indirect method, by sterilization in 70% ethanol (30 s) and 1% sodium hypochlorite (1 min), rinsed in sterile water and placed in Petri dishes containing PDA media (Potato-Dextrose-Agar) + 0,05 g of tetracycline per L. Plates were maintained in a Biochemical Oxygen Demand (BOD) incubator for seven days at 28 ± 2 °C. After one week, the grown colonies were morphologically identified under an optical microscope and separated into morphotypes. Then, two representative isolates from each morphotype group were identified at the molecular level. The identification of isolated phytopathogenic fungi followed the same steps as in the greenhouse experiment, and the occurrence of these isolates was quantified.

2.2.3 Soil respirometry

To estimate the soil respirometry in the collected soil samples in each treatment, we used the respirometry evaluation method in the function of incubation time (VIVIAN et al. 2006). For this, samples containing 100 g of moist soil (at field capacity) were sieved and incubated for 21 days in hermetically sealed flasks connected through a thin hose to an Erlenmeyer containing 100 mL of NaOH (0.25 mol. L^{-1}). A flask without soil was used as an alternative to control the air quality, which served as a mock in relation to the others. The samples were kept at room temperature (± 28 °C).

These soil-containing flasks were subjected to a sealed system. A compressor was used to push the air to make the system work. Before the air leaving the compressor enters

the system, it passes through an Erlenmeyer containing silica (to avoid humidity in the system) and three Erlenmeyer containing concentrated NaOH (1 mol. L^{-1}) so that there is no influence of CO_2 from the environment and only the CO_2 from the respiration of microorganisms is quantified. Two empty Erlenmeyer (Reflux) were added to prevent NaOH from entering the system. This way, the CO_2 released from the soil samples is carried by a continuous flow of air (CO_2-free) to the NaOH solution (0.25 mol. L^{-1}) (VIVIAN et al. 2006). The system works when bubbles are slowly formed in the final NaOH solution. This indicates that the evolved CO_2 reacts with NaOH from the solution and forms sodium carbonate (Na_2CO_3). The NaOH that was not consumed during the reaction is quantified (VIVIAN et al. 2006).

2.2.4 Carbon from soil basal respiration

For a better understanding of the long-term effect of treatments on soil respiration, 21 days after the start of the soil respirometry experiment, the amount of CO_2 that evolved from the soil samples was estimated from the titration of 50 mL of the NaOH (0.25 mol. L^{-1}) solution with one acid HCl solution (0.1 mol. L^{-1}) previously standardized. The indicator used was phenolphthalein (1%), two drops. At the end of the titration, the color solution changes from pink to colorless, highlighting an acid-base neutralization reaction. At the end of each analysis, the Erlenmeyer was filled with a new 100 mL NaOH solution (0.25 mol. L^{-1}) (VIVIAN et al. 2006).

To evaluate microbial activity in the soil in response to the treatments studied, the carbon amount from basal soil respiration was estimated by the formula (SOUZA et al. 1999): BRS (mg C-CO_2 kg^{-1} soil) = (((Vm – Vs). M. 6. 1000) / DSM), where BRS = Carbon from basal respiration of the soil; Vm (mL) = volume of HCl spent in the titration of the mock solution; Vs (mL) = volume of HCl spent on samples titration; M = exact molarity of HCl; DSM (g) = Dry soil mass.

2.2.5 Soil microbial biomass carbon

At the end of 21 days incubation period in a closed system, the soil was removed from the flasks, and the carbon was extracted from the soil to calculate the microbial biomass carbon (MBC). These parameters were quantified by the methodology described by Islam & Weil (1998). Two samples of 20 g of soil each were collected from each flask. One of the samples was treated for 30 s with microwave radiation (irradiated), and the other (non-irradiated) was not. The energy radiated by microwaves causes microbial cells

to rupture, releasing intracellular compounds, including carbon, which was be extracted. The balance of irradiated x non-irradiated samples was quantified.

The MBC was extracted from the samples (irradiated and non-irradiated) of soil by adding 80 mL of a 0.5 mol L^{-1} K_2SO_4 solution to the samples, followed by shaking for 30 min on a horizontal shaker table and resting for another 30 min. Then, the samples were filtered through filter paper. In a digester tube, 10 mL of the filtrate was taken, and the following reagents were added in this order: 2.0 mL of $K_2Cr_2O_7$ (0.0667 mol. L^{-1}), 5 mL of concentrate H_3PO_4, and 10 mL of H_2SO_4 (0.2 mol. L^{-1}). The tubes were heated for 30 min at 100 °C, allowing them to cool until reaching room temperature (±28 °C). The volume was adjusted to 100 mL in a calibrated cylinder. The sample was transferred to 250 mL Erlenmeyer, the diphenylamine indicator was added (5 drops), and the titration was carried out with a 0.033 mol. L^{-1} solution of $(NH_4)_2Fe(SO_4)_2$ until the blue-dark/purple color changed to green (reduction-oxidation volumetrics) in the samples (ISLAM & WEIL, 1998). At the end of this step, the carbon extracted from the soil of each sample was calculated using the formula (DE-POLLI & GUERRA, 1997): $C = (Vm - Vs) \times N \times 0.003 \times 50 \times (8 \times Ps)^{-1} \times 10^6$, with C = carbon extracted from the soil (mg. kg^{-1}); Vw = volume of $(NH_4)_2Fe(SO_4)_2$ spent in the titration of the mock solution (mL); Vs = volume of $(NH_4)_2Fe(SO_4)_2$ spent in the titration of the sample solution (mL); N = normality of $(NH_4)_2 Fe(SO_4)_2.6H_2O$, and DSM = Dry soil mass (g).

From the estimated amount of carbon at 21 days of incubation (C), the MBC (mg. kg^{-1} soil) was calculated by the formula (DE-POLLI & GUERRA, 1997): $MBC = FC. kc^{-1}$, with FC = Flow obtained from the difference between the amount of C retrieved in the extract of the irradiated and that of the non-irradiated sample, kc = correction factor: 0.33 (SPARLING & WEST, 1988).

2.2.6 Soil microorganisms

Soil samples were prepared by serial dilution (1 g of soil in 9 mL of sterile distilled water) until finding the best dilution ranges that obeyed the counting limit of 25 to 250 CFU. Therefore, an aliquot (0.1 mL) was distributed in Petri dishes containing specific culture media for each target microorganism using the spread plate method. For quantification of total fungi, Martin's culture medium (MARTIN, 1950) was used (10 g of glucose; 5 g of peptone, 1 g of $KH_2 PO_4$, 15 g of agar, 3.3 mL of rose Bengal, and 1 L of water distilled). A nutrient agar culture medium was used to quantify total and

sporulating bacteria. For the analysis of sporulating bacteria, the diluted soil samples were kept in a water bath (80 °C per 20 min) prior to placing the sample aliquots in Petri dishes to kill non-sporulating bacteria (BETTIOL, 2007). The starch-casein culture medium (10 g of soluble starch, 0.3 g of hydrolyzed acid casein, 2 g of KNO_3, 2 g of NaCl, 2 g of K_2HPO4, 0.05 g of $MgSO_4.7H_2O$, 0.02 g of $CaCO_3$, 0.01 g of $FeSO_4.7H_2O$, 18 g of agar, and 1 L of water distilled) was used to quantify actinomycetes (KÜSTER; WILLIAMS, 1964). The Petri dishes were maintained in a Bio-Oxygen Demand (BOD) incubator at 28 ± 2 °C. After the growth of microorganisms (fungi five days, bacteria two days, and actinomycetes five days), quantification was performed using the colony counting technique, calculating: Number of colonies on the plate x reciprocal of the dilution of the sample = number of colonies forming unit per gram of soil ($CFU.g^{-1}$). Microbiological analyses, analyzed as response variables to the field experiment, were performed in a design completely randomized with four replications. Each replicate consisted of three Petri dishes.

2.3 Statistical analyses

Data were submitted to the Shapiro-Wilk normality test ($p<0.05$) and Bartlett's homogeneity of variances ($p<0.05$). The effect of the treatments used in the greenhouse experiment was observed from the significance of the F test and the means compared by the Tukey test ($p<0.05$). In the field experiment, the joint analysis of the variance of the variables was performed to verify the homogeneity of the experiment, that is, if they are identical. The effect of the treatments was observed by the F test, and the means were compared by the Duncan test ($p<0.05$). The effect of the treatments used in the soil microbial experiment was observed from the significance of the F test and the means compared by the Tukey test ($p<0.05$). The nonparametric Kruskal-Wallis's test was performed for the variable (total fungi) that did not show the normal distribution and/or homogeneity of variances ($p<0.05$). All analyzes were performed using R software version 4.02. (R CORE TEAM, 2020).

3 RESULTS

3.1 Greenhouse experiment

At the end of the experiment, at 60 DAP, no difference between the treatments was found for the variables related to plant growth, NL, SD, and HE. However, there was a difference in the variables FM, FRM, RV, DM, INC and SEV (Table 1).

Table 2. Variables related to plant growth and disease epidemiology at 60 DAP of melon plants, submitted to different treatments, in a greenhouse. Mossoró, RN, Brazil.

Treat.	NL[1]	SD (mm)	PH (m)	FM (g)	FRM (g)	RV (dm^3)	DM (g)	INC (%)	SEV
CT[2]	111.2 a[3]	7.00 a	155.6 a	377.2 a	10.18 b	7.28 b	34.2 abc	80 bc	1.8 abc
PM	90.6 a	7.55 a	144.0 a	367.4 a	9.29 b	9.85 b	29.8 bc	100 c	3.0 c
C	85.2 a	7.36 a	162.2 a	364.6 a	12.09 b	11.96 ab	38 ab	60 bc	1.6 abc
C+CA	86.8 a	7.20 a	165.2 a	318.8 ab	12.53 ab	8.01 b	33.6 bc	40 b	0.8 abc
C+CC	73.0 a	5.91 a	129.0 a	178.4 b	5.91 b	8.07 b	17.2 c	80 bc	2.2 bc
JB	117.4 a	7.58 a	164.8 a	453.6 a	13.64 ab	8.84 b	32.8 bc	20 b	0.2 ab
JB+CA	112.8 a	7.67 a	164.0 a	396.2 a	9.28 b	13.39 ab	41.4 ab	40 b	0.8 abc
JB+CC	110.2 a	7.65 a	172.0 a	460.4 a	23.29 a	21.09 a	54 a	0 a	0 a

[1] NL: number of leaves, SD: stem diameter, PH: plant height, FM: fresh mass, FRM: fresh root mass, RV: root volume, DM: dry mass, INC: disease incidence, and SEV: disease severity. [2] CT: Control treatment, PM: Polyethylene mulch, C: Crambe incorporation, JB: Jack bean incorporation, CA: Compost Aid®, CC: Copper Crop®. [3] The averages followed by the same letters in the column did not differ statically from each other by Tukey's test ($p<0.05$).

For the epidemiological variables (INC and SEV), treatments containing only polyethylene mulch (PM), incorporation with crambe (C, C+CA, and C+CC), and the treatment containing JB+CA did not differ statistically from the control (Table 1). For these variables, treatments containing JB incorporation stood out, mainly JB+CC, in which there was no appearance of root diseases in plants in this treatment.

The treatments that best controlled root rot positively affected the root volume (RV) of melon plants. The treatment with the lowest incidence and severity of the disease, (JB+CC) was also the one with the highest root volume, about 21.09 dm^3, equivalent to approximately three times the root volume of the control (7.28 dm^3) (Figure 1).

