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DARLAN FERREIRA BORGES

ALTERNATIVE MANAGEMENT OF SOILBORNE PATHOGENS OF MELON CROP AND DETECTION OF MULTIPLE VIRUSES FROM FRUIT CROPS

MOSSORÓ

2020

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

Linha de Pesquisa: Proteção de Plantas

Orientadora: Profª. D. Sc. Márcia Michelle de Queiroz Ambrósio

Coorientador: Prof. Ph. D. Washington Luís da Silva

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Martin Luther King

ABSTRACT

BORGES, Darlan Ferreira. **Alternative management of soilborne pathogens of melon crop and detection of multiple viruses from fruit crops**. 2020. 115f. Thesis (Doctorate in Plant Science) - Universidade Federal Rural do Semi-Árido (UFERSA), Mossoró-RN, 2020.

The cultivation of melon (*Cucumis melo* L.) is extremely important for the Brazilian economy. The country is the thirteenth largest melon producer in the world and the third in Latin America. However, even with high melon production, the damages triggered by soilborne pathogens cause losses in production and yield and risk the abandonment of melon cultivated areas. Thus, in order to improve quality and productivity, it is necessary to develop new techniques that can help in the management of soilborne pathogens, which are the main drive of losses in melon cultivation. Many control techniques are already being used to manage such pathogens, such as chemical, biological control, and the use of natural products; however, little is known about the use of green manure associated with polyethylene mulch to that end. Therefore, the objective of this research was to evaluate the effect of incorporating plant materials (*Crotalaria juncea* L. and *Pennisetum glaucum* L.) and commercial products (Compost-Aid® + Soil-Set®) to efficiently control pathogens transmitted via soil in melon production. In addition, we evaluated the responses of the soil microbiota (total fungi, total bacteria, sporulating bacteria, and total actinomycetes) to the above treatments. Two identical greenhouse experiments were conducted in a completely randomized design with seven treatments and seven replications. In conclusion, with part of this work, we were able to generate practical and theoretical information for producers to facilitate the management of the main soilborne pathogens that cause damage to the melon root system, optimizing techniques (mulch and green manure) that are already being used by the melon producers. During the second part my research, which was carried out in the United States of America (USA), different viruses in fruit trees were studied. In this study, our main goal was to determine the incidence and prevalence of grapevine viruses in the New England region vineyards. These viruses can cause losses of $ 25,000 to $ 40,000 per hectare and even replacement of entire vineyard in cases of high incidence of the viruses. Many of the grapevine viruses investigated in our study had previously been found in the state of New York (NY). Based on the proximity of NY to the New England region and the fact that grape growers from this region buy much of their plant material from NY nurseries, it was expected that the viruses already

described in NY would be detected in New England. To address this hypothesis, we partially sequenced specific genes from each virus species identified in our study and phylogenetically compared those sequences with virus sequences from different origins in the world and in the U.S. The second part of the research carried out in the USA was the writing of a review on fig mosaic disease. The USA is the eighth largest fig producer in the world, around 28,300 tons is produced each year, with the state of California being the largest producer. Typical fig mosaic disease symptoms such as chlorotic and yellowish spots, discoloration, deformation, and mosaic patterns on leaves and fruits have been observed in fig trees for almost a century, but the etiological agents associated with fig mosaic disease have only been investigated in the last decade. Twelve viruses - fig leaf mottle-associated virus 1 (FLMaV-1), fig leaf mottle-associated virus 2 (FLMaV-2), fig leaf mottle-associated virus 3 (FLMaV-3), Arkansas fig closterovirus-1 (AFCV-1), Arkansas fig closterovirus-2 (AFCV-2), fig mosaic emaravirus virus (FMV), fig latent virus 1 (FLV-1), fig mild mottle-associated virus (FMMaV), fig cryptic virus (FCV), fig fleck-associated virus (FFKaV), and fig badnavirus 1 (FBV-1) - and three viroids - apple dimple fruit viroid (ADFVd), citrus exocortis viroid (CEVd), ands hop stunt viroid (HSVd) - are associated with the disease. In this review, we proposed the standardization of the name of each virus, the conduction of geographic studies of the disease, and the development of isolation protocols to study these viruses and viroids *in vivo*. Furthermore, we suggested the conduction proper disease resistance tests, development of diagnoses assays and phylogenetic studies.

Keywords: *Cucumis melo* L. Disease management. Microbiota. Grapevine viruses. Fig mosaic disease.

RESUMO

BORGES, Darlan Ferreira. **Manejo alternativo de patógenos do solo da cultura do melão e detecção de vários vírus em fruteiras**. 2020. 115f. Tese (Doutorado em Fitotecnia) - Universidade Federal Rural do Semi-Árido (UFERSA), Mossoró-RN, 2020.

O cultivo de melão (*Cucumis melo* L.) é de extrema importância para a economia brasileira, especialmente para a região Nordeste. O país é o décimo terceiro maior produtor de melão do mundo e o terceiro da América Latina. Porém, mesmo com elevada produção, os danos provocados por patógenos habitantes do solo causam perdas no rendimento da cultura e riscos de abandono de áreas de produção. Desta forma, para que haja melhorias de qualidade e produtividade, é necessário o desenvolvimento de novas técnicas que possam ajudar no manejo de fitopatógenos habitantes do solo, principais responsáveis por perdas no cultivo do meloeiro. Muitas técnicas de controle já são utilizadas visando ao manejo dos patógenos de solo, como o controle químico, o biológico e o uso de produtos naturais, mas pouco se sabe do uso da adubação verde associada ao *mulch* de polietileno. Sendo assim, o objetivo deste projeto foi avaliar o efeito da incorporação de materiais vegetais (*Crotalaria juncea* L. e *Pennisetum glaucum* L.) e os produtos comerciais (Compost-Aid® + Soil-Set®) em controlar com eficiência os patógenos transmitidos pelo solo na produção de melão, além de avaliar a resposta da microbiota do solo (fungos totais, bactérias totais e esporulantes e actinomicetos). Dois experimentos idênticos foram conduzidos em casa de vegetação, em delineamento inteiramente casualizado, com sete tratamentos e sete repetições. Com parte deste trabalho, foi possível gerar informações práticas e teóricas para os produtores, visando a facilitar o manejo de patógenos habitantes do solo que causam danos no sistema radicular do meloeiro, otimizando técnicas (*mulch* e a adubação verde) já utilizadas pelos principais produtores de melão. Durante a segunda parte do meu estudo, realizada nos Estados Unidos da América (EUA), foram estudadas diferentes viroses em frutíferas. Neste estudo, nosso principal objetivo foi determinar a incidência e prevalência de vírus da videira nos vinhedos da região da New England. Estas viroses podem causar perdas de $25.000 a $40.000/ha, além da necessidade do replantio de toda a videira, em casos de alta incidência de viroses. Muitos vírus investigados em nosso estudo já haviam sido identificados anteriormente no estado de Nova York (NY). Com base na proximidade de NY com a região de New England e o fato de os produtores de uvas dessa região comprarem grande parte de seu material vegetal de viveiros de NY,

esperava-se que os vírus já descritos em NY fossem identificados na região de New England. Para analisarmos essa hipótese, sequenciamos parcialmente genes específicos de cada espécie de vírus identificada em nosso estudo e comparamos filogeneticamente essas sequências com sequências de vírus de diferentes origens do mundo e dos EUA. Na segunda parte do estudo, também realizada nos EUA, foi feita uma revisão sobre o mosaico da figueira. Os EUA são o oitavo maior produtor de figo do mundo, produzindo cerca de 28,3 mil toneladas por ano, sendo o estado da Califórnia o maior produtor. Sintomas típicos da doença, como manchas cloróticas e amareladas, descoloração, deformação e padrões de mosaico nas folhas e frutos foram observados em figueiras por quase um século, porém os agentes etiológicos associados à doença do mosaico da figueira foram investigados apenas na última década. Doze viroses - fig leaf mottle-associated virus 1 (FLMaV-1), fig leaf mottle-associated virus 2 (FLMaV-2), fig leaf mottle-associated virus 3 (FLMaV-3), Arkansas fig closterovirus-1 (AFCV-1), Arkansas fig closterovirus-2 (AFCV-2), fig mosaic emaravirus virus (FMV), fig latent virus 1 (FLV-1), fig mild mottle-associated virus (FMMaV), fig cryptic virus (FCV), fig fleck-associated virus (FFKaV), fig badnavirus 1 (FBV-1) - e três viroides - apple dimple fruit viroid (ADFVd), citrus exocortis viroid (CEVd), and hop stunt viroid (HSVd) – são associados à doença. Com base na revisão feita, nós propomos a padronização dos nomes de cada vírus, a realização de estudos geográficos da doença, o desenvolvimento de protocolos de isolamento para estudar esses vírus e viroides *in vivo*. Além disso, sugerimos a realização de testes adequados de resistência a doenças, desenvolvimento de ensaios diagnósticos e estudos filogenéticos.

Palavras-chave: *Cucumis melo* L. Manejo de doenças. Microbiota. Viroses da videira. Mosaico da figueira.

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SUMMARY

GENERAL INTRODUCTION

Melon crop (*Cucumis melo* L.) is extremely important for the economy of the region of Northeastern of Brazil, where several intensive cultural practices linked to monoculture of melon were adopted (SALES JUNIOR et al., 2017). Due to the steadily growing of the area planted in recent years, melon cultivation generates many jobs in the region (ABRAFRUTAS, 2018). It is the country's second most exported fresh fruit (DA FRUTICULTURA, 2017) and Brazil is the thirteenth largest producer of melon in the world and the third in Latin America (FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS - FAO, 2019).

According to Bedendo (2011), several arable areas in Brazil are contaminated by soilborne pathogens. This is due in part by the presence of resistance structures produced by those pathogens, which facilitate their survival for years, and also because those pathogens have a wide host range. The increase in these pathogens' populations and, consequently, the loss in the production of fruits of a certain crop can be propped by the use of monoculture practices (HUANG et al., 2013). The main disease-causing agents in the root system of melon crop are: *Fusarium solani* (Mart.), *Didymella bryoniae* (Auersw.) Rehm, *Lasiodiplodia theobromae* (Pat.) Grif. and Maubl, *Macrophomina phaseolina* Tassi (Goid.), *Myrothecium roridum* Tode, *Monosporascus cannonballus* Pollack and Uecker, and *Rhizoctonia solani* Kühn (PORTO et al., 2016).

Many techniques are used to control soilborne pathogens, such as chemical and biological control, the use of natural products, among others. According to Sales Junior (2017), all these measures are superficial and that the development of new techniques to carry out this management is extremely important, even if all of them have to be used concurrently. Furthermore, melon importing countries, mostly Europeans, have strict regulations in place that limit the use of chemicals, which adds another layer of complexity on soilborne pathogen control in melon fields.

One of the techniques used is green manure, which, from a previously studied species, has its plant material deposited or incorporated on the soil, aiming at the management of phytopathogens (BARRADAS, 2010). The incorporation of some green manure into the soil has already been proved to reduce the inoculum of soilborne pathogens in the melon crop (DANTAS et al., 2013; CRUZ et al., 2016; PORTO et al., 2016). The materials behave differently, some of them stand out in the management of

diseases of the root system; however, when applied alone, the aimed result is slow and not satisfactory.

Soil solarization is a very promising technique for the control of pathogens that affect the root system; however, it is more efficient when associated with the incorporation of plant materials. This technique, besides controlling phytopathogens, benefits beneficial soil microorganisms, and it consists of covering the soil previously moistened with transparent plastic (ROCHA; CARNEIRO, 2016).

Mulching is a technique widely used in the production of strawberries and pineapples and, more recently, it has also been widely used in the production of melons and watermelons, in order to increase the quality of the fruit and also reduce the need for irrigation, the incidence of weeds, and leaf diseases (LIMA JUNIOR; LOPES, 2009). Polyethylene plastics are widely used for mulching and they can be of different colors: black-white, black-silver, and black-black, the color choice is determined by the variation of the planting time and the place of cultivation (LAMBERT et al., 2017). In the Northeast of Brazil, black-white polyethylene plastic has been commonly used in the cultivation of melon.

Based on the need to develop new control alternatives, we expect this research to provide a body of evidence showing the efficacy of using polyethylene mulch concomitantly with the incorporation of plant materials (*Crotalaria juncea* L. and *Pennisetum glaucum* L.) and commercial products (Compost-Aid® + Soil-Set®) in the management of melon soilborne pathogens and benefiting the soil microbiota. To that end, the use of polyethylene mulch starts from a principle similar to that of solarization, which is already used in the cultivation of melons but for other purpose. Polyethylene mulch can be associated with previous incorporation of plant materials to better control of soilborne pathogens without eliminating the population of beneficial microorganisms and providing better plant development and, consequently, greater melon production.

During the second part of our research, which was carried out in the United States of America (USA), studies were developed involving viruses in fruit trees in the USA. Based on various surveys, there are over 60 virus species known to infect grapevines and several of those can cause severe yield and vigor reduction, alter the grape juice chemistry, and decrease the vineyards lifespan - resulting in critical economic losses to growers (MARTELLI et al., 2017). In USA, the grape industry is blooming, mainly due to the high value price aggregated to grape and the agritourism opportunities provided by the attractive wineries (WILCOX et al., 2015).

The main concern is grapevine leafroll disease that can cause losses of $25,000 to $40,000 per hectare and its economically recommended to replace the entire vineyard if the disease prevalence is greater than 25% (ATALLAH et al., 2012). Surveys have indicated that grapevine leafroll-associated virus (GLRaVs), grapevine fanleaf virus (GFLV), and tomato ringspot virus (ToRSV) are the major virus threat to USA wineries (WILCOX et al., 2015). Those viruses have all been found in the state of New York (NY). We know that the grape growers from New England region buy much of their plant material from NY nurseries, then viruses expected to be first detected in our surveys in this region. Sequences of each virus found in our study were compared with virus sequences from different sources in the world and in the USA.

Fig mosaic disease (FMD) is a major disease complex affecting fig trees (*Ficus carica* L.) throughout the world, and it was first described in the early 1930s (CONDIT, 1933). Interestingly, from all 12 virus and three viroids associated with FMD, only fig latent virus-1 (FLV) and fig cryptic virus (FCV) are seed transmissible (CASTELLANO et al., 2009; FAUQUET et al., 2005). The majority of the viruses and viroids associated with FMD are transmitted via vegetative propagation of infected plant material and vectors such as mite, *Aceria ficus* (FLOCK, 1955).

The U.S. ranks as the eighth largest fig producer in the world, approximately 28,300 tons; the majority of U.S. production occurs in California (FOOD AND AGRICULTURE ORGANIZATION - FAO 2019). Although symptoms, such as chlorotic and yellowish spots, discoloration, deformation, and mosaic patterns on the leaves and fruit have been observed in fig trees for almost a century, the etiological agents associated with FMD have been investigated only within the past decade (ELBEAINO et al., 2006, 2007b, 2009a, 2010). Twelve viruses - fig leaf mottle-associated virus 1 (FLMaV-1), fig leaf mottle-associated virus 2 (FLMaV-2), fig leaf mottle-associated virus 3 (FLMaV-3), Arkansas fig closterovirus-1 (AFCV-1), Arkansas fig closterovirus-2 (AFCV-2), fig mosaic emaravirus virus (FMV), fig latent virus 1 (FLV-1), fig mild mottle-associated virus (FMMaV), fig cryptic virus (FCV), fig fleck-associated virus (FFKaV), and fig badnavirus 1 (FBV-1) - and three viroids - apple dimple fruit viroid (ADFVd), citrus exocortis viroid (CEVd), ands hop stunt viroid (HSVd) - are associated with the disease. In addition, most of them have being detected in various fig producing areas of the world.

The main form of disease control is prevention, which relies on the production of tested pathogen-free plant material. Fig seedlings should be tested to ensure that the

plants are virus-free at the time of purchase and or before transplantation. Our review explains in detail these and other important points regarding each virus and viroid associated with FMD.

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CHAPTER I - INCIDENCE AND SEVERITY OF ROOT ROT OF MUSKMELON ARE REDUCED BY THE INCORPORATION OF VEGETABLE RESIDUE INTO SOIL COVERED WITH POLYETHYLENE MULCH

ABSTRACT

Melon (*Cucumis melo* L.) is one of the most important fruit crops in the Northeastern region of Brazil and nearly all production is exported to European countries. From the indiscriminate use of monoculture, the incidence of soilborne pathogens in melon fields is on the rise, resulting in increasing losses in fruit production. The objective of this study was to investigate if the incorporation of different vegetable materials (*Crotalaria juncea* L. and *Pennisetum glaucum* L.) in the soil, combined with polyethylene mulch, and the application of commercial products (Compost-Aid® + Soil-Set®) can efficiently control soilborne pathogens in melon production. Two greenhouse experiments were identically set up using soil naturally infested with various phytopathogenic fungi, including *Fusarium* spp. and *Macrophomina* spp. The experimental design was completely randomized, with seven treatments and seven replications. The pathogens' occurrence, disease incidence and severity were evaluated, as well as fruit quality characteristics (weight, firmness, and brix). Two treatments showed great potential for decreasing disease incidence, severity, and the occurrence of the pathogens. One of the treatments had pearl millet (*Pennisetum glaucum*) incorporated into the soil that was covered with polyethylene mulch. The other treatment was when crotalaria was incorporated into the soil, covered with polyethylene mulch. Commercial products (Compost-Aid® and Soil-Set®) were applied in high temperature and lower humidity, in both treatments. These treatments also yielded fruits with higher weight and brix than the control treatment.

Keywords: *Fusarium*. *Macrophomina*. *Cucumis melo* L. Disease management. Alternative control.

1 INTRODUCTION

The cultivation of melon (*Cucumis melo* L.) is extremely important for the Brazilian economy, as it is the country's second most exported fresh fruit (DA FRUTICULTURA, 2017). Brazil is the thirteenth largest producer of melon in the world and the third in Latin America (FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS - FAO, 2019). However, the continuous practice of monoculture by many Brazilian farmers has directly influenced the surge of soilborne pathogen populations in melon fields and consequently increased the reduction in fruit quality, yield, and fruit production - jeopardizing the capability of growers to meet export contracts (HUANG et al., 2013).