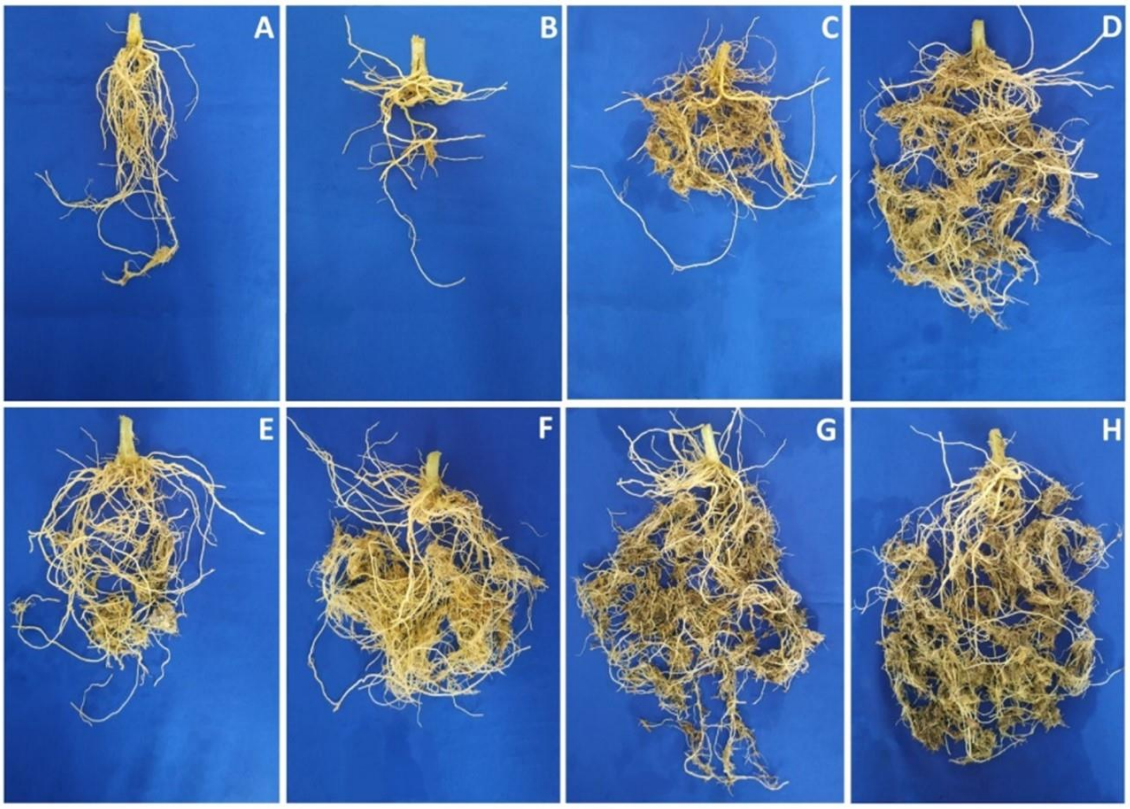

Figure 1. Melon roots at 60 days, treated with the following treatments: (A) Control; (B) PM: Polyethylene mulch; (C) Crambe incorporation; (D) Crambe incorporation and Compost Aid® application; (E) Crambe incorporation and Copper Crop® application; (F) Jack bean incorporation; (G) Jack bean incorporation and Compost Aid® application; (H) Jack bean incorporation and Copper Crop® application.

Despite a reduction in disease and increased plant-related variables, the treatment containing crambe was not taken to be tested in the field. The crambe culture, despite being reported as adapted to adverse climatic conditions, did not demonstrate the expected effect when planted in the semi-arid region of northeastern Brazil, not reaching satisfactory fresh mass values that justify its use as material for incorporation into the soil.

3.2 In-field experiments

Treatments containing jack bean incorporation, whether associated with alternative products or not, stood out in both experiments compared to the control treatment and the use of polyethylene mulch alone (PM).

In both experiments, more than 80% of melon plants evaluated showed characteristic symptoms of the disease. The symptoms included necrosis and rot of the root, plant wilting, and plant death, especially around fruit formation (PORTO et al.

2016). However, the plants differed in terms of disease severity. Plants that were grown in soils treated with JB and JB + alternative products (CC or CA) showed lower disease severity compared to control and PM treatments (Table 2). The average severity score was below 2, which according to the scale described by Ambrósio et al. (2015) means that only 3 to 10% of plant tissues are infected.

Table 3. Incidence (INC) and severity (SEV) of root rot in melon plants in two field experiments. Mossoró, RN, Brazil.

Treatments	*Experiment 1*		*Experiment 2*	
	INC[1] (%)	SEV	INC (%)	SEV
Control	96.88 a[3]	2.69 a	100.00 a	3.56 a
PM[2]	84.38 ab	2.40 a	93.75 ab	3.18 a
JB	62.50 b	1.63 b	75.00 abc	2.13 ab
JB+CA	37.50 c	1.09 bc	59.38 bc	1.63 b
JB+CC	25.00 c	0.72 c	50.00 c	1.34 b

[1]INC: disease incidence, SEV: disease severity. [2]PM: Polyethylene mulch, JB: Jack bean incorporation, CA: Compost Aid®, CC: Copper Crop®. [3]Averages followed by the same letters in the column did not differ statically from each other by Tukey's test ($p<0.05$).

In experiment 1, the treatment JB+CA and JB+CC reduced the incidence of the disease by 61.29% and 59.48%, respectively, compared to the control treatment. There was also a reduction in the severity of the disease in the same treatments by 74.19% (JB+CA) and 73.25% (JB+CC), respectively, compared to the control treatment. In experiment 2, the treatment JB+CA reduced the incidence of the disease by 40.62% and 54.21% in severity, respectively, compared with control management. The treatment JB+CC reduced the incidence by around 50% and the severity of the disease by 62.36% compared to the control treatment. The treatment containing JB+PM also stood out, promoting a 40.17% reduction in disease severity compared with the control treatment.

Isolations in PDA media attempted from the roots and soil baseline region in the stem of infected plants showed an occurrence of pathogens of different nature. Overall, fungus of the genus *Fusarium* and *Macrophomina* had the highest occurence, but in some plants *Monosporascus* and *Rhizoctonia* were also present in both experiments (Figure 2). No significant difference was found between treatments when the occurrence of fungi was analyzed by Tukey's test ($p<0.05$).

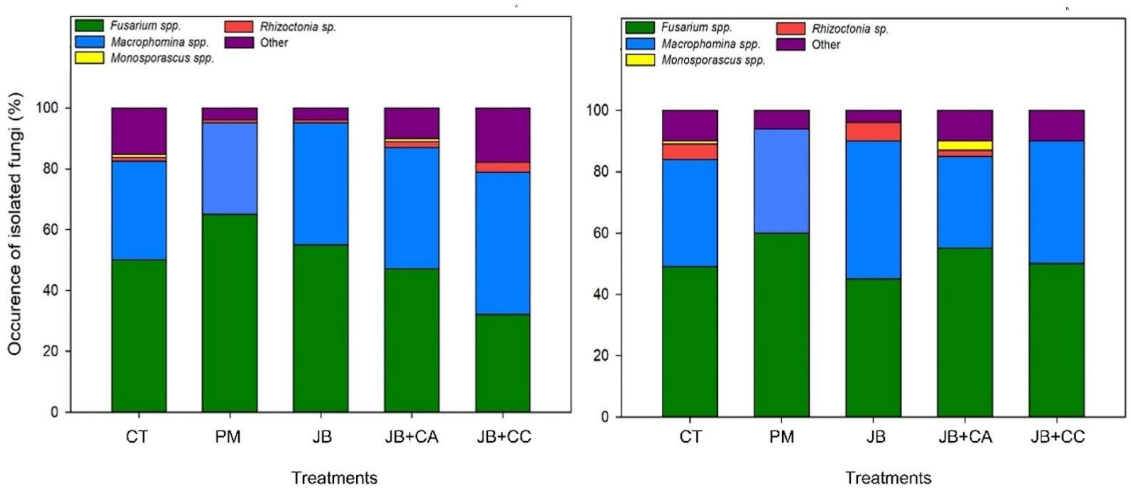

Figure 2. Occurrence of fungi isolated from roots and stem soil baseline of melon plants with root rot symptoms; (A) Experiment 1 and (B) Experiment 2. CT: control treatment, PM: Polyethylene mulch, JB: Jack bean incorporation, CA: Compost Aid®, CC: Copper Crop®.

The sequences obtained were analyzed in the NCBI databases (National Center for Biotechnology Information) through nucleotide comparison. *Macrophomina* isolates showed 100% nucleotide identity with *M. phaseolina*, and *Fusarium* isolates showed 100% nucleotide identity with *F. oxysporum*, *F. pernambucanum*, *F. solani*, *F. equiseti*, and *F. spinosum* species.

Treatments which jack bean (JB) material were incorporated had a significant increase of the final fruit yield. In experiment 1, the JB+CA and JB showed the best results for melon productivity (Table 3). The JB+CA treatment doubled the melon production compared to the control management but did not differ statically from the JB treatment, which increased melon productivity by 58.6%. In experiment 2, the treatments JB, JC+CA, and JB+CC increased by more than 80% melon yield compared to the control treatment and were not statistically different from each other (Table 5). Although there was no significant difference in the fruit weight in any of the experiments, there was a difference in the number of fruits and the number of fruits per plant, directly reflecting the difference in final melon yield observed (Tables 4 and 5).

In experiment 1, soluble solids content (SS) did not show statistical differences between treatments (Table 4). In experiment 2, fruits from the control treatment, JB, and JB+CC treatment showed the highest soluble solids content compared to other treatments. There was no difference between treatments in the fruit firmness variable, ranging from 23.04 to 25.79 N.

Table 4. Melon production components in experiment 1. Mossoró, RN, Brazil.

Experiment 1						
Treatments	Fruit weight (kg)	Number of fruits	Fruits per plant	Productivity (kg/ha)	SS[1] (°Brix)	Firmness (N)
CT[2]	1.01 a[3]	13.00 bc	1.63 bc	20.314,70 c	11.98 a	23.44 a
PM	1.34 a	11.25 c	1.41 c	23.666,97 bc	11.99 a	23.44 a
JB	1.36 a	15.25 ab	1.91 ab	32.220,35 ab	12.07 a	25.79 a
JB+CA	1.49 a	17.75 a	2.22 a	41.334,31 a	12.56 a	24.22 a
JB+CC	1.43 a	14.50 b	1.81 b	31.609,44 b	12.57 a	23.04 a

[1]SS: soluble solids content. [2]CT: Control treatment, PM: Polyethylene mulch, JB: Jack bean incorporation, CA: Compost Aid®, CC: Copper Crop®. [3] Averages followed by the same letters in the same column did not differ statically from each other by Duncan's test ($p<0.05$).

Table 5. Melon production components in experiment 2. Mossoró, RN, Brazil.

Experiment 2						
Treatments	Fruit weight (kg)	Number of fruits	Fruits per plant	Productivity (kg/ha)	SS (°Brix)[1]	Firmness (N)
CT[2]	1.32 a	11.00 c	1.38 c	22,116.25 b	12.37 ab	20.10 a
PM	1.24 a	12.50 bc	1.56 bc	21,743.75 b	11.77 b	21.18 a
JB	1.49 a	17.00 a	2.12 a	39,368.75 a	13.42 a	21.67 a
JB+CA	1.54 a	16.00 ab	2.00 ab	38,183.75 a	13.46 a	21.57 a
JB+CC	1.56 a	17.25 a	2.16 a	37,042.50 a	11.99 b	20.59 a

[1]SS: soluble solids content. [2]CT: Control treatment, PM: Polyethylene mulch, JB: Jack bean incorporation, CA: Compost Aid®, CC: Copper Crop®. [3] Averages followed by the same letters in the same column did not differ statically from each other by Duncan's test ($p<0.05$).

From the carbon from soil basal respiration assessments carried out during 21 days of soil incubation, a positive effect of the treatments on the microorganism's respirometry was verified (Table 6). At 25 days, in experiment 1, the accumulated C-CO_2, in treatment JB, was the highest but did not differ from the PM, JB+CC, and JB+CA treatments. In experiment 2, the JB, JB+CA, and JB+ CC treatments also stood out, which did not differ from each other statistically in both experiments. In this same evaluation, the carbon generated from the soil samples in the control treatment had the lowest averages in both experiments, with values of about 400 mg.kg-1 of soil. Noteworthy, at the time of opening

the polyethylene mulch (25 days), the commercial products Copper Crop® and Compost Aid® had not yet been applied; only the biofumigation simulation had been applied.

Table 6. Values of soil basal respiration in different treatments.