Fusarium solani (Mart.) and *Macrophomina phaseolina* Tassi (Goid.) are among the major pathogens that cause diseases in melon plant root systems (PORTO et al., 2016). They produce resistance structures, chlamydospores (*F. solani*) and sclerotia (*M. phaseolina*), which can help the pathogens to survive in the soil for long periods of time, threating crop production (LOPES; DE ÁVILA, 2005). Therefore, it is necessary to adopt and combine different integrated disease management strategies to help control those persistent soilborne pathogens.

Several control techniques are widely used to manage soilborne pathogens in crop fields (e.g. chemical, biological control, and the use of natural products). However, for melon crop there is no chemical registered in the Brazilian Department of Agriculture (Ministério da Agricultura, Pecuária e Abastecimento) to control *F. solani* and *M. phaseolina* in the field. The only recommendations are to use resistant varieties and cultural practices to manage these pathogens (BRASIL - MAPA, 2019). However, as Sales Junior et al. (2017) stated, all these measures are superficial and the development of new techniques to complement these management strategies is extremely important, even if all of them need to be used concomitantly. Additionally, melon importing countries, mostly European, have restricted regulations in place that limit the use of chemicals, which adds another layer of complexity on soilborne pathogen control in melon fields. Therefore, there is an eminent need to develop soilborne pathogen management strategies that use less chemicals, are sustainable, and suitable to the Northeastern region of Brazil that melon growers can readily adopt.

One of the complementary techniques used is the application of green manure, which can be deposited over the ground or incorporated into the soil, to efficiently manage phytopathogens (BARRADAS, 2010). This technique has been successfully tested to reduce the inoculum of soilborne pathogens in melon crop (AMBROSIO et al., 2016; DANTAS et al., 2013; PORTO et al., 2016). However, optimal results are achieved when green manure is incorporated into the soil with cover crops, such as crotalaria (*Crotalaria juncea*) (NETO et al., 2016) and pearl millet (*Pennisetum glaucum*) (ASMUS et al., 2016). When applied alone, green manure application yields slow results over time, and thus it is not attractive to growers to adopt.

Another promising technique for the control of pathogens that affect plant root system is soil solarization, that has great efficiency when associated with the incorporation of vegetal materials, mainly because it helps raise the soil temperature (ROCHA; CARNEIRO, 2016), in addition to releasing volatile and non-volatile compounds, which can be toxic to soilborne pathogens. Polyethylene mulch, unlike other type of plastics used in solarization, can be of various colors (black-white, black-silver, and black-black), being the choice of color determined by the variation of the planting season and the place of cultivation (LAMBERT et al., 2017). This technique is already being used to reduce weeds, increase irrigation efficiency, decrease nutrient losses through leaching caused by rain, accelerate plant development, improve fruit hygiene and quality, and also to increase productivity (LAMBERT et al., 2017). In the Northeast region of Brazil, white-black polyethylene mulch with holes for transplanting seedlings has been widely used and it is recommended for melon cultivation.

Soilborne pathogens are very common in melon production fields in the Northeast region of Brazil. It is possible that the incorporation of plant materials into the soil associated with polyethylene mulch may reduce the inoculum potential of these pathogens. This approach can accelerate the decomposition of organic matter, raise the soil temperature, and release compounds from plant materials that are toxic to pathogens. However, during the treatment, the polyethylene mulch should not contain holes in order to stimulate and harness the benefits in controlling soilborne pathogens. Therefore, in addition to the incorporation of plant material and treatment time, the used polyethylene mulch with predrilled holes, which is widely used by melon growers to facilitate the transplantation of seedlings, will have to be replaced with one without holes.

The overall goal of this study was to develop new alternatives to manage soilborne pathogens in melon fields. We hypothesized that the incorporation of vegetal materials in the soil combined with polyethylene mulch and with the application of commercial products will reduce soilborne pathogens initial inoculum in the soil. We tested this hypothesis in soil naturally infested with soilborne pathogens, and added inoculum of *F. falciforme* and *M. phaseolina*, in order to reduce the damage caused by these fungi throughout the melon cycle, from planting to harvest.

2 MATERIAL AND METHODS

2.1 Experiment setup

In order to achieve preliminary results for field application and mainly to ensure high infestation and viability of the studied pathogens, the experiments were performed in a controlled environment (greenhouse). Two experiments were conducted concomitantly, the second experiment was implanted 30 days later the first experiment, using soil that has long been cultivated with muskmelon, with up to three crop cycles per year in the same field, and also with a long history of natural infestation of soilborne pathogens. The soil had the following chemical characteristics: pH(H_2O)=6.10, P(mg dm^{-3})=101.00, sum of bases (SB) (cmolc dm^{-3})=2.99, K^{+}(mg dm^{-3})=85.10, Mg^{+2}(cmolc dm^{-3})=0.50, Al^{+3}(cmolc dm^{-3})=0.00, cation exchange capacity (CEC) (cmolc dm^{-3})=3.65, O.M=3.56 (g Kg^{-1}), and base saturation (V%)=82.00. The experiments were conducted in 14L pots, with a diameter of 0.28m, in a greenhouse located in the city of Mossoró in the state of Rio Grande do Norte, Brazil (5° 11' 17" South, 37° 20' 39" West). Both trials had the same treatments.

2.2 Experimental design

A completely randomized design with seven treatments and seven replications was used. The treatments were: (C) - Control (pots were not covered with polyethylene mulch neither with vegetal material), (M) - polyethylene mulch (pots were covered with polyethylene mulch but not with vegetal material), (C+M) - incorporation of *Crotalaria juncea* L. + polyethylene mulch, (P+M) - incorporation of *Pennisetum glaucum* L. + polyethylene mulch, (M+CS) - polyethylene mulch + (Compost-Aid® + Soil-Set®), (C+M+CS) - incorporation of *Crotalaria juncea* L. + polyethylene mulch + (Compost-Aid® + Soil-Set®), and (P+M+CS) - incorporation of *Pennisetum glaucum* L. + polyethylene mulch + (Compost-Aid® + Soil-Set®).

2.3 Inoculum

Inoculum were prepared by cultivating the fungi in flasks containing arenorganic substrate (LEFÈVRE; SOUZA, 1993). The substrate was composed of three parts of cow manure, one part of washed sand, and 2% of oats (v/w); 20mL of distilled water was added to each 100mL of substrate. Then, the substrate was autoclaved twice, at 24 hours intervals, for one hour each at 1.27 Kg/cm^2 (18 psi) and 121°C. Subsequently, five 5mm diameter discs were transferred in a laminar flow cabinet from the colonies growing in Petri dishes to the flasks containing the arenorganic substrate. The fungi used were, *M. phaseolina* (CMM-1531, deposited in the *Coleção* de Culturas de *Fungos* Fitopatogênicos "Profª. *Maria Menezes*", at the Universidade Federal Rural de Pernambuco, Brazil, GenBank code MN136199) and *F. falciforme* (CML 3946, deposited at the *Coleção* micológica de Lavras, at the Universidade Federal de Lavras, Brazil, GenBank code MH709261). The soil naturally infested with soil pathogens was also artificially infested on the same day that the pots were filled and after 20 days of growth in the arenorganic substrate in the laboratory, 54g of substrate from each fungus per pot, totalizing 108g of *M. phaseolina* and *F. falciforme* inoculated per pot.

The incorporation of the vegetal materials (leaves and branches) and the covering of the pots with the polyethylene mulch were done 17 days before transplanting. The plant materials, crotalaria (*Crotalaria juncea* L.) and pearl millet (*Pennisetum glaucum* L.), were incorporated in the first 10cm of the soil at the amount of 4kg/m² of plant material per pot, the pots were kept for 15 days in the greenhouse (AMBROSIO, 2003). After the treatment period (15 days), holes were drilled in the polyethylene mulch to remove toxic gases and to lower the soil temperature. The melon seedlings were transplanted two days later. Seedlings were cultivated in trays using topsoil mix and hybrid yellow melon Goldex Topseed seeds, the seedlings were transplanted 12 days after sowing.

In treatments (M+CS), (C+M+CS) and (P+M+CS), Compost-Aid® and Soil-Set® (Table 1) were applied once at day one after transplanting at the dosage of 3 kg ha^{-1} and 2 L ha^{-1}, respectively. Those two products were applied twice again, at 7 and 14 days after transplanting, at the concentrations of 2 kg ha^{-1} (Compost-Aid®) and 1.5 L ha^{-1} (Soil-Set®) - considering a population of 12,500 plants ha^{-1} and one plant per pot for each experiment. Plants were watered by drip irrigation and fertigation was conducted according to soil analysis to meet the crop needs (CAVALCANTE et al., 2008). The maximum temperature of the soil in each pot was measured by a mercury thermometer

and the maximum temperature and humidity of the air was measure by digital higro-thermometer, every day at 1.00 p.m.

Table 1. Composition of biofertilizers used in this study.