Treatments	Carbon from soil basal respiration			
	Experiment 1		*Experiment 2*	
	25 days[1]	60 dsp	25 days	60 dsp
	---------- mg C-CO_2 kg^{-1} soil ----------			
CT[2]	426.67 b[3]	1,172.79 b	458.08 c	914.82 b
PM	763.32 ab	1,471.02 ab	1,019.47 bc	1,311.84 ab
JB	1,400.03 a	1,986.98 a	1,758.64 a	2,016.25 a
JB+CA	1,244.51 ab	1,926.60 a	1,597.63 ab	2,030.89 a
JB+CC	1,220.73 ab	1,467.37 ab	1,542.74 ab	1,405.16 ab

[1]25 days: On the opening day of polyethylene mulch (25 days after applying polyethylene mulch); 60 dsp: Immediately after the melon harvest (60 days after seedlings planting). [2]CT: Control treatment, PM: Polyethylene mulch, JB: Jack bean incorporation, CA: Compost Aid®, CC: Copper Crop®. [3]Averages followed by the same letter in the same column do not differ statistically by Tukey's test ($p<0.05$).

In soil samples submitted to standard management (CT) collected at 60 dsp, the values were lower than expected compared to the other treatments, ending the evaluation period with 1,172.8 (Exp. 1) and 914.82 (Exp. 2) mg of CO2.kg-1 of soil. However, the control treatment did not differ statistically from the PM and JB+CC treatments in both experiments. The JB and JB+CA treatments, which did not differ statistically at this time, tended to increase the carbon content in the soil, increasing more than 60% (Exp. 1) and 160% (Exp. 2) compared to the control treatment.

The initial C concentration in the microbial biomass was similar in both experiments: 2.75 and 2.4 mg of C.kg^{-1} of soil in experiments 1 and 2, respectively (Table 7). As the experiment progressed, the tendency was for C to accumulate in the microbial biomass in the last two assessments times for all treatments studied compared to the initial sample (0 days).

At 25 days after polyethylene mulch application, an increase in MBC was observed in all treatments compared to the control treatment in both experiments. In experiment 1, the JC+CC treatment presented a carbon concentration of 35.34 mg of C.kg-1 of soil, an increase of 414.4% compared to the control treatment; however, it did not differ statistically from the PM treatment, which had an increase in the carbon accumulated in the microbial biomass by 216.6%, compared to the control treatment. In experiment 2, the same treatment (JB+CC, and PM) stood out but they did not differ

statistically from JB and JB+CA treatments, an increase of at least 139.91% of the carbon in the microbial biomass carbon compared to the control treatment.

Comparing the MBC at 25 days with the values found at 60 dsp, there was a decrease in its values for some treatments, such as the treatments CT, PM, JB, and JB+CC (Exp. 1), and PM, JB, JB+CC (Exp. 2). At 60 dsp, in both experiments, there were no statistical differences for MBC between the studied treatments.

Table 7. Microbial biomass carbon in different treatments.

Treatments	**Microbial biomass carbon**					
	Experiment 1			Experiment 2		
	0 days[1]	25 days	60 dsp	0 days	25 days	60 dsp
	----------mg.kg^{-1} soil----------					
CT[2]	2.75	6.87 b[3]	4.90 a	2.40	4.91 b	7.85 a
PM	2.75	21.75 ab	5.87 a	2.40	16.61 ab	8.82 a
JB	2.75	14.76 b	13.75 a	2.40	24.09 a	21.60 a
JB+CA	2.75	10.79 b	18.65 a	2.40	11.78 ab	19.65 a
JB+CC	2.75	35.34 a	7.87 a	2.40	19.63 ab	18.67 a

[1]0 days: before applying polyethylene mulch; 25 days: On the opening day of polyethylene mulch (25 days after applying polyethylene mulch); 60 dsp: Immediately after the melon harvest (60 days after seedlings planting). [2]CT: Control treatment, PM: Polyethylene mulch, JB: Jack bean incorporation, CA: Compost Aid®, CC: Copper Crop®. [3]Averages followed by the same letter in the same column do not differ statistically by Tukey's test ($p<0.05$).

The initial population of the evaluated microorganisms (total bacteria, sporulating bacteria, total actinomycetes, and total fungi) was similar in both experiments. After introducing jack bean incorporation at the beginning of the experiment, there was an increasing trend for all microorganisms evaluated compared to the initial period.

The initial total bacteria population for the two experiments was similar. In the first experiment, it was 0.49 x 10^5; in the second, it was 0.5x10^5 (Table 8). At 25 days, after incorporating the Jack bean into the soil, covering the soil with polyethylene mulch, and submitting the soil to the biofumigation process was an increase in the total bacteria population in all treatments. The treatment in which there was jack bean incorporation (JB, JB+CA, and JB+CC) stood out in both experiments for having the highest number of CFUs (Table 8). This behavior was maintained until the 60 dsp, with the JB, JB+CA, and JB+CC standing out compared to the control treatment (CT) and using only the polyethylene mulch (PM). The JB+CA treatment is noteworthy. Compared to the control treatment, it increased total bacteria CFUs by more than 64% in both experiments at 60 dsp.

Table 8. Colonies forming units (CFUs) of total bacteria in different treatments.

Treatments	Total bacteria					
	Experiment 1			Experiment 2		
	0 days[1]	25 days	60 dsp	0 days	25 days	60 dsp
	----------10[5] of the number of CFUs g[-1] ----------					
CT[2]	0.49	3.63 b[3]	4.90 b	0.50	3.65 b	6.36 c
PM	0.49	5.17 ab	4.04 b	0.50	3.54 b	3.31 c
JB	0.49	6.49 ab	7.00 a	0.50	6.62 a	16.6 bc
JB+CA	0.49	7.00 a	8.06 a	0.50	6.68 a	58.6 a
JB+CC	0.49	8.08 a	7.35 a	0.50	6.65 a	36.5 ab

[1]0 days: before applying polyethylene mulch; 25 days: On the opening day of polyethylene mulch (25 days after applying polyethylene mulch); 60 dsp: Immediately after the melon harvest (60 days after seedlings planting). [2]CT: Control treatment, PM: Polyethylene mulch, JB: Jack bean incorporation, CA: Compost Aid®, CC: Copper Crop®. [3]Averages followed by the same letter in the same column do not differ statistically by Tukey's test ($p<0.05$).

Following the same behavior of the total bacteria population, there was an increase in sporulating bacteria CFUs compared to the initial population (0 days) (Table 9). In experiment 1, the JB+CC treatment stood out at 25 days, increasing the bacteria CFUs by 141.7% compared to the control treatment; however, it did not differ statistically from the treatments PM, JB, and JB+CA, which increased bacteria CFUs by 28.8, 84.43, and 53.7% compared to the control treatment, respectively. In experiment 2, the treatments JB, JB+CC, and JB+CA did not differ statistically from each other, increasing the sporulating bacteria CFUs by more than 104.8% compared to the control treatment.

At 60 dsp, the JB+CC treatment continued to stand out. In experiment 1, JB+CC treatment increased the sporulating bacteria population by 98.2% compared to the control, statistically differing from the other treatments studied. In experiment 2, the treatments JB, JB+CA, and JB+CC stood out, increasing the population by more than 345% compared to the control treatment, which did not differ statistically between them.

Table 9. Colonies forming units (CFUs) of sporulating bacteria in different treatments.

Treatments	Sporulating bacteria					
	Experiment 1			*Experiment 2*		
	0 days[1]	25 days	60 dsp	0 days	25 days	60 dsp
	----------10[4] of the number of CFUs g[-1] ----------					
CT[2]	0.67	5.75 b[3]	5.70 b	0.13	4.11 b	0.71 b
PM	0.67	7.41 ab	4.39 b	0.13	3.98 b	0.43 b
JB	0.67	10.6 ab	6.16 b	0.13	10.5 a	5.18 a
JB+CA	0.67	8.84 ab	6.48 b	0.13	10.08 a	5.65 a
JB+CC	0.67	13.9 a	11.3 a	0.13	8.42 ab	3.16 ab

[1]0 days: before applying polyethylene mulch; 25 days: On the opening day of polyethylene mulch (25 days after applying polyethylene mulch); 60 dsp: Immediately after the melon harvest (60 days after seedlings

planting). [2]CT: Control treatment, PM: Polyethylene mulch, JB: Jack bean incorporation, CA: Compost Aid®, CC: Copper Crop®. [3]Averages followed by the same letter in the same column do not differ statistically by Tukey's test ($p<0.05$).

The initial total actinomycetes population in soil samples collected at 0 days differed between the two experiments (Table 10). In experiment 1, it was $0.55x10^4$ CFUs, and in experiment 2, it was $0.99x10^4$ CFUs. During the experiment, there were no statistical differences between them at 25 days and 60 dsp.

Table 10. Colonies forming units (CFUs) of total actinomycete in different treatments.

Treatments	**Total actinomycete**					
	Experiment 1			*Experiment 2*		
	0 days[1]	25 days	60 dsp	0 days	25 days	60 dsp
	----------10^4 of the number of CFUs g^{-1} ----------					
CT[2]	0.55	0.66 a[3]	6.34 a	0.99	2.46 a	1.96 a
PM	0.55	0.81 a	5.79 a	0.99	2.20 a	3.26 a
JB	0.55	0.64 a	6.80 a	0.99	4.66 a	3.97 a
JB+CA	0.55	0.99 a	6.10 a	0.99	2.75 a	4.23 a
JB+CC	0.55	0.43 a	6.38 a	0.99	5.43 a	3.95 a

[1]0 days: before applying polyethylene mulch; 25 days: On the opening day of polyethylene mulch (25 days after applying polyethylene mulch); 60 dsp: Immediately after the melon harvest (60 days after seedlings planting). [2]CT: Control treatment, PM: Polyethylene mulch, JB: Jack bean incorporation, CA: Compost Aid®, CC: Copper Crop®. [3]Averages followed by the same letter in the same column do not differ statistically by Tukey's test ($p<0.05$).

The initial fungi population in both experiments was similar: $1.1x10^2$ (Exp. 1) and $1.45x10^2$ CFUs (Exp. 2) (Table 10). The behavior of the fungi population during the experiment increased compared to the initial population (0 days) in the same way as the population of the other microorganisms that we studied in this work. The treatments in which jack beans were incorporated into the soil (JB, JB+CA, and JB+CC) stood out in at 25 days by increasing the fungal CFUs population by more than 55.50% in experiment 1 and 55.12% in experiment 2, compared to the control treatment. Over the course of the experiment, there was a tendency to equalize the fungi population in the soil; therefore, at 60 dsp, there were no statistical differences between treatments.

Table 11. Colonies forming units (CFUs) of total fungi in different treatments.

Treatments	Total fungi					
	Experiment 1			Experiment 2		
	0 days[1]	25 days	60 dsp	0 days	25 days	60 dsp
	----------10[2] of the number of CFUs g[-1] ----------					
CT[2]	1.1	4.5 ab[3]	8.48 (6.5) a[4]	1.45	3.90 b	7.93 (7.75) a[4]
PM	1.1	3.7 b	10.38 (13.2) a	1.45	4.33 ab	25.2 (15.5) a
JB	1.1	7.0 ab	8.86 (10.2) a	1.45	6.05 a	28.2 (14.75) a
JB+CA	1.1	8.5 a	9.65 (11.1) a	1.45	6.54 a	18.1 (9.25) a
JB+CC	1.1	8.1 a	9.67 (11.5) a	1.45	6.53 a	6.59 (5.25) a

[1]0 days: before applying polyethylene mulch; 25 days: On the opening day of polyethylene mulch (25 days after applying polyethylene mulch); 60 dsp: Immediately after the melon harvest (60 days after seedlings planting). [2]CT: Control treatment, PM: Polyethylene mulch, JB: Jack bean incorporation, CA: Compost Aid®, CC: Copper Crop®. [3]Averages followed by the same letter in the column do not differ statistically by Tukey's test ($p<0.05$).[4]Averages followed by the same letter in the same column do not differ statistically by Kruskal-Wallis non-parametric test ($p<0.05$) – Rank data are in parentheses.

It is important to emphasize that in all populations of microorganisms evaluated (total bacteria, sporulating bacteria, total actinomycetes, and total fungi) the PM treatment did not differ statistically from the control treatment at all evaluation times (0 days, 25 days, and 60 dsp).

4 DISCUSSION

In the last decade, studies designed to investigate alternative management strategies for diseases in the melon culture in the biggest producing region of Northeast Brazil have gained prominence due to the importance of the culture for the region. Understanding how soil management affects fruit production, plant health, stability, and diversity of the soil microbiota is extremely important as the balance of attributes related to the plant and the soil dictates the ability of the system to react to any external changes (MUNOZ et al., 2007). In this work, we shed light on the effects of different soil management approaches in melon-growing areas on melon productivity and plant and soil health.

In the field experiment, the assays were different regarding the potential of the disease. In experiment 2, for example, there were higher incidence and disease severity rates than in experiment 1. This fact can be explained by the behavior of the soil-borne pathogens, which manifest themselves in a non-uniform way, causing symptoms in plants through clumps (TÖFOLI and DOMINGUES, 2022). Despite this difference, the treatments behaved similarly in both experiments. The treatment feature of behaving equally at different disease levels in the field is desirable because it proves its efficiency.

In this study, using polyethylene mulch alone for 25 days had no positive effects on suppressing root rot disease as normally occurs in solarization using polyethylene plastic. Traditionally, this plastic cover brings numerous benefits for melon cultivation in the Brazilian semi-arid region, and it is used to mitigate water loss through evaporation and control weeds in the melon field. In some cases, the polyethylene mulch remains for several cycles in the soil, and it can increase disease incidence and exacerbate already poor soil conditions by lowering the biological balance of the soil (CHALKER-SCOTT, 2007; QI et al. 2022; DE MOURA et al. 2022). This affects the microbial communities and play a key role in improving soil fertility; they help in organic material degradation and consequently increase the availability of N, P, K, and Fe in the soil, making them readily available to the plant (CAESAR-TONTHAT et al., 2014; RASHID et al., 2016). This process of recognizing and exploring the mechanisms of soil microorganisms combined with the addition of organic materials can reduce the application of chemical fertilizer and become an essential tool for improving soil fertility (SONG et al., 2015; RASHID et al., 2016) and minimize the effect of damage caused by the constant use of polyethylene mulch on the soil microbial community.

The Jack bean used in soil incorporation treatment provided promising results in the experiment conducted in a greenhouse and the field. The benefits of this culture targeting this technique are widely publicized since they are already known to facilitate the suppression of plant diseases, promoting the control of soil-borne pathogens (FERREIRA et al. 2015). By combining the incorporation technique with the use of polyethylene mulch, this effect can be optimized, as there is the release of substances such as enzymes, glycoproteins, polypeptides, and compounds from amino acids that, when concentrated below the polyethylene mulch, can reduce the population of the pathogen (PORTO et al., 2016). Silva López (2012) adds that the inhibition of the soil pathogen due to the incorporation of Jack beans into the soil may occur due to the isomorphic form of the urease enzyme, which directly inhibits the vegetative growth and germination of some pathogens, such as *R. solani*, *F. solani*, and *F. oxysporum*, produce lesions in the cell wall and membranes and plasmolysis of fungal cells (POSTAL et al. 2012).

Although the use of plants of the Brassicaceae family to control soil-borne pathogens has been extensively studied (TAVARES-SILVA et al., 2017; RONCATO et al., 2018). When compared to the use of Jack bean, the incorporation of the crambe into the soil does not demonstrate efficiency to control melon root rot pathogens in our studies. There are reports of Crambe growing tolerance in semi-arid conditions. However, when

this crop is submitted to extreme stress conditions (*e.g.*, high salt and water stress), its development may be compromised (SILVA et al., 2019) as well as its ability to produce compounds that aid in disease suppression. The climatic conditions where the experiments with crambe were conducted were extreme (high temperatures, high luminosity, and low humidity), which may have influenced the results in this study. Furthermore, even though our pathogenicity tests, prior to starting the experiment, showed that *Fusarium* sp. and *Macrophomina* sp. don't cause rot in crambe, little is known about the susceptibility of this culture to other pathogens species that cause melon root rot disease complex. Therefore, further study is needed to evaluate its effects, after incorporation in the soil, on the development of melon plants and possible host of root rot pathogens.

The bacteria, enzymes, and nutrients in Compost Aid®, combined with the incorporation of green material, can benefit the plant, the soil, and suppress the disease. In a previous experiment carried out by De Moura et al. (2022), Compost Aid®, by itself or in association with another alternative products, was unable to inhibit the growth of *M. phaseolina*. But in this study, with the possibility of the polyethylene mulch accelerating the decomposition process of the green material, the Compost Aid's compounds can create a system with different beneficial capacities, ranging from providing nutrients to plants to the production of phytohormones (*e.g.*, auxin) and antibiotics (KHALID et al. 2011; KALANTARI et al. 2018). This means that in the presence of organic materials in the soil, the effect of Compost Aid® is amplified. Besides, they can improve crop root growth, increase nutrient absorption (KHALID et al. 2011), and strengthen plants' resistance to biotic stresses, such as root rot caused by soil-borne pathogens (FREITAS et al. 2018). Thus, based on the theory of trophobiosis, growth promotion mediated by any treatment that benefits plant growth, even if it has no direct effect on the pathogen, may represent a compensatory effect on developing diseases, including root diseases. In this experiment, the use of Compost Aid® also promoted a greater amount and rate of respiration of microorganisms, which means that the management of the association of bioactive inducer in association with the incorporation of jack bean to the soil favored the community and microbial activities.

Despite increasing some biological attributes studied in this work on the control treatment, such as RBS, MBC, and soil microbiology, the Cooper Crop® alternative product exhibited non-standard behavior compared JB and JB+CA treatments. For example, for sporulating bacteria, in experiment 1, the JB+CC treatment had the highest

average, 11.3 x 10^4 CFUs.g^{-1} of soil. In experiment 2, it presented an average of 3.16 x 10^4 CFUs.g^{-1} of soil, not statistically different from the control treatment. The copper present in Copper Crop® composition is beneficial for plant health in small amounts because the presence of this micronutrient is essential for processes related to vegetative growth and the formation of grains, seeds, and fruits (REN et al., 2009). Copper also has shown positive effects in the control of soil-borne pathogens for causing membrane damage, loss of enzyme activity, and protein dysfunction in these pathogens (REN et al., 2009). However, copper is not selective (CHIBUIKE; OBIORA, 2014), and the microbial biomass may have been affected after applying Copper Crop® as a treatment. Our results are similar to those of Wang et al. (2019), in which the number of bacteria, actinomycetes, fungi, and the gene expression of ammonia-oxidizing microorganisms can vary with the addition of copper to the soil. These data assume that copper doses beyond those required by the plant can affect biological health and microbial functions, and its use in the field must be well evaluated with existing copper levels in the soil and those applied in the form of chemical fertilizers.

Incorporating Jack beans into the soil associated with alternative products in melon management techniques proved to be effective in reducing the occurrence of root rot pathogens and improving melon productivity. It is worth mentioning that these techniques must be continuous so that the reduction in the incidence of the disease can last for several crop cycles. This prolonged effect will result from a significant decrease in the amount of inoculum and restoration of the biological balance of the soil (BETTIOL et al., 2005).

The union of soil management techniques in the melon crop areas in the Brazilian northeast region studied in this research benefited the soil. Introducing elements that are beneficial to melon management, which promotes plant growth and biological soil health, is an innovation for agricultural enterprises seeking management based on sustainability (MEENA et al., 2020). Thus, techniques sub as the incorporation of green materials, such as Jack beans, and the use of alternative products based on microorganisms and enzymes, as we did in this work, must be used and disseminated in association with the management used by melon growers in the Brazilian northeast.

5 CONCLUSION

The treatments containing jack bean incorporation (JB, JB + CA, and JB + CC) were superior in the variables related to the management of the diseases (incidence and

severity of the disease), the plant development (number of fruits per plant, fruits weight, and productivity), and the microbiology of the soil (Soil basal respiration carbon, soil microbial biomass carbon, and bacterial and fungal CFUs) that we evaluated in our work, compared to the control treatment, which has similar characteristics to the management commonly used by melon growers. The treatment JB + CA stood out, as it reduced the severity of the disease, compared to the control. In the same treatment, productivity increased compared to the control. JB, JB + CA, and JB + CC also provided good results by optimizing the biological characteristics of the soil, improving the number of microorganisms in the soil and its biological efficiency, compared to the control treatment, being the most suitable treatments for managing root rot in melon and increase microbial communities.

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CHAPTER 2

The format of this chapter is following the rules of the journal Plos Pathogens in which the manuscript will be submitted.

The genomic region matters when synthesizing dsRNA for plant virus suppression via RNAi

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ABSTRACT

Potato virus Y (PVY) is one of the most economically important plant viruses in the world. Since no efficient control methods are available, RNA interference (RNAi)-based crop protection strategy has become a promising option for the control of viral plant pathogens. In this context, the aim of this study was to test the hypothesis that dsRNA molecules derived from different genomic regions would induce different viral suppression by RNAi, and evaluate the fate and movement of the dsRNA molecules inside tobacco plants. Regions with a high concentration of 21nt siRNAs, detected by a small RNA profiles analysis, were selected to synthesize dsRNA (~600 bp) from each cistron in suppressing PVY infection. The dsRNAs synthesized from CP, HC-PRO, and NIb cistrons were applied and two weeks after viral inoculation, PVY symptoms were assessed. The translocation of the applied dsRNA molecules was also evaluated for 14 days, and in this step no virus inoculation was performed. RT-qPCR was performed on the total RNA extracted from the localized and systemic leaves, and to investigate whether dsRNAs move as dsRNAs or siRNAs within the plant, we used the stem-loop RT-PCR methodology. The movement of dsRNA molecules within the plant was also investigated by labeling the dsRNA with the fluorescent dye cyanine 3-UTP (Cy3-UTP). The dsRNAs applied protected treated plants against PVY infection, and HC-PRO-dsRNA induced greater protection, entered and moved fast compared to CP and NIb-dsRNAs. This fact was confirmed by the fluorescence signal of dsRNA-cy3 in treated leaves. DsRNAs and siRNAs were detected in systemic leaves after 24 hours of dsRNA application, demonstrating that these molecules translocated systemically inside the plant, and remained for at least 14 days. The synthesis and application of exogenous dsRNAs targeting the HC-PRO genomic region of PVY proved to be a promising technique for controlling this disease.

AUTHOR SUMMARY

Potato virus Y is responsible for loss production of various *Solanaceae* family members, such as potato, tobacco, and tomato. PVY has a single-stranded positive-sense RNA genome, which encodes a polyprotein that is cleaved into 11 multifunctional proteins vital for viral replication, movement, transmission, and infection. Based on this knowledge, we focused on using some of these proteins as a basis for the synthesis of dsRNA molecules that, when applied to the plant, will activate the RNA interference mechanism, and suppress the disease. In this work, we also investigated the fate and movement of the dsRNA molecules inside tobacco plants, and whether these molecules move in the form of dsRNA or siRNA. Here, we found that HC-Pro-dsRNA provided greater plant protection against PVY infection than CP-dsRNA and NIb-dsRNA, and this can be explained by the fact that it enters and moves faster inside the plant, proving that the genomic region matters when we use RNAi-based control to plant virus suppression. Furthermore, the movement of these molecules can occur both in the form of dsRNA and siRNA. Our study is a starting point for several other studies to improve the action of RNAi-based technology to control plant viruses for field application.