Compost-Aid®		
Bacteria	UFC g^{-1}	Enzymes
Lactobacillus plantarum	1.25 x 10^8	Protease
Bacillus subtilis	1.25 x 10^8	Cellulase
Enterococcus faecium	1.25 x 10^8	Xylanase
Soil-Set®		
Minerals	%	g L^{-1}
Sulfur	3.70	45.51
Zinc	3.2	39.36
Copper	2.00	24.60
Iron	1.60	19.68
Manganese	0.8	9.84

***Compost-Aid®** and **Soil-Set®** are trade names of compounds produced by Alltech Crop Science.

2.4 Disease evaluation

At the end of the cycle of the melon, all plants were collected to evaluate the occurrence of root rot. Fragments from the border of the disease lesions were removed from all plants that presented symptoms (BUENO et al., 2004). Five fragments from each plant were surface disinfested. The five fragments were placed in a Petri dish containing potato dextrose agar (PDA) + antibiotic (tetracycline 0.05 g L^{-1}) and incubated for seven days, in a BOD at 28 ± 2 °C. Then, the plates were evaluated for the presence of the pathogens, if the pathogen was present in the five fragments, it was evaluated as 100% occurrence, if it was present in just one fragment it was evaluated as 20% occurrence. For instance, it is possible to have on the same plate 80% occurrence of *Fusarium* sp., 40% occurrence of *Macrophomina* sp., and 40% occurrence of *Rhizoctonia* sp. Pathogens were identified using standard identification keys, according to morphological characteristics (BARNETT; HUNTER, 1998; LESLIE; SUMMERELL, 2008).

The incidence of the disease was assessed by the percentage of plants showing symptoms. In our case, we evaluated seven plants, if the seven showed symptoms, it

meant 10% disease. If there were four plants with symptoms, it totaled 57% of disease in this treatment. Disease severity was determined by using a disease note scale (AMBROSIO et al., 2015), following the classifications: (0) - asymptomatic, (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, and (5) - more than 50% of infected tissues. All steps involved in the experiments are depicted in figure 1.

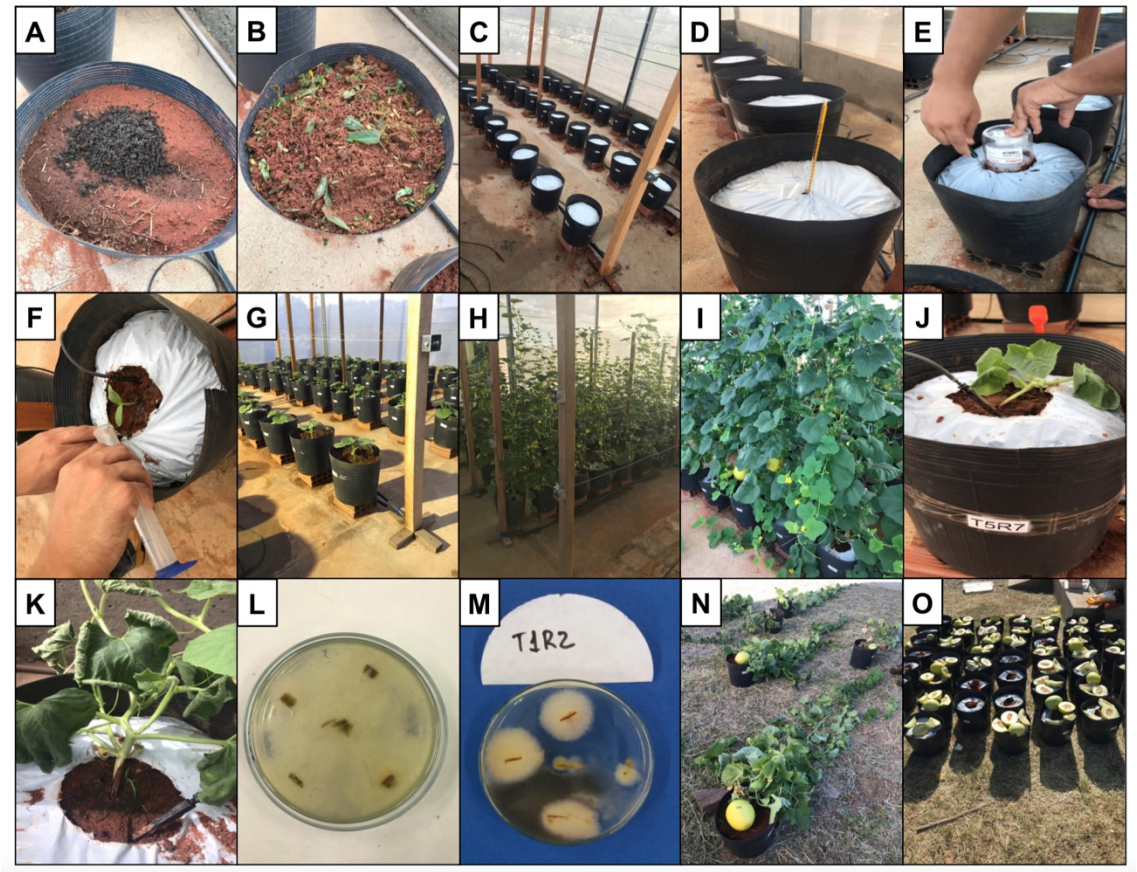

Figure 1. Steps depicting the implementation, conduction, and evaluation of the experiments. A – Fungal inocula being added to the soil. B – Incorporation of plant material. C - Pots covered with polyethylene mulch and going through the 15-days soil treatment period. D – Recording the soil temperature. E – Drilling holes on the polyethylene mulch. F – Seedlings planting and application of Compost-Aid® + Soil-Set®. G, H, and I – Different growth stages of melon plants during the 60 days of the experiment duration. J and K – Plants that did not survive the high disease pressure used in the experiments. L and M – Petri dishes illustrating the fungal isolation assay from infected plant tissue for the evaluation of incidence and occurrence of soilborne pathogens. N and O - End of the experiments, last evaluations and harvesting.

2.5 Fruits evaluation

Fruit weight, firmness, and brix content were measured respectively, using a digital hook scale, an analogical penetrometer PTR 100, and a portable refractometer RT-30 ATC.

2.6 Statistical analysis

The values for weight, firmness, and brix of the fruits were analyzed with the generalized linear mode using the glm function (DOBSON, 2002). Because incidence and severity were not normally distributed, a nonparametric Kruskal-Wallis test was used to analyze those variables (HOLLANDER et al., 2013). Pairwise correlation analysis were performed on the dataset with the nonparametric Kendall's τ rank correlation coefficient to measure the strength of the relationship between each type of symptom using the packages Hmisc (version 4.2-0) (HARRELL JUNIOR, 2016) and corrplot (version 0.84) (WEI; SIMKO, 2016). All statistical analyzes and plotting for data visualization was performed in R program version 3.1.1 (R CORE TEAM, 2019).

3 RESULTS

The maximum soil temperature in all treatments was higher than the greenhouse air temperature in both experiments (Figure 2). However, at the end of experiment 2 – starting at 41 days after the holes were punctured on the polyethylene mulch - both temperatures (air and maximum soil temperature) had similar measurements (Figure 2D). In both experiments, the maximum soil temperature did not exceed 41 °C and it didn't go below 32 °C, except in (C+M), (P+M), (C+M+CS) and (P+M+CS) at 7 days of treatment which reached 42 °C (Figure 2A). Overall, all treatments had similar maximum soil temperature throughout the experiments (Figure 2), with the exception of the control (C) treatment in the first 15 days of the experiment 1, it had lower maximum soil temperature in comparison to the other treatments, with average of 36.63 °C and all other treatments between 37.11 and 37.47 °C. The relative air humidity trend was similar until day 41 in both experiments, then it became higher in experiment 1 in comparison to experiment 2 (Figure 3).