1 INTRODUCTION

Potato virus Y is one of the top ten most economically important plant viruses in the world (Rybicki 2015). This is an evolving virus that exists in multiple strains that can cause different levels of diseases including mosaic, wrinkling of the leaves, chlorosis, and necrosis, and in severe cases, plants become stunted and die (Karasev and Gray 2013; da Silva et al. 2020). Some strains of PVY can cause potato tuber necrotic ringspot disease (PTNRD), a tuber deformity that makes the potato tubers unmarketable (da Silva et al. 2017). Crop yield losses caused by PVY worldwide are unspecified due to global economic data limitations. However, few studies reported an estimated range of 10-80% loss production of various *Solanaceae* family members including potato (*Solanum tuberosum* L.), tobacco (*Nicotiana tabacum* L.), and tomato (*Solanum lycopersicum* L.) (Hameed et al. 2017). These high rates of losses in agriculture are due to the worldwide distribution of the virus and its insect vector, aphids, and the wide host range of the virus (Lacomme and Jacquot 2017). Chemical control practices targeting PVY insect vectors are often unfeasible due to high cost, environmental hazards, and being inefficient in reducing the virus spread - as PVY is transmitted in a non-persistent manner which only takes seconds for the vector to acquire and transmit the virus from an infected plant to a new host (Hameed et al. 2017). Since no efficient PVY control methods are available, there is an urgent need for developing disrupted strategies to manage PVY infections.

Potyviruses, such as PVY, bear a single-stranded positive-sense RNA genome, which encodes a polyprotein that when mature, is cleaved into 11 multifunctional proteins vital for viral replication, movement, transmission, and infection (Urcuqui-Inchima et al. 2001). For instance, the coat protein (CP) is the viral structural protein, it forms the virion capsid that protects the viral genome and is also involved in virus movement inside the plant and vector transmission. The HC-PRO protein is involved in regulating virus entry and exit from host plant vascular tissues, transmission via aphids, and enhancing viral pathogenicity by suppressing post-transcriptional gene silencing in the host plant (Valli et al. 2018). Another important potyviral protein is the nuclear inclusion protein b (NIb), which is the RNA-dependent RNA polymerase that is responsible for viral replication. NIb is also involved in multifunctional roles of viral-host interactions and it is responsible for recruiting host proteins into the viral replication complexes (VRCs) to enable the virus replication (Shen et al. 2020). These all highlight that strategies developed to inhibit the translation or function of those proteins would directly disrupt virus infection.

Recently, RNA interference (RNAi)-based crop protection strategy has become a promising option for the control of plant viruses and other pathogens (Hernández-Soto and Chacón-Cerdas 2021). This natural mechanism, also known as post-transcriptional gene silencing, regulates gene expression and is highly conserved among eukaryotes organisms (Fire et al. 1998), which relies on double-stranded RNA (dsRNA) or hairpin RNA (hpRNA) molecules as precursors (Shidore et al. 2021). Once those molecules are in the cell, dicer-like enzymes recognize and process them into small fragments, the so-called small interfering RNAs (siRNAs), usually 20-25nt in length (Shidore et al. 2020). The processed siRNAs are recruited by Argonaute proteins (AGOs), forming the RNA-induced silencing complex (RISC), to guide the cleavage of target RNAs, RNA molecules that share complementarity with the internalized siRNA sequences. Thus, antiviral therapeutics could be developed on the bases of such a mechanism by synthesizing RNAi inducers (dsRNA, hpRNA, or siRNA), that target viral RNA sequences, and applying them to plants to trigger RNAi and prime the plant to suppress target virus pathogens.

Several recent studies have shown the potential of using RNAi-based technology to control plant viruses. For example, the application of dsRNA molecules targeting the nucleocapsid (N) gene of tomato spotted wilt virus (TSWV) delayed the symptom expression in tobacco and tomato plants infected with TSWV by at least 40 days (Tabein et al. 2020). They also showed that the application of dsRNAs targeting the movement protein (NSm) gene resulted in a poor suppression of the virus, demonstrating the importance of choosing the right genomic region for virus control via RNAi. The HC-Pro and NIb genes were also used as a target to synthesize dsRNA molecules that protected the tobacco (*Nicotiana benthamiana* Domin.) plants against pepper motlle virus (PepMoV) by inhibiting viral development (Yoon et al. 2021). Similarly, dsRNA synthesized from the CP and 2b genes protected tobacco (*Nicotiana tabacum* L.) and quinoa (*Chenopodium quinoa* Willd.) plants against cucumber mosaic virus (CMV) (Holeva et al. 2021) by reducing local damage caused by virus expression in these hosts. Proving this efficiency, other factors such as concentration, length, and movement of dsRNA inside the plant, as well as the time of application, should be investigated to improve the efficiency in suppressing viral diseases (Das and Sherif 2020). The aim of this study was to test the hypothesis that dsRNA molecules derived from different genomic regions would induce different viral suppression by RNAi. We also evaluated the fate and movement of the dsRNA molecules inside tobacco plants.

2 MATERIAL AND METHODS

2.1 Plant material and PVY strain

The PVY isolate used in this study was obtained from infected potato plants (Isolate MN21, strain PVY^{N-Wi}, cordially provided by Dr. Stewart Gray from USDA-ARS/Cornell University). The isolate was maintained in lyophilized tobacco plant tissue and stored at -80 ˚C. For inoculum reactivation, tobacco plants (*Nicotiana tabacum* cv. Samsun), propagated via seed, were kept in 1-gallon pots containing Cornell potting mix growth (Boodley and Shelldrake 1982) and maintained in an insect-free growth chamber under controlled conditions at 25 ±3˚C and 16 h photoperiod. When the plants were at the three to five-leaf stage, they were mechanically inoculated with PVY using carborundum (325 mesh) as abrasive - the inoculum was prepared from lyophilized tissue homogenized in phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 2 mM KH_2PO_4, with pH adjusted to 7.4 with HCl) at 1:5 w/v (1 g of leaf to 5 mL of PBS). Seven days after inoculation, characteristic symptoms of PVY infection could be observed on inoculated plants and the PVY strain identity was confirmed using a multiplex RT-PCR (Chikh-Ali et al. 2013). Infected plants were maintained in a growth chamber, *per* the conditions described above, and used as viral inoculum sources in subsequent experiments.

2.2 Small RNA profiling analyses

Small RNA profiles were analyzed to aid the selection of PVY genomic regions for testing as perfomed by our group previously (Shidore et al. 2020). Infected tobacco leaves from source plants were harvested, ground in PBS at 1:5 w/v (1 g of leaf to 5 ml of PBS) and used as PVY inoculum. Then, three tobacco plants (at the three- to five-leaf stage) were inoculated by using a cotton swab to lightly rub the PVY inoculum using carborundum as an abrasive. The top two fully mature leaves were treated with PVY inoculum and PBS alone as a mock, on individual plants as two different treatments, each treatment had three replicates. One week after inoculation, a systemically infected leaf was harvested from each individual plant and immediately frozen in liquid nitrogen followed by total RNA extraction using the Purelink Plant RNA reagent following the manufacturer's instructions (Invitrogen, Waltham-MA, USA).

Small interfering RNAs (siRNAs) were isolated from the total RNA using polyacrylamide gel electrophoresis, followed by adapter ligation to the 5′ and 3′ ends of the siRNAs. The NEBNext Small RNA Library Prep Set for Illumina (NEB, Ipswich – MA, USA), was then used according to the manufacturer's protocol. Libraries were barcoded, pooled in equal molarity, quality checked using a 2100 Bioanalyzer, and then sequenced on an Illumina HiSeq4000 platform (generating 76 bp single-end reads) at Yale Center for Genome Analysis, Yale University, New Haven – CT, USA. Then, Illumina's CASAVA pipeline v1.8.2 software was used to demultiplex the raw reads, followed by reads quality enhancement using Trimmomatic software (Bolger et al. 2014). Identification of distinctive siRNAs reads was performed by aligning the reads with the PVY genome with a specific pipeline (Donaire and Llave 2019). Finally, siRNA orientation and graphical representation across the viral genome were performed using MISIS (Seguin et al. 2014) and R software (R Core 2022).

2.3 dsRNA synthesis

Total RNA extracted from an infected tobacco plant was reverse transcribed using the ProtoScript II First Strand cDNA Synthesis Kit (NEB, Ipswich, MA), following the manufacturer's instructions. The cDNA obtained was amplified using PVY-specific primers designed for the CP, HC-PRO, and Nib cistrons (Table 1), using the Phusion® High-Fidelity PCR Master Mix with HF Buffer (NEB, Ipswich, MA). These genomic regions were selected based on the siRNA mapping analyses data, we selected regions with a high concentration of 21nt siRNAs. Amplicons generated (~600 bp) for each genomic region were resolved in 1.5% agarose gel electrophoresis, cleaned using ChargeSwitch PCR Clean-Up Kit (Thermo Fisher Scientific, Waltham, MA), quantified using Nanodrop 2000 (Thermo Fisher Scientific, Waltham, MA), and Sanger sequenced at the Keck Biotechnology Resource Laboratory, Yale University, New Haven – CT, USA, to confirm the amplicon sequences prior to dsRNA synthesis. Then, PCR amplicons were used as templates for dsRNA synthesis using the T7 Ribomax Express RNAi System kit (Promega, Madison, WI), following the manufacturer's instructions. The T7 RNA polymerase promoter (TAATACGACTCACTATAGGG) was added at the 5'-end of the amplification primers (Table 1) to increase the effectiveness of the RNA synthesis.

2.4 Viral inoculum standardization

To determine the minimum inoculum concentration required to induce PVY infection symptoms in tobacco plants, a sequential inoculum dilution experiment was conducted. Infected tobacco leaves were collected and macerated at 1:1 w/v (1 gram of leaf to 1 mL of PBS) in an extraction bag (Bioreba, Reinach BL, Switzerland), which possesses a synthetic intermediate layer for optimal filtration, to create an undiluted stock solution. Then, serial dilutions (1:100, 1:200, 1:400, 1:800, 1:1200, 1:1600, and 1:2000) from the stock were made by adding PBS. A double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) was performed on the diluted samples to determine the absorbance value for each diluted point, ELISA was duplicated following the manufacturer's instructions (Bioreba, Reinach BL, Switzerland). Then, a bioassay was set up where five tobacco plants (at the three- to five-leaf stage) were inoculated per dilution point. The top two fully mature leaves were inoculated by using a cotton swab to lightly rub the PVY inoculum, 50 µL of inoculum per leaf, using carborundum as an abrasive, five plants were inoculated with PBS only as the mock. PVY symptoms on the plants were evaluated at seven and 14 days post-inoculation and correlated to the absorbance values for each dilution. ELISA was performed on inoculated plants at every evaluation time to determine the plant infection status.

2.5 Effect of synthesized dsRNAs on suppressing PVY infection

To test the effect of synthesized dsRNA from each cistron in suppressing PVY infection in tobacco plants, 50 µg of dsRNAs were applied per leaf, and two leaves were treated per plant. Plants were mechanically inoculated with PVY either along with the dsRNA (0 days) or at five days after dsRNA treatment. The experiment included two controls, a positive control consisting of plants inoculated with PVY only (no dsRNA), and a negative control where plants were treated with dsRNA only (no PVY) – we did set up a pilot experiment using dsRNA from the *Fusarium oxysporum FpPPR1* gene to very if a non-PVY-target dsRNA would induce some level of PVY suppression, and since we detect no effect of applying such dsRNA on PVY suppression, we did not include this control in our experiment. Plants were maintained in an insect-free growth chamber at 25 ±3 ◦C and 16 h photoperiod. Two weeks after viral inoculation, PVY symptoms were

assessed, and ELISA was conducted to check the infection status of each plant (Figure 1). This experiment was repeated three times with five replicates per treatment.

2.6 DsRNA translocation in tobacco plants

To shed light on how exogenous application of dsRNA molecules behave inside the plant and their potential effects in suppressing PVY infection via RNAi, the translocation of the applied dsRNA molecules inside the plants was evaluated. The dsRNAs synthesized from CP, HC-PRO, and NIb cistrons were applied as previously mentioned (50 µg of dsRNA were applied per leaf, and two leaves were treated per plant); with the exception that no virus inoculation was performed (Fig 1A and 1B). The mock treatment used was only nuclease-free water without dsRNA and there were three plants per treatment. Since symplastic movement of mobile RNA in plants occurs from the photosynthetic source tissue (old leaves) to photosynthetic sink tissue (young leaves) (Melnyk et al. 2011), therefore dsRNA was applied to the two fully formed leaves of plants at the three- to five-leaf stage. Three mm discs were collected every 24 hours (Fig 1C) from the dsRNA-treated leaves (localized leaf) and from the subsequent young leaf to the application site (systemic leaf), this was performed on the same leaf for 14 days. Immediately after collection, the discs were washed in a 0.05% tween 20 solution, to remove potential dsRNAs present on the outside of the discs, and dried on a paper towel. Leaf discs were placed in 1.5 mL tubes, immediately frozen in liquid nitrogen, and stored at -80◦C for downstream analyses.

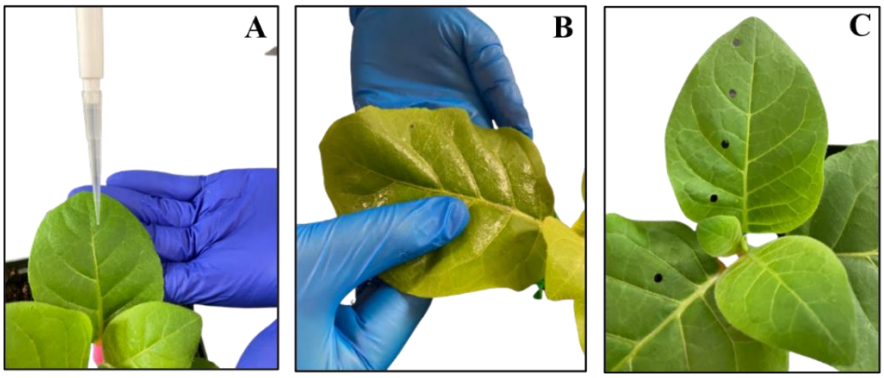

Figure 1. Experiment set up. Application of dsRNA (50 µg per leaf) (A) and mechanical application of dsRNA (B) on tobacco leaves. Collection of leaf discs (C) for total RNA extraction.

Aiming to quantify the translocation of applied dsRNA molecules from localized to the systemic leaves, RT-qPCR was performed on the total RNA extracted from the collected samples. Due to the small size of discs collected from leaves, the small-scale

RNA isolation protocol from PureLink® Plant RNA Reagent kit (Thermo Fisher Scientific, Waltham, MA) was used for total RNA extraction. Reverse transcription (RT) was carried out with 1 µg of the total RNA in a 20 µL reaction volume using the ProtoScript II First Strand cDNA synthesis kit (NEB, Ipswich - MA, USA). The RNA was denatured at 95°C for 5 min in the presence of random primer mix and nuclease-free H_2O, before adding the remaining RT reagents and conducting the RT steps. Then, 2 µL of cDNA was used for qPCR using the SsoAdvanced Universal SYBR Supermix (BIO-RAD, Hercules-California, USA) in a 10 µL reaction volume, following the manufacturer's specifications, in a C1000 touch CFX96 real-time PCR system (BIO-RAD, Hercules-California, USA). The qPCR primers (Table 1) were designed to target 250 bp of the central regions of each applied dsRNA (CP, HC-PRO, and Nib) because of the potential degradation of the applied dsRNA in their extremities by exoribonucleases (Arraiano et al. 2013), which could mask the assay if primers target the dsRNA extremities. Newly synthesized dsRNAs from each region were added as a template for the positive control in each corresponding reaction, and the mocks (samples from plants not treated with dsRNA) were used as the negative control. The cycle threshold (Ct) values were analyzed to correlate with the dsRNA load in each sample.

Table 12. Primers designed for the amplification of specific regions of the PVY genome.

Primer	Type	Sequence (5' - 3')	Target region	Amplicon size
CPT7for	F	TAATACGACTCACTATAGGGGCAACTCAATC ACAGTTTGATACGT	CP	600 bp
CPT7rev	R	TAATACGACTCACTATAGGGACATCCTCGGT GGTGTGCCTCTC		
MN21HCT7 F	F	TAATACGACTCACTATAGGGAGCCAAACGA GTCAACCAC	HC-Pro	600 bp
MN21HCT7 R	R	TAATACGACTCACTATAGGGAGCCAAACGA GTCAACCAC		
NIbT7for	F	TAATACGACTCACTATAGGGGCGATTGTATA AAGGCTTGC	NIb	600bp
NIbT7rev	R	TAATACGACTCACTATAGGGGCAATCAGCA AATCATCACC		
CPq_F	F	AGTCGAGTACCCGTTGAAACC	CP	250 bp
CPq_R	R	TGCGGCCTTCATTTGAATGTG		
HCq_F	F	TGCACTACACTTGATGATGGCT	HC-Pro	251 bp
HCq_R	R	CCAAGCTTTGGCACACACAT		
NIbq_F	F	TGCGGCGTTTACCTGAGAAT	Nib	250 bp
NIbq_R	R	TCCACAACAGTAGAAGGCTGAC		

2.7 Standard curves to assess the dsRNA concentration inside the plant

To estimate the concentration (ng/µL) of dsRNA in leaf tissue, we generated standard curves based on the Ct values obtained from RT-qPCRs run on serial dilutions of the synthesized dsRNA from each genomic region. Aliquots from serial dilutions of dsRNA concentrations (1:10, 1:100, 1:100, $1:10^4$, $1:10^5$, $1:10^6$, $1:10^7$, $1:10^8$, and $1:10^9$) were amplified by RT-qPCR as previously described. A standard curve for each dsRNA was prepared by plotting a linear regression curve with $\log_{10}$ of dsRNA dilutions on the X-axis and Ct values on the Y-axis (n=3 for each dilution point). Thus, by obtaining the Ct values from the dsRNA translocation experiment, we can enter the data to the Y-axis from the equation obtained from the linear regression estimated above and estimate the dsRNA concentration (X) in the sample collected (Fig S1). Statistical analyzes and graph plotting were performed in R (R Core 2022) using the RStudio interface (RStudio Team 2022).

2.8 Stem-loop RT-PCR to detect siRNAs

To investigate whether exogenously applied dsRNAs move in the form of dsRNA or siRNAs inside the plant, or how long those molecules remain in the plant, we used the stem-loop reverse transcription PCR (RT-PCR) methodology proposed by (Kaldis et al. 2018). This technique is used for detecting and amplifying siRNAs, where the stem-loop is designed in the shape of a hairpin loop and has a 3' complementary overhang to the siRNA. Based on the siRNA profiling analyses described above, a 21-nt siRNA was selected from the sense strand of each dsRNAs synthesized (Table 2), for the design of dsRNA-specific stem-loop reverse primers (Kaldis et al. 2018). The siRNAs were selected from hotspots, genomic regions with a high abundance of reads with different unique siRNA sequences (Shidore et al. 2020), detected on each of the genomic regions where the dsRNAs were designed. For this procedure, total RNA extraction was performed as described above. In the RT step, total RNA (1µg RNA in 20 µL of reaction) was denatured at 65 ˚C for 5 min in the presence of specific reverse primers for each siRNA (Table 3). Then, Protoscript II Reaction Mix and Enzyme Mix were added. To increase the RT efficiency, the pulsed RT-reaction was conducted as follows: 60 cycles of 30 ◦C for 30 s, 42 ◦C for 30 s, 50 ◦C for 1 s, and 85 ◦C for 5 min to inactivate the enzyme. The PCR conditions were 95 ◦C for 3 min followed by 35 cycles of 95 ◦C for 30 s and 61

◦C for 1 min. The resultant PCR amplicons were resolved in 2% agarose gel electrophoresis.

Table 2. Sequence of siRNAs hotspots originating from the sense strand of synthesized dsRNAs from PVY genome.

siRNA	Sequence (5'- 3')	Position*	Target region	Size
CP_si	CGACTTTTCGGGTTGGACGGT	9,165 to 9,185	CP	21 nt
HC-Pro_si	AAGAAGGTTCGTGACATGTGT	2,034 to 2,054	HC-Pro	21 nt
NIb_si	CGTGTTCTTTGTTAATGGTGA	7,952 to 7,972	Nib	21 nt

* This position refers to the sequence of the specific gene using the PVY sequence GenBank KY847982 as reference.

Table 3. Primers designed in this study for the detection of siRNAs by stem loop RT-PCR.

Primer	Step	Sequence (5'- 3')	Target region	Amplicon size
CP_SL	RT	GTCGTATCCAGTGCAGGGTCCGAGGTA TTCGCACTGGATACGACACCGTC	CP	-
HC_SL	RT	GTCGTATCCAGTGCAGGGTCCGAGGTA TTCGCACTGGATACGACACACAT	HC-Pro	-
NIB_SL	RT	GTCGTATCCAGTGCAGGGTCCGAGGTA TTCGCACTGGATACGACTCACCA	NIb	-
CP_SL_F	PCR	CGACCGACTTTTCGGGTTG	CP	50 bp
HC_SL_F	PCR	AAGAAAGAAGGTTCGTGAC	HC-Pro	50 bp
NIB_SL_F	PCR	CGT GCG TGT TCT TTG TTA A	NIb	50 bp
Universal reverse primer	PCR	GTGCAGGGTCCGAGGT	-	-

2.9 Tracking the dsRNAs with cy3 fluorophore

To track the movement of dsRNA molecules inside the plant, dsRNA molecules were labeled with cyanine 3-UTP fluorescent dye (Cy3-UTP) by adding 1 µL of Cy3-UTP to the T7 Ribomax reaction. Then, the dsRNA-cy3 was purified using a ChargeSwitch PCR Clean-Up Kit (Thermo Fisher Scientific, Waltham, MA). The dsRNA-cy3 molecules were applied by the same method used in the dsRNA experiment described above. After 24 hours, localized (treated) and systemic (untreated) leaves were collected to verify the dsRNAs distribution. Tissue preparation was performed using a

solution of 4% formaldehyde for 30 minutes to fix and preserve the tissues, followed by tissue visualizing and imaging under a confocal laser scanning microscopy (LSM 880, Zeiss).

3 RESULTS

The small RNA profiling analyses performed revealed regions of high "hotspots" and low "coldspots" abundance of siRNAs on the PVY genome. The hotspots could be indicative of regions of the virus genome that are highly targeted during RNAi (Shidore et al. 2020). Therefore, we selected those regions in each of the cistrons chosen (HC-PRO, Nib, and CP) to be used as templates for the synthesis of dsRNAs for further experiments. Among the chosen cistrons, HC-PRO and NIb had a higher incidence of hotspots when compared with the CP cistron. HC-PRO had the second-highest number of hotspots among all cistrons on the PVY genome, behind only the CI cistron.

The sequential inoculum dilution experiment revealed that the dilution 1:400, with an absorbance value of 0.1 determined by DAS-ELISA, was the highest dilution that would still get all plants infected by PVY (Table 4). In lower dilution points, inoculated plants showed strong PVY symptoms within seven days, suggesting that there was an excess of inoculum, which could mask the ability of RNAi to suppress the virus infection. Therefore, the absorbance of 0.1 was chosen as the standard for downstream experiments in this research.

Table 4. Plant infection status after inoculation with different dilutions of PVY inoculum.

Dilutions	Absorbance	Number of infected plants	
		7 DPI	14 DPI
1:1	1.5	5**	5***
1:100	0.4	NA	NA
1:200	0.2	5**	5***
1:400	0.1	5*	5**
1:800	0.07	NA	NA
1:1200	0.05	0	3
1:1600	0.03	NA	NA
1:2000	0.02	NA	NA
Mock	0	0	0

3.1 HC-PRO-dsRNA induced greater protection against PVY than dsRNAs from CP or NiB cistrons

In the three experiments carried out, the dsRNAs synthesized and exogenously applied in tobacco leaves protected treated plants against PVY infection. However, the level of protection depended on the dsRNA applied. HC-PRO-dsRNA induced significantly greater protection against PVY in tobacco plants compared to CP-dsRNA and NIb-dsRNA, 100% of the treated plants were protected against PVY when the dsRNA was applied with the virus inoculum (0 days), and over 86% of the plants were protected when plants were challenged five days after dsRNA treatment (Fig 2). NIb-dsRNA induced 100% protection when applied alongside the virus (0 days); however, 40% of the plants inoculated with PVY five days after NIb-dsRNA treatment developed PVY symptoms. On the other hand, CP-dsRNA induced the lowest level of protection against PVY compared to the other dsRNAs tested. Over 20% of the plants treated on the same day of virus inoculation (0 days) developed PVY symptoms, and over 73% of the plants inoculated with the virus five days after CP-dsRNA treatment developed the disease. Furthermore, the negative controls (plants treated with each dsRNA alone and not challenged with PVY and plants not treated with dsRNA nor inoculated with PVY) did not develop and disease. All positive controls (plants not treated with dsRNA but

challenged with PVY) showed clear symptoms of the viral disease, which include leaf mosaic and chlorosis, with 100% of infected plants, in both treatment times, 0 and 5 days (Fig 3).