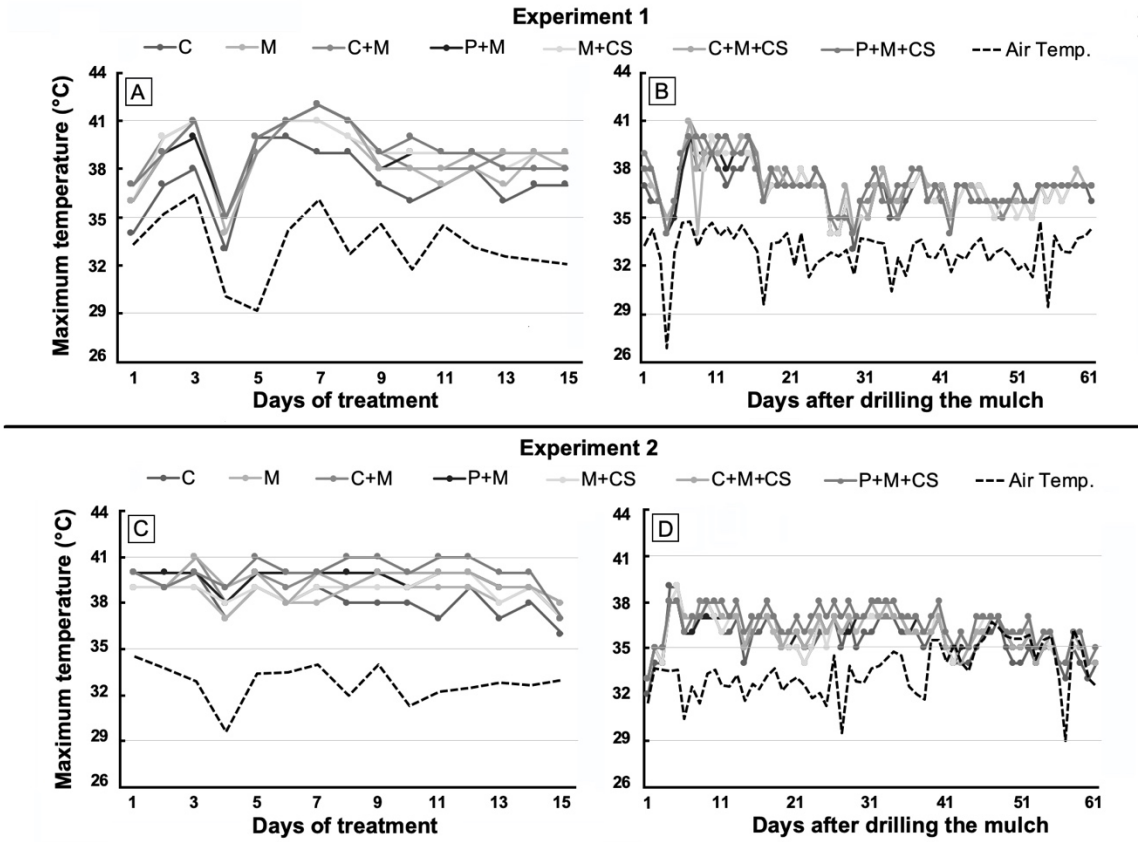

Figure 2. Maximum soil temperature measured in experiment 1 (A and B) and in experiment 2 (C and D). Graphs A and C display soil temperatures measured during the curing treatment (15 days before holes were drilled on polyethylene mulch). Graphs B and D represent temperature measured after the curing treatment. Treatments: (C) – Control, (M) - polyethylene mulch, (C + M) - incorporation of Crotalaria juncea L. + polyethylene mulch, (P + M) - incorporation of *Pennisetum glaucum* L. + polyethylene mulch, (M+CS) - polyethylene mulch + (Compost-Aid® + Soil-Set®), (C+M+CS) - incorporation of *Crotalaria juncea* L. + polyethylene mulch + (Compost-Aid® + Soil-Set®), and (P+M+CS) - incorporation of *Pennisetum glaucum* L. + polyethylene mulch + (Compost-Aid® + Soil-Set®).

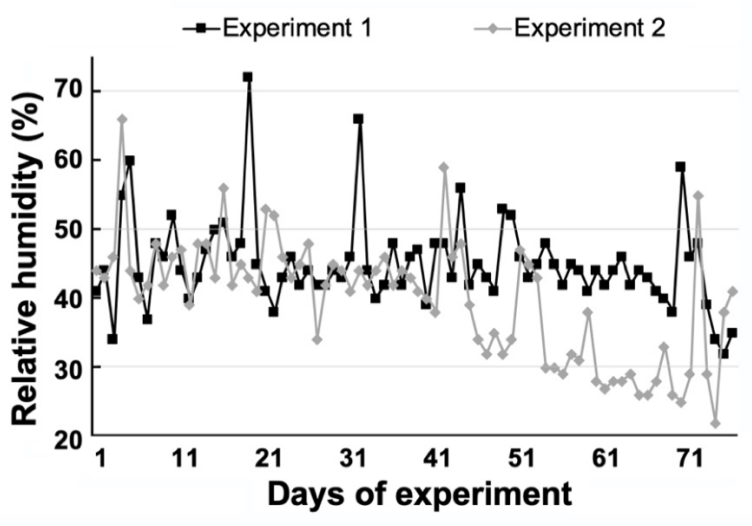

Figure 3. Relative humidity measured inside the greenhouse during experiments 1 and 2.

In this study, we infested the soil with the same amount of inoculum of *M. phaseolina* (GenBank MN136199) and *F. falciforme* (GenBank MH709261) and intentionally used a soil from a field with previous history of melon root rot, so that other pathogenic fungi could occur during the assessments. *Fusarium* occurred more frequently in both experiments in comparison to other fungi isolated, with less occurrence in experiment 2 than experiment 1. Overall, *Macrophomina* occurred with less frequency in experiment 1 than in experiment 2, but it did occur in low incidence in experiment 2 in most treatments. Furthermore, we isolated *Rhizoctonia* spp. in (P+M) and (C+M+CS) treatments in experiment 1, but we did not detect this fungus in any of the treatments in experiment 2 (Figure 4).

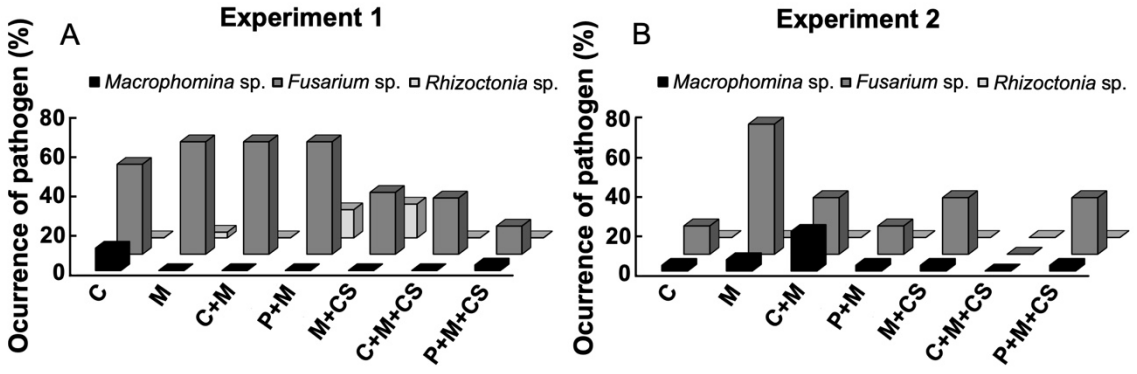

Figure 4. Occurrence of pathogens isolated from muskmelon plants in experiment 1 (A) and experiment 2 (B). Treatments: (C) – Control, (M) - polyethylene mulch, (C+M) - incorporation of *Crotalaria juncea* L. + polyethylene mulch, (P+M) - incorporation of *Pennisetum glaucum* L. + polyethylene mulch, (M+CS) - polyethylene mulch + (Compost-Aid® + Soil-Set®), (C+M+CS) - incorporation of *Crotalaria juncea* L. + polyethylene mulch + (Compost-Aid® + Soil-Set®), and (P+M+CS) - incorporation of *Pennisetum glaucum* L. + polyethylene mulch + (Compost-Aid® + Soil-Set®).

Overall, the disease incidence was higher in experiment 1 than in experiment 2, except for the treatments (M) and (P+M+CS), which had higher disease incidence in experiment 1 than in experiment 2 (Figure 5). The disease incidence in (M) treatment was consistently high in both experiments. In experiment 1, the treatments (C+M+CS) and (P+M+CS) had the lowest disease incidence but only (P+M+CS) was statistically different from the other treatments ($p<0.05$). No disease was observed in (C+M+CS) treatment in experiment 2 (Figure 5).

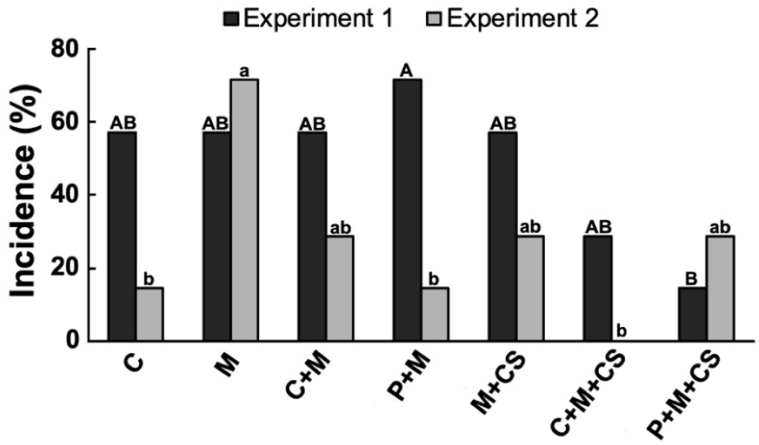

Figure 5. Root rot disease incidence on melon plants in different treatments. Treatments: (C) – Control, (M) - polyethylene mulch, (C+M) - incorporation of *Crotalaria juncea* L. + polyethylene mulch, (P+M) - incorporation of *Pennisetum glaucum* L. + polyethylene mulch, (M+CS) - polyethylene mulch + (Compost-Aid® + Soil-Set®), (C+M+CS) - incorporation of *Crotalaria juncea* L. + polyethylene mulch + (Compost-Aid® + Soil-Set®), and (P+M+CS) - incorporation of *Pennisetum glaucum* L. + polyethylene mulch + (Compost-Aid® + Soil-Set®). Bars with the same letter in the same experiment do not differ statistically by Kruskal-Wallis test ($p<0.05$), the upper case in experiment 1 and the lower case in experiment 2.

The highest disease severity was observed in the treatment (P+M) followed by (M+CS) and the lowest in (P+M+CS) treatment, in experiment 1 (Figure 6). However, in experiment 2, the highest disease severity was found in (M) treatment and the lowest in treatment (C+M+CS) followed by (P+M) (Figure 6).

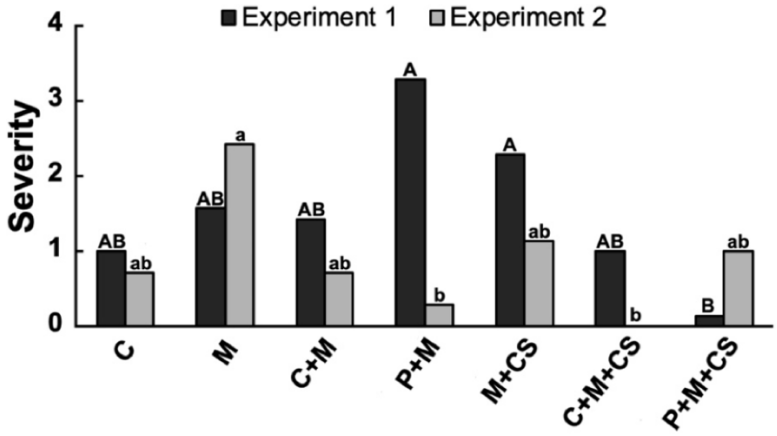

Figure 6. Severity of melon root rot disease in different treatments in melon plants evaluated by a disease rating scale: (0) - asymptomatic, (1) - less than 3% of infected tissues, (2 – 3) - 10% of infected tissues, (3 – 11) - 25% of infected tissues, (4 – 26) - 50% of infected tissues, and (5) - more than 50% of infected tissues. Treatments: (C) – Control, (M) - polyethylene mulch, (C+M) - incorporation of *Crotalaria juncea* L. + polyethylene mulch, (P+M) - incorporation of *Pennisetum glaucum* L. + polyethylene mulch, (M+CS) - polyethylene mulch + (Compost-Aid® + Soil-Set®), (C+M+CS) - incorporation of *Crotalaria juncea* L. + polyethylene mulch + (Compost-Aid® + Soil-Set®), and (P+M+CS) - incorporation of *Pennisetum glaucum* L. + polyethylene mulch + (Compost-Aid® + Soil-Set®). Bars with the same letter in the same experiment do not differ statistically by Kruskal-Wallis test ($p<0.05$), the upper case in experiment 1 and the lower case in experiment 2.

Fruit weight and brix were lower in control treatment (C) in both experiments in comparison to the other treatments, but brix was statistically significant just in experiment 2 ($p <0.05$). On the other hand, fruit firmness was higher in the control treatment (C) in experiment 1 in comparison to the other treatments ($p<0.05$) (Table 2).

Table 2. Effect of the treatments on the characteristics of the melon fruits at harvest

Treatments	Experiment 1			Experiment 2		
	Weight (kg)	**Firmness** (Kgf)	**Brix** (°Bx)	**Weight** (Kg)	**Firmness** (Kgf)	**Brix** (°Bx)
C	0.88 a[x]	4.54 a	9.57 a	0.50 a	6.60 b	7.14 a
M	1.10 ab	4.11 a	10.71 ab	0.87 b	5.55 a	11.50 d
C+M	1.42 b	4.17 a	11.93 b	0.85 b	5.34 a	10.77 cd
P+M	1.37 b	4.28 a	12.00 b	0.79 b	5.80 ab	9.03 b
M+CS	1.16 ab	3.82 a	10.56 ab	0.77 b	4.92 a	9.31 bc
C+M+CS	1.20 ab	3.83 a	10.69 ab	0.78 b	5.74 a	10.29 bcd
P+M+CS	0.89 ab	3.93 a	10.86 ab	0.70 b	5.15 a	9.83 bcd

Treatments: (C) – Control, (M) - polyethylene mulch, (C+M) - incorporation of *Crotalaria juncea* L. + polyethylene mulch, (P+M) - incorporation of *Pennisetum glaucum* L. + polyethylene mulch, (M+CS) - polyethylene mulch + (Compost-Aid® + Soil-Set®), (C+M+CS) - incorporation of *Crotalaria juncea* L. + polyethylene mulch + (Compost-Aid® + Soil-Set®), and (P+M+CS) - incorporation of *Pennisetum glaucum* L. + polyethylene mulch + (Compost-Aid® + Soil-Set®). [x]Tukey's Honest Significant Difference (Alpha = 0.05). Averages with the same letter within the same column are not significantly different.

Root rot incidence and severity were negatively correlated with fruit weight, dry matter, and fresh matter in both experiments. Fruit firmness was negatively correlated with root rot incidence and severity in experiment 2, but it had a low correlation in experiment 1 with those symptoms. It was noteworthy that the higher the fruit firmness the lower the brix of the fruit is, and that *Fusarium* sp. was negatively correlated to *Macrophomina* sp. in experiment 1, but the correlation was positive between those fungi in experiment 2 (Figure 7).

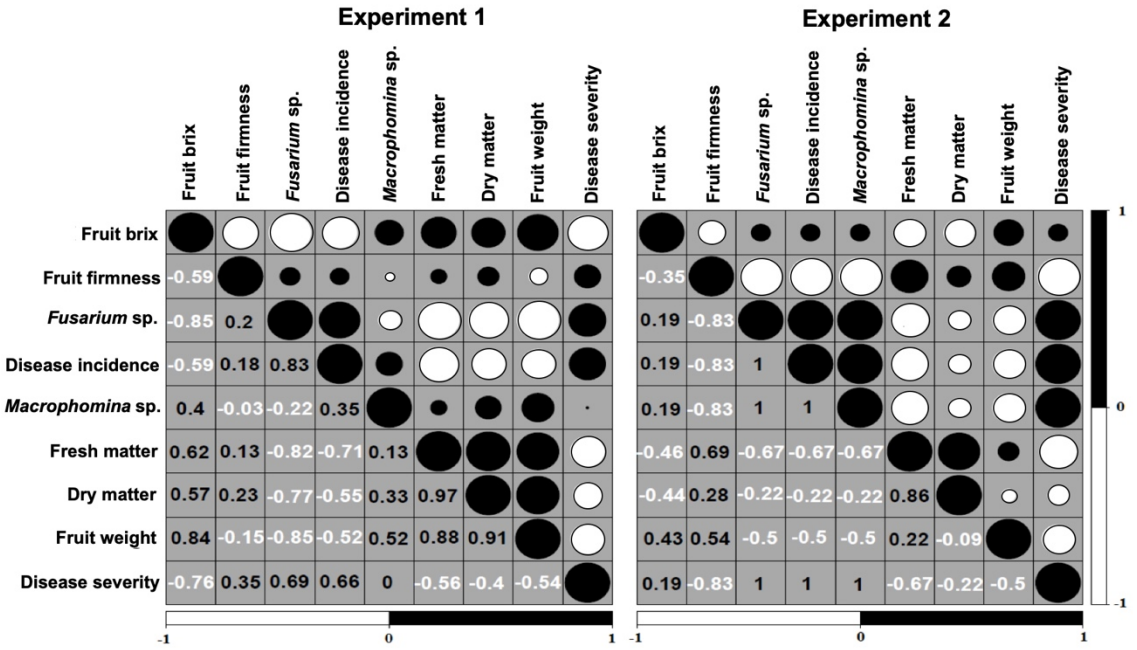

Figure 7. Pairwise correlation analysis using the nonparametric Kendall's t rank correlation coefficient to measure the strength of the relationship between each variable in experiments 1 and 2. Positive correlations are displayed in black and negative correlations in white. Circle size is proportional to the correlation coefficients.

4 DISCUSSION

The maximum soil temperature achieved in all treatments throughout the phase one, before drilling a hole on the polyethylene mulch, in both experiments (Figure 2), was above the optimal temperature for the development of *Macrophomina* spp. (30-35 °C) (GHOSH et al., 2018) and *Fusarium* spp. (28-30 °C) (PAPIZADEH et al., 2018). However, the treatment period of only 15 days may have caused just a fungistatic effect (fungal growth inhibition) on the root rot pathogens. Experiments conducted at temperature ranges close to what we achieved in this study concluded that the fungicide affect in *Fusarium* spp. is achieved only after 21 days of treatment and the same temperature range had little to no effect on *Macrophomina* spp. development (BASSETO et al., 2011).

Studies have shown that polyethylene mulch can be used in several crops for leaf disease suppression and the choice of its color is according to the planting season and the place of cultivation (LAMBERT et al., 2017). In the Northeast region of Brazil, white-black polyethylene plastic has been widely used and it is recommended for melon

cultivation. There is evidence that polyethylene mulch can also be used for weed control and soil moisture retention (SILVA; FELIPE, 2014). Soil solarization, preferably transparent plastic, is mainly used to control pathogens and weeds, but this technique is not used in melon crops in Brazil, due to the large areas of production that makes the use of such technology difficult to accept by producers. However, the use of polyethylene mulch, which is already used in Brazil for melon production, can be adapted to reduce soil inoculum potential, which has been increasing year after year due to the adoption of monoculture. Furthermore, because this technique is similar to solarization and widely used in the control of soilborne pathogens, it may be beneficial to microorganisms living in the soil, especially those that thrive in high temperatures (ROCHA; CARNEIRO, 2016).

Soilborne pathogen control efficacy achieved by using polyethylene mulch can be boosted by concomitantly incorporating organic material. Organic material has some natural antipathogen compounds that are released into the soil and may help to suppress pathogen growth (DANTAS et al., 2013). Besides, the incorporation of organic material helps to elevate the soil temperature higher than when polyethylene mulch is used alone (WONG et al., 2011). However, we did not observe significant increase of the maximum soil temperature using polyethylene mulch with or without the incorporation of organic materials. The maximum soil temperature was similar among all treatments throughout the experiments, even in the control (C) treatment where the soil was not covered with polyethylene mulch or vegetal material (Figure 2). Comparing several studies, Pramanik et al. (2015) demonstrate that the coloration of plastics is fundamental for soil temperature increase, especially transparent and black plastics. Furthermore, Ibarra-Jiménez et al. (2011) concluded that white plastic, used in our experiments, helps to increase productivity, but causes minimal increase in soil temperature, which corroborates with our soil temperature results.

The low occurrence of *M. phaseolina* found in our experiments can be explained by the environmental conditions (Figure 4). In both experiments, the soil temperature and air humidity were high (Figure 3). The soil moisture was also high because of the drip irrigation system that was activated hourly. It is possible that this microclimate was unfavorable for the development of the pathogen. Although *M. phaseolina* thrives in high temperature (30–35 °C) (GHOSH et al., 2018), it develops better in low humidity (LINHARES et al., 2016). On the other hand, *Fusarium* spp. develops better in high humidity (PANWAR et al., 2017), which is exactly what we observed in our first

experiments – high humidity and high levels of occurrence of this pathogen. Moreover, in the second experiment the air relative humidity was lower than registered in experiment 1 towards half of the duration of the experiment (Figure 3). This helps to explain why we observed lower occurrence of *Fusarium* spp. in experiment 2 than registered in first experiment (Figure 4).

Based on the disease incidence and severity, which were positively correlated (Figure 7), the treatments that achieved the overall best control of melon root rot in both experiments were (C+M+CS) and (P+M+CS). However, the microclimatic conditions in the greenhouse, which were different in the two experiments, seemed to play an important role in the efficiency of each treatment in controlling melon root rot disease. For instance, the lower relative humidity observed throughout experiment 2 in comparison to experiment 1 seems to create the optimum condition for the efficacy of (C+M+CS) as no disease developed in this treatment (Figure 4B).

It is noteworthy that the treatments with vegetal material incorporation achieved the best results overall. This may be due to the release of antifungal compounds from the vegetal material, as found by Linhares et al. (2016), when using pearl millet as coverage there was a low survival of *M. phaseolina*. Furthermore, it has also been found that when pearl millet is used as coverage there is a significant reduction of the pathogen *Sclerotium rolfsii* in bean plants. The reduction is due to the increase of the natural population of *Pseudomonas* (NETO; BLUM, 2010). Therefore, these research results support the low pathogen survival observed in the treatments with *Crotalaria juncea* in our studies.

The efficacy of the incorporation of vegetal material on controlling melon root rot was boosted by the addition of Compost-Aid® and Soil-Set®. It is possible that the bacteria that compose the product (Table1) had a direct effect on inhibiting the growth of the root rot pathogens in our experiments. Studies have demonstrated that the application of Compost-Aid® on the soil can successfully control *Meloidogyne javanica* (MIAMOTO et al., 2017) and when it is applied together with Soil-Set® decreases the germination rate of *Cercospora coffeicola* more than 20% (LABORDE, 2014). According to our results, the best use of the formulations (Compost-Aid® and Soil-Set®) is to apply them simultaneously with the incorporation of vegetal material and polyethylene mulch. The results achieved by this combination emphasizes the current managing plant pathogens recommendations, that is, the efficiency in controlling plant pathogens is maximized when several techniques are combined (KIMATI, 2011).

The significantly higher fruit firmness and lower Brix obtained in the control treatment (C) in comparison to the other treatments evaluated during the two experiments (Table 2), corroborate other studies designed to evaluate productivity and fruit quality. This may be due to the absence of the polyethylene mulch, since the only treatment that it was not used in was the control treatment (C) (LAMBERT et al., 2017; LIMA JUNIOR; LOPES, 2009; SERAFIM et al., 2015; SILVA; FELIPE, 2014).

5 CONCLUDING REMARKS

Collectively, our research shows that the incorporation of plant material (crotalaria or pearl millet), associated with the use of polyethylene mulch and commercial products (Compost-Aid® and Soil-Set®), greatly reduces the incidence and severity of melon root rot and the occurrence of its causing pathogens. However, in conditions of high soil temperature and high relative humidity, the combination containing pearl millet yields the best results. On the other hand, when the soil temperature is high and the relative humidity is low, the combination in which crotalaria is incorporated into the soil, yields the lowest disease incidence, severity and pathogens' occurrence.

Finally, the greenhouse experiments conducted in this study are extremely important, as the results achieved can now be easily transferred and tested in field conditions. Moreover, in this research we focused on testing technologies that are cost efficient and readily available to melon growers in the Northeastern region of Brazil to adopt. With these promising results in hand, we will be able to efficiently communicate our research to stakeholders to offer them more alternative strategies to control this devastating disease in melon crops. The obtaining of alternative strategies will support the achievement of our ultimate goal: to shrink the gap between lab research and farm application to strengthen the "lab to farm" concept.

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CHAPTER II - EFFECTS CAUSED BY ALTERNATIVE CONTROL TECHNIQUES FOR ROOT ROT PATHOGEN ON SOIL MICROBIAL COMMUNITIES

ABSTRACT

Growers have long adopted monoculture to maintain the high melon (*Cucumis melo* L.) production demand in the Northeastern region of Brazil. This cultivating strategy culminates in up to three crop cycles per year being used. Little is known about the effect of monoculture on the soil microbiota, which can interfere with populations of soilborne plant pathogens and result in crop losses. The main objective of this study was to evaluate if the incorporation of plant material (*Crotalaria juncea* L. and *Pennisetum glaucum* L.) used with polyethylene mulch and/or in association with commercial soil amendment products (Compost-Aid® + Soil-Set®) can help to condition an environment that is beneficial to soil microbial communities. Two identical greenhouse experiments were conducted using a completely randomized design with seven treatments and seven replications. The treatment (P+M+CS) (incorporation of *P. glaucum* + polyethylene mulch + (Compost-Aid® + Soil-Set®)) showed the most promising results. Overall, in the second experiment, it provided a significant increase in the fungi population and a numeric increase in the population of actinomycetes, sporulating bacteria, and total bacteria in post-harvest. In sporulating bacteria populations, in the second experiment, after drilling a hole in the polyethylene mulch (pre-planting), (P+M+CS) also provided the highest population count. Collectively, the incorporation of *P. glaucum* together with the use of polyethylene mulch and the soil amendment products (Compost-Aid® and Soil-Set®), the treatment (P+M+CS) increased the total fungi population in 183%, total bacteria in 55%, sporulating bacteria in 21%, and actinomycetes in 146% in relation to the control treatment. Based on our results, we strongly recommend this management strategy as a soil health conditioner for agricultural lands widely used for melon production, especially under monoculture practices, where melon is cultivated three times per year.

Keywords: Soil microorganisms. Mulching. Cover crop. Biological control. *Cucumis melo* L.

1 INTRODUCTION

Agriculture is a vital industry in Brazil and the country ranks in third position among the largest producers of melon (*Cucumis melo* L.) in Latin America and thirteenth in the world (FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS - FAO, 2019). According to IBGE (INSTITUTO BRASILEIRO DE GEOGRAFIA E ESTATÍSTICA, 2017), 94% of the Brazilian melon production is concentrated in the state of Rio Grande do Norte, mainly in the Mossoró city microregion. Monoculture has been widely used to keep up with the high production demand and growers usually reach three melon production cycles in a single year. All the agricultural practices associated with the high production pressure (*e.g.,* high use of agrochemicals) and monoculture may impact the soil microbiota abundance, diversity and species richness. In addition, it interferes with plant growth and with severity of diseases caused by soilborne pathogens (HUANG et al., 2013).

Monoculture tends to be unsustainable as the lack of genetic diversity in the crop planted contributes for the selection and rapidly raise of pathogens that compromise crop yield (SHEN et al., 2018). Furthermore, this practice promotes changes in the soil microbial community that culminates in high incidence of soilborne diseases (MUELLER et al., 2016). There is a close link between the increase of the incidence of soilborne pathogens and the loss of beneficial microbial groups for plants and in the composition of the soil microbiota (SHEN et al., 2018). Agreeable in the scientific community, high diversity and appropriate composition of soil microbiota foster soil health and mitigates the upsurge of plant pathogens populations (LING et al., 2011).

Different techniques are used to manage soilborne diseases, such as chemical and biological control and the use of natural products - all these agricultural practices can be used alone or concomitantly (SALES JUNIOR et al., 2017). In fact, the use of soil solarization associated with the incorporation of plant materials raises the soil temperature higher than when each technique is used alone, which has been shown as a promising practice in controlling soilborne pathogens (ROCHA; CARNEIRO, 2016). Thus, polyethylene mulch, which is already used in melon cultivation in the Northeast of Brazil for controlling weeds, if modified to be used without holes before transplanting (such as solarization treatment) may be an alternative approach for disease control. Polyethylene mulch can be applied in combination with other techniques (*e.g.,* incorporation of vegetal material) to raise the soil temperature to levels lethal to

soilborne plant pathogens (WONG et al., 2011), and the containment by polyethylene mulch of volatile and non-volatile substances, which can cause effects on soil microorganisms. However, practices to raise soil temperature need to be taken with caution as such high temperature can be detrimental to the beneficial soil microorganisms that are important for agriculture (NASCIMENTO et al., 2016a).

The most abundant groups of soil microbes are bacteria and fungi, which are regulators of various biological, chemical and physical processes in the soil (MATTOS, 2015). These microbes promote soil health and consequently plant growth by catalyzing unique and indispensable transformations in soil formation, soil biogenesis, organic matter decomposition, toxins degradation and biogeochemical cycling (SHEN et al., 2018). The composition of the soil microbial community is influenced by several factors, such as temperature, moisture, soil aeration, organic substrates and nutrient availability (NASCIMENTO et al., 2016a). These factors are likely to be affected when soilborne pathogen management strategies are performed.

In order to minimize these disturbance in the soil, commercial products were used such as Compost-Aid® (*Lactobacillus plantarum* - 1.25 x 10^8 UFC g^{-1}; *Bacillus subtilis* - 1.25 x 10^8 UFC g^{-1}; *Enterococcus faecium* - 1.25 x 10^8 UFC g^{-1}), which is composed of microorganisms beneficial to the soil, and Soil-Set® (Sulfur – 45.51 g L^{-1}; Zinc – 39.36 g L^{-1}; Copper – 24.60 g L^{-1}; Iron – 19.68 g L^{-1}; Manganese – 9.84 g L^{-1}) as source of micronutrients, which together have already shown positive results in the control of nematodes (MIAMOTO et al., 2017). Moreover, the application of Compost-Aid® alone was shown to inhibit 100% and 98.57% of the growth of the fungi *Macrophomia phaseolina* and *Sclerotium rolfsii*, respectively (NASCIMENTO et al., 2016b). There is a lack of studies designed to investigate how soil microbial communities respond to the use of polyethylene mulch concomitantly with the incorporation of plant materials added to commercial products to control soilborne pathogens.

Thus, the goal of this study was to investigate if the techniques used for raising soil temperature, to manage root rot pathogens, impact the communities of the soil microbes: actinomycetes, sporulating bacteria, total bacteria and total fungi. To address our research questions, we incorporated plant materials (crotalaria, *Crotalaria juncea* L., and millet, *Pennisetum glaucum* L.) with polyethylene mulch, used alone or concomitantly with commercial products (Compost-Aid® + Soil-Set®). Then, we evaluated the development of target soil microbial communities under those systems.

2 MATERIALS AND METHODS

2.1 Experiment setup

The experiments were conducted twice (the second trial was set up 30 days after the first trial was finished) in a greenhouse located in the city of Mossoró, in the state of Rio Grande do Norte, Brazil (5° 11' 17" South, 37° 20' 39" West). We used 14 L plastic pots of 0.28 m in diameter. The soil used in the experiments was collected from an area extensively cultivated with melon plants, up to three crop cycles per year in the same field. This soil has a long history of natural infestation by root rot pathogens, and has the following chemical characteristics: pH(H_2O)=6.10, P(mg dm^{-3})=101.0, sum of bases (SB) (cmolc dm^{-3})=2.99, K^{+}(mg dm^{-3})=85.1, Mg^{+2}(cmolc dm^{-3})=0.50, Al^{+3}(cmolc dm^{-3})=0.0, cation exchange capacity (CEC) (cmolc dm^{-3})=3.65, O.M=3.56 (g Kg^{-1}), and base saturation (V%)=82.0. The same treatments and soil were used in both trials.

2.2 Experimental design

A completely randomized design with seven treatments and seven replications was used. The treatments were: (C) - Control (pots were not covered with polyethylene mulch neither with vegetal material), (M) - polyethylene mulch (pots were covered with polyethylene mulch but not with vegetal material), (C+M) - incorporation of *C. juncea* L. + polyethylene mulch, (P+M) - incorporation of *P. glaucum* L. + polyethylene mulch, (M+CS) - polyethylene mulch + (Compost-Aid® + Soil-Set®, trade names of products produced by Alltech Crop Science), (C+M+CS) - incorporation of *C. juncea* L. + polyethylene mulch + (Compost-Aid® + Soil-Set®), and (P+M+CS) - incorporation of *P. glaucum* L. + polyethylene mulch + (Compost-Aid® + Soil-Set®).

2.3 Plant cultivation

Seeds of hybrid yellow melon GOLDEX TOPSEED were sown in trays with substrate for 12 days, then seedlings were individually transplanted in pots. Exactly 17 days before transplanting, the vegetal materials (*C. juncea* L. and *P. glaucum* L.) were incorporated in the first 10 cm of the soil and applied at rate of 4 kg/m² of plant material per pot (AMBROSIO, 2003). The pots were covered with the polyethylene mulch and kept covered for 15 days. On the fifteenth day, holes were drilled on the polyethylene mulch to remove toxic gases and to low the soil temperature to condition de soil for the melon seedlings that were transplanted two days later.

Throughout the experiment, plants were watered by drip irrigation and fertigation was conducted according to soil analysis to meet the crop needs

(CAVALCANTI et al., 2008). In treatments (M+CS), (C+M+CS) and (P+M+CS), Compost-Aid® (*Lactobacillus plantarum* - 1.25 x 10^8 UFC g^{-1}; *Bacillus subtilis* - 1.25 x 10^8 UFC g^{-1}; *Enterococcus faecium* - 1.25 x 10^8 UFC g^{-1}) and Soil-Set® (Sulfur – 45.51 g L^{-1}; Zinc – 39.36 g L^{-1}; Copper – 24.60 g L^{-1}; Iron – 19.68 g L^{-1}; Manganese – 9.84 g L^{-1}) were applied once at one day after transplanting, according to the manufacturer's recommendations, at the dosage of 3 kg ha^{-1} and 2 L ha^{-1}, respectively. Those two products were applied twice again, at seven and 14 days after transplanting, at the concentrations of 2 kg ha^{-1} (Compost-Aid®) and 1.5 L ha^{-1} (Soil-Set®) - considering a population of 12,500 plants ha^{-1} and one plant per pot. The maximum temperature of the soil in each pot was measure by a mercury thermometer and the maximum temperature and humidity of the air was measured by a digital hygro-thermometer, daily at 1.00 p.m.

2.4 Microbiota evaluation

Before filling up the pots with soil to set up the experiments, three soil samples were randomly collected from the bulk homogenized soil. Then the single samples were combined into a composite sample of 300 g per experiment. Two other soil samples were collected in each pot (a sample per pot each time), one on the day the mulch was drilled (Pre-planting) and another at harvest (60 days post-transplanting). Soil samples were kept in transparent plastic bags and stored at 10 °C to perform microbial community evaluations.

In order to quantify the target soil microbiota (fungi, bacteria, sporulating bacteria, and actinomycetes), isolations were attempted on selective culture media specific for each group of microorganisms. For total fungi counting, we used Martin's medium (K_2HPO_4 – 1.00 g; $MgSO_4.7H_2O$ - 0.50 g; peptone – 5.00 g; dextrose – 10.00 g; rose bengal - 0.03 g; agar – 16.00 g; distilled water - 1,000 mL) (MARTIN, 1950) plus 0.05 g L^{-1} of tetracycline. For total and sporulating bacteria, the agar nutrient medium was used (nutrient agar - 23.00 g; distilled water - 1,000 mL). For actinomycetes, we used the culture medium starch casein (starch - 10.00 g; casein - 0.30 g; KNO_3 - 2.00 g; NaCl – 2.00 g; K_2HPO_4 - 2 g; 0.05 g; $MgSO_4.7H_2O$ - 0.01 g; agar – 16.00 g; distilled water - 1,000 mL) (CUNHA et al., 2014).

Soil microorganism isolations were performed by using serial dilution technique. One gram of soil was taken from each sample and placed in test tubes containing 9 mL of sterile distilled water. Each tube was homogenized in a vortex tube shaker and serial dilutions were performed by factor of 10 (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5}). For each

dilution point, 100 µL of the solution were collected and placed individually on a 9.0 cm diameter Petri dish, containing the specific selective media for the corresponding microbe group analyzed, and dispersed with a Drigalski spatula. For the analysis of sporulating bacteria, the samples were kept for 20 minutes in a water bath at 80 °C, prior to placing the sample aliquots in Petri dishes, to kill non-sporulating bacteria (BETTIOL, 2007). Three plates were plated per sample per dilution point and after counting, the values were converted to colony forming units per gram of soil (CFU g^{-1}). Only the dilution points that had 20 to 200 colonies per plate were considered for the calculations because of colonies saturation that occur when too many microbial colonies grow together in a Petri dish, which inhibits the growth of other colonies and underestimate the results (TORTORA et al., 2016). All plates were inverted and kept in a biochemical oxygen demand (BOD) incubator for six days at 28 ± 2 °C. The quantification of microbial communities by the plate count method was chosen because this technique has the advantage of providing the quantification of viable microbe cells (TORTORA et al., 2016). All steps involved in the experiments are depicted in figure 1.

Figure 1. Steps depicting the implementation, conduction, and evaluation of the experiments. A - Collection of soil (with a long history of natural infestation by soilborne pathogens) to be used in the experiments. B – Incorporation of plant material. C - Pots covered with polyethylene mulch during the 15-days soil treatment period (solarization). D – Recording the soil temperature. E – Drilling holes on the polyethylene mulch. F – Seedlings planting. G – Application of Compost-Aid® + Soil-Set®. H – Plants at 45 days after transplanting. I – Soil weighing for dilution. J – Serial dilution step. K – Petri dishes with each specific culture media. L – Plates containing Martin