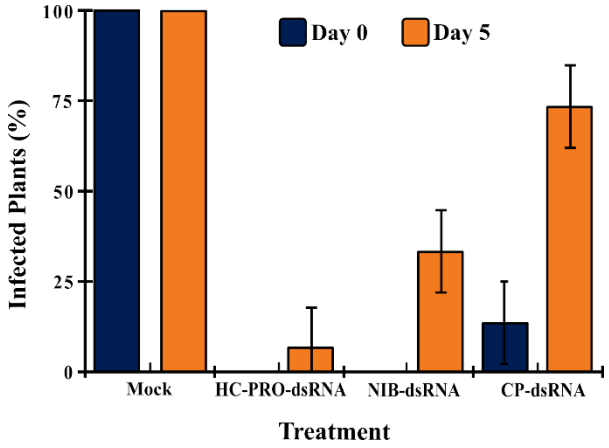

Figure 2. Percentage of tobacco plants displaying typical PVY symptoms. Plants were inoculated with PVY at 0 or five days after dsRNA treatment (n=15).

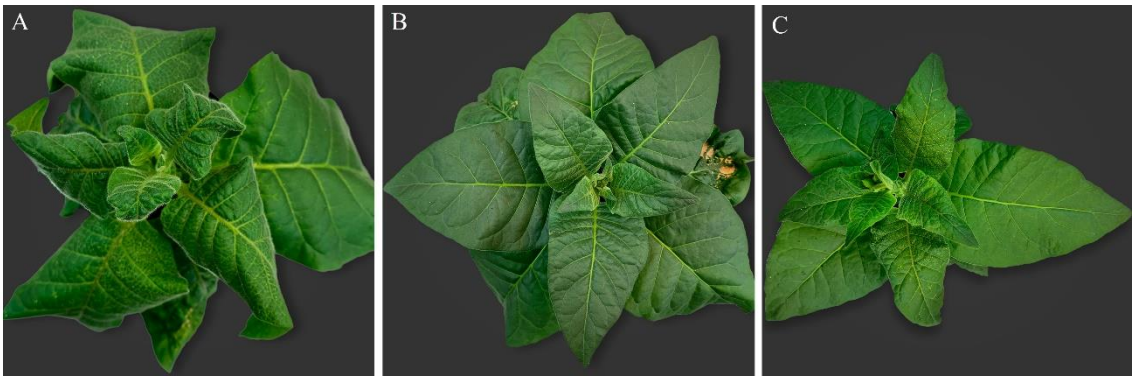

Figure 3. The effectiveness of protection trigged by CP-dsRNA (A), HC-Pro-dsRNA (B), NIb-dsRNA(C).

3.2 HC-PRO-dsRNA entered and moved faster inside tobacco plants than Nib-dsRNA and CP-dsRNA

We investigated the systemic movement of all three different dsRNAs, exogenously applied to tobacco leaves, inside the plants over time by RT-qPCR. This is an indirect indication of the concentration of dsRNA in the samples analyzed - the lower the Ct value, the greater the dsRNA in that sample. Noteworthy, the Ct values on day six for HC-PRO-dsRNA and day 10 for CP-dsRNA are not reported due to sample loss. DsRNAs were detected in systemic leaves (untreated) after 24 hours of dsRNA application, demonstrating that the dsRNAs applied translocated systemically inside the plant, and remained for at least 14 days (Fig 4). These results were confirmed by the RT-

PCR products run on a 1.5% agarose electrophoresis gel for all the dsRNAs applied (Fig 5). Utilizing data obtained from the dsRNA standard curve (Table S1) to predict the dsRNA concentration in the samples, the calculated concentrations of CP-dsRNA, HC-PRO-dsRNA, and NIb-dsRNA were 1.04 x 10^{-8} ng/µL, 7.87 x 10^{-9} ng/µL, and 1.4 x 10^{-7} ng/µL, respectively - after 24 hours of treatment in systemic leaves.

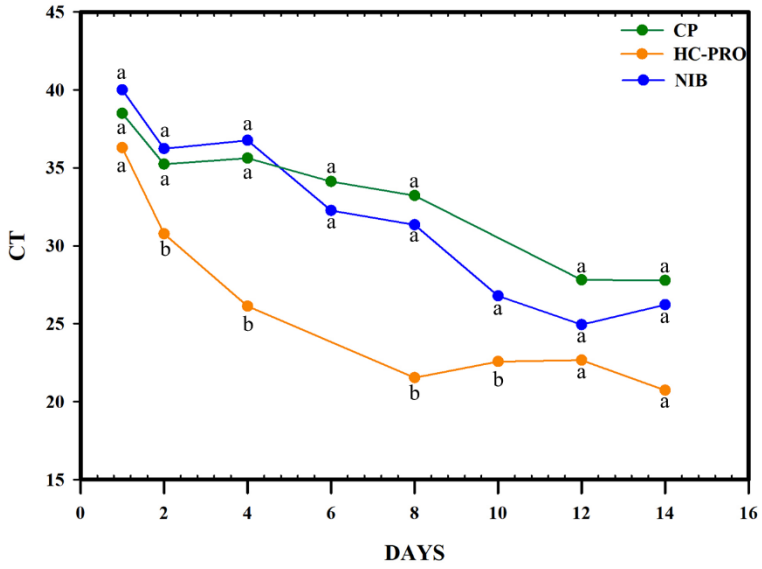

Figure 4. Tracking dsRNAs in systemic leaves (n=5) via RT-qPCR. Averages followed by the same letters on the same day did not differ statically from each other by Tukey's test (P<0.05).

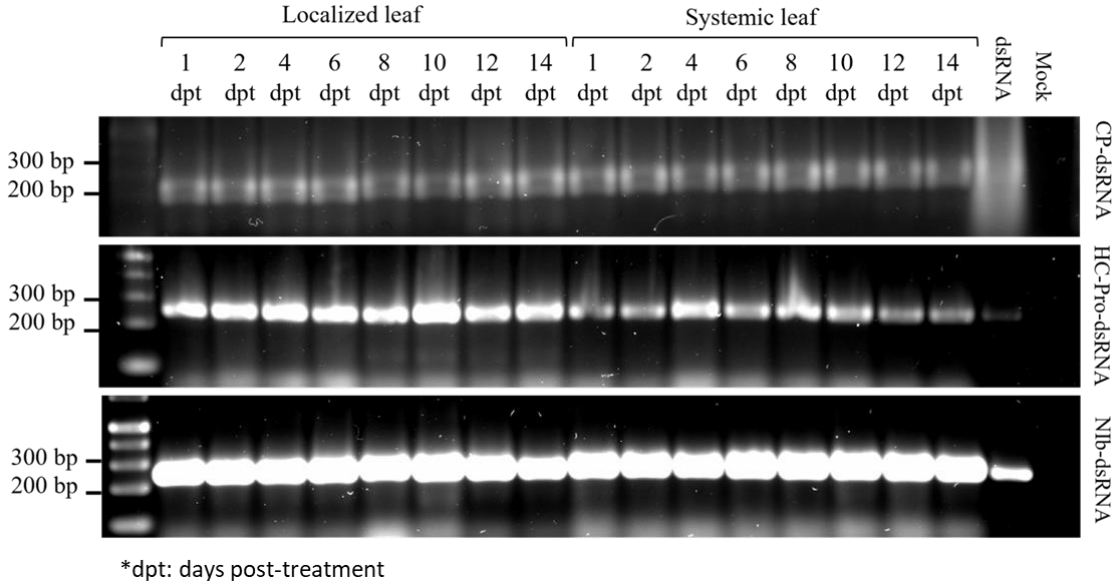

Figure 5. RT-PCR product of CP-dsRNA (250 bp), HC-Pro-dsRNA (251 bp) and NIb-dsRNA (250 bp) target sequences derived from Potato Virus Y over 14 days after treatment on localized (treated) and systemic (untreated) leaves, revealed by 1.5% agarose gel electrophoresis.

Although all dsRNAs were detected, at low concentrations, in systemic leaves after 24 hours, on the 4th day of evaluation we observed significant differences between the Ct values from samples of plants treated with HC-PRO-dsRNA compared to samples treated with the other dsRNAs (Fig 4 and 5). On that point, HC-PRO-dsRNA had an estimated concentration of 1.8 x 10^{-4} ng/µL, a much higher value than the concentration of NIb-dsRNA (2.1 x 10^{-5} ng/µL) and CP-dsRNA (2.89 x 10^{-7} ng/µL) (Table S1). This significant difference remained up to eight days after the treatments and began to equalize after 10 days. Hence, our data highly suggested that HC-PRO-dsRNA moved fast inside the plants and gave the highest disease suppression among the other dsRNA tested (Fig 4 and 5).

3.3 SiRNAs can be detected in localized and systemic leaves

We performed stem-loop RT-PCR on RNA samples from each plant every 24 hours from day 1 until day 14 after dsRNA treatment. The products of this amplification (50 bp) were confirmed by 2.0% agarose electrophoresis gel (Fig 6). Data showed that there was a systemic transport not only of dsRNAs inside the plant but also of siRNAs. The siRNA selected from each cistron was detected in localized (treated) and systemic (untreated) leaves at all points tested (Fig 6).

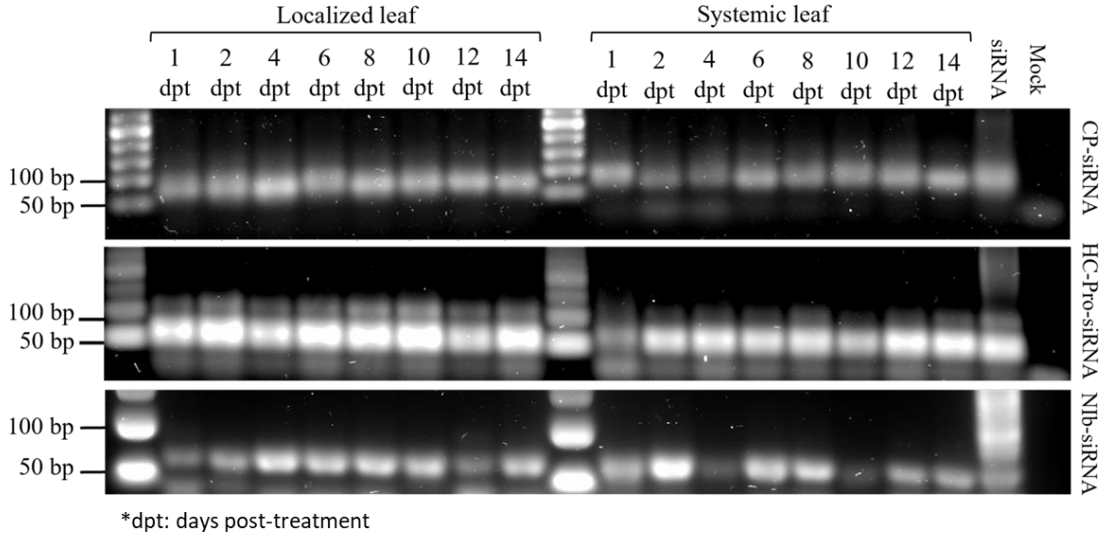

Figure 6. Stem loop RT-PCR products of CP, HC-Pro, and NIb-siRNAs (50 bp) target sequences derived from Potato Virus Y over 14 days after treatment on localized (treated) and systemic (untreated) leaves, revealed by 2.0% agarose gel electrophoresis.

3.4 Fluorescence microscopy confirmed the movement of dsRNAs inside plants

The treated and systemic leaves, stem, and root was imaged by confocal laser scanning microscope 24h after treatment. Dsrna-cy3 was strongly detected in treated leaves for up to 24 hours, after which the fluorescence signal was quenched and couldn't be detected after two days of treatment. On the other hand, in systemic leaves and other parts of treated plants a weak fluorescence signal could be observed and as for localized leaves, the signal quenched over time and couldn't be detected after two days of treatment. (The pictures are being finalized to be published).

4 DISCUSSION

Plant diseases caused by viruses are difficult to control, and their management strategies are mainly based on the use of resistant and tolerant crop varieties and chemical control of insect vectors. These strategies are not always efficient, economically profitable, or environmentally sound (Rubio et al. 2020). In the quest for environmentally safe and efficient disease control alternatives, RNAi-based approaches have been studied as important and promising tools in managing plant diseases (Jin et al. 2021; Worrall et al. 2019; Shidore et al. 2021). However, a deep understanding about the effects of the exogenous application of dsRNA molecules on plants and how they move systemically within the plant is needed. Herein, we unveiled significant knowledge of the application of dsRNA from different target viral genomic regions on priming tobacco plants to suppress PVY infection.

We selected three different cistrons to target with RNAi, HC-PRO, Nib, and CP. And then we performed siRNA profiling analyses to pinpoint the regions within the cistrons to design the dsRNAs, based on the siRNA hotspot concentration - we targeted regions that had the highest siRNA hotspot concentration within each cistron. The rationale to choose the cistrons was based on the major function of the proteins translated by each cistron. HC-PRO acts suppressing RNAi, Nib is the RNA-dependent RNA polymerase, and CP is the coat protein; therefore, disrupting the translation of these proteins would likely suppress the virus infection. However, potyvirus's genome codes for a polyprotein that when matured is cleaved into functional proteins (Urcuqui-Inchima et al. 2001). In this case, the disruption of any genomic region would theoretically yield the same outcome as it would block the translation of the entire polyprotein and not individual proteins as we first rationalized. However, it was very surprising to find out

that dsRNAs from cistrons yielded different levels of viral suppression. A possible explanation for such results may be that the cleavage of specific PVY genome locations by RNAi may result in truncated protein translation. That is, cleaving the HC-PRO cistron would not disrupt the translation initiation from the viral RNA VPg cap 5'end entirely – the ribosome complex would still be able to start the translation and would stop when it gets to the site cleaved via RNAi. In that case, disrupting the HC-PRO cistron would yield a truncated polyprotein containing the P1 protein that could still be functional, but it is not known to interfere with the RNAi machinery. On the other hand, blocking Nib or CP cistrons would not stop the translation of a truncated polyprotein that contains the HC-PRO, which blocks the RNAi machinery and wane the virus suppression over time. In other words, using RNAi to target the HC-PRO cistron or regions before that cistron, closer to the viral RNA 5' end, would inhibit the translation of HC-PRO and the RNAi pathway would not be suppressed. Whereas, blocking regions after the HC-PRO cistron, closer to the 3' end, would not be able to stop HC-PRO translation and as a result, RNAi would be inhibited, and the virus would be able to produce the necessary proteins for replication and cause infection. This hypothesis is quite plausible and needs to be tested in future research to shed light on this intricate system.

Our working hypothesis for why HC-PRO-dsRNA was more effective in suppressing PVY infection than Nib- or CP-dsRNA is that HC-PRO-dsRNA circulates rapidly inside the plant, triggering RNAi systemically and quickly priming the plant to fight the viral infection. To test this hypothesis, we investigated the systemic movement of all three different dsRNAs, exogenously applied to tobacco leaves, using RT-qPCR and fluorescence microscopy. We indeed confirmed that HC-PRO-dsRNA spread and accumulated systemically faster than the other dsRNAs tested. And to corroborate this hypothesis further, when we applied the dsRNA and challenged with the viruses at the same time, we got nearly 100% protection with all three dsRNAs. We believe that is because the virus was exposed to a high concentration of dsRNA and the RNAi machinery in the plant produced a response strong enough to suppress the virus RNAs present. Whereas, challenging the plant after five days from dsRNA application, the dsRNA hasn't moved and accumulated enough in the systemic leaves inoculated with the virus to produce a strong antiviral response at that site. In fact, when analyzing the RT-qPCR results on the dsRNAs, we noticed that the concentration of HC-PRO-dsRNA in just two days is much higher than the other dsRNAs. And that trend holds until 12 days after dsRNA treatment when the concentration of the other dsRNAs doesn't differ statistically

from HC-PRO-dsRNA. Therefore, HC-PRO-dsRNA moved systemically and accumulated faster in our treated plants than Nib-dsRNA and CP-dsRNA, priming the plants to efficiently fight PVY infection.

DsRNAs produced in vitro derived from CP, HC-PRO, and NIb cistrons have shown positive results in the control of other plant viruses (Konakalla et al. 2016; Petrov et al. 2022; Kaldis et al. 2018). A good example is the study carried out by Vadlamudi et al. (2020), in which the application of dsRNA derived from CP and HC-PRO cistrons conferred 100% resistance in papaya (*Carica papaya* L) cv. Nanha against papaya ring spot virus (PRSV) caused by the PRSV-Tirupati isolate. When papaya plants were infected with the PRSV-Delhi isolate and treated with CP-dsRNA and HC-PRO-dsRNA, 94% and 81% of the plants were resistant, respectively. These results suggest that dsRNAs derived from these regions can act as vaccines and confer protection against several viral diseases in cultivated plants, but their effectiveness may vary according to the isolate and also to the host. Therefore, basic research, *e.g.,* dsRNA movement and dsRNA efficacy in controlling the target virus, on the pathosystem (virus and host) needs to be conducted prior to adopting the application of dsRNA as a management tool to fight target viral infections in agricultural systems.

In this study, we attempted to use the stem-loop RT-PCR technique to investigate the movement of siRNA derived from applied dsRNA inside the plants. We were interested in finding out if the dsRNA would indeed be processed into siRNA by the plant Dicers and if the siRNA tracked would be found systemically in the treated plants. We use our siRNA profiling analysis data to select the siRNA, one *per* dsRNA used, to track. *A priori* the technique worked, we are able to track the target siRNAs in localized and systemic leaves. However, after some reflection, we decided to test the technique to see if it would also work on pure dsRNA and viral RNA or if it was specific to the target siRNA. The stem-loop RT-PCR also amplified bands from dsRNA and viral RNA templates, demonstrating that this technique is not specific to siRNA – we replicated the assay several times with different samples targeting different siRNAs and got the same results. It works for the detection of siRNAs, as long as only this molecule is applied to the plants, if dsRNA or viral RNA containing the siRNA sequence is present, false positives will arise. In other words, in the presence of the target virus or the dsRNA, it is not possible to determine whether the siRNA was generated from the Dicer processing the dsRNA in the RNAi pathway or from the template dsRNA or the viral genome. It is important to disseminate this piece of knowledge because there are many studies in the

literature using this technique to amplify target siRNAs. Several of these studies applied this technique in viral-infected samples and in samples containing the applied dsRNA from where the target siRNA is derived, it is likely that some of the results from these studies may be false positives.

The synthesis and application of exogenous dsRNAs targeting the HC-PRO genomic region of PVY proved to be a promising technique for controlling this disease. More studies are needed to determine the dynamics of these molecules in different hosts and environments. There is also an urgent need to develop a delivery system for the controlled delivery of RNAi inducers in plant cells to prolong the protection window against target plant viruses for field applications.

5 SUPPLEMENTARY FILES

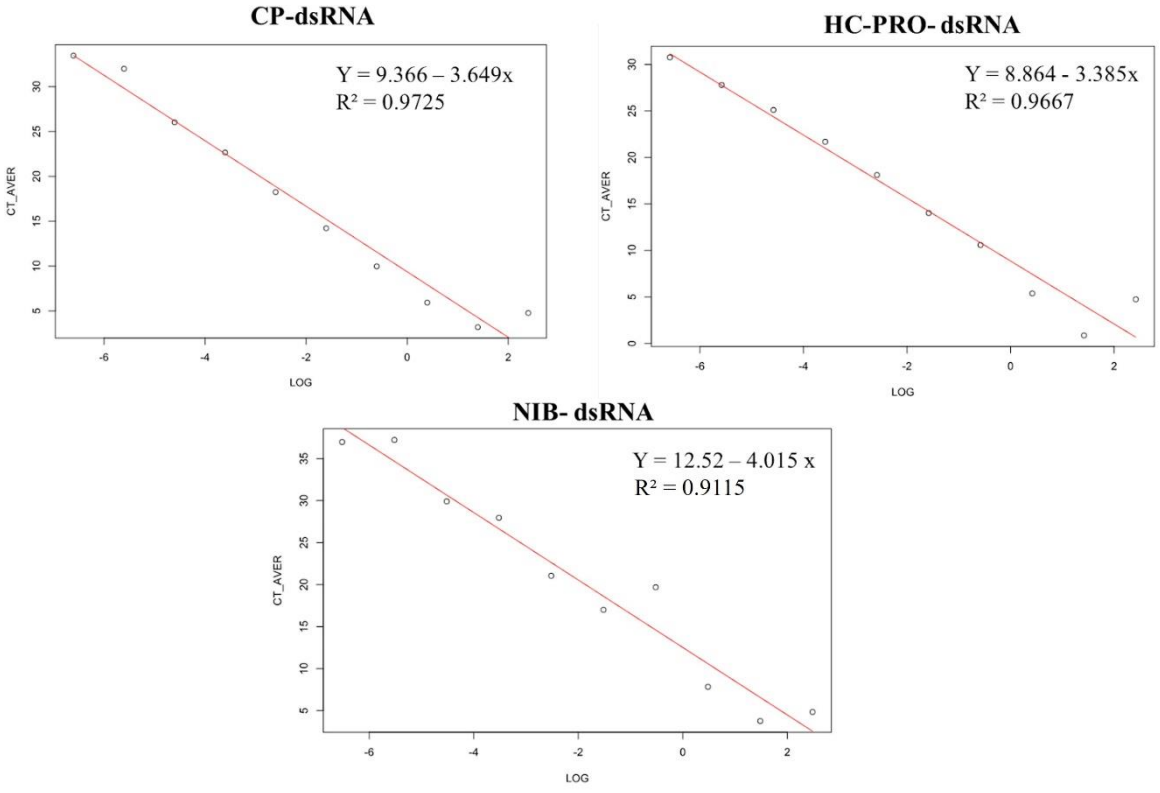

Figure S1. Standards curves for each dsRNA synthesized from the RT-qPCR data.

Table S1. Estimation of dsRNA concentration inside the plant from standard curves.

Day	CP		HC-PRO		NIB	
	Ct	**Concentration (ng/µL)**	**Ct**	**Concentration (ng/µL)**	**Ct**	**Concentration (ng/µL)**
1	38.49 a[1]	1.04×10^{-8}	36.29 a	7.87×10^{-9}	40.00 a	1.4×10^{-7}
2	35.23 ab	8.15×10^{-8}	30.77 ab	3.36×10^{-7}	36.23 ab	1.2×10^{-6}
4	35.62 ab	6.38×10^{-8}	26.12 bc	7.97×10^{-6}	36.76 ab	9.2×10^{-7}
6	32.14 bc	5.73×10^{-7}	-	-	32.26 ab	1.2×10^{-5}
8	33.2 abc	2.89×10^{-7}	21.54 bc	1.8×10^{-4}	31.34 ab	2.1×10^{-5}
10	-	-	22.57 bc	8.89×10^{-5}	26.78 b	2.8×10^{-4}
12	27.81 c	8.80×10^{-6}	22.66 bc	8.37×10^{-5}	24.94 b	8.0×10^{-4}
14	27.78 c	8.99×10^{-6}	20.73 c	3.13×10^{-4}	26.22 b	3.9×10^{-4}

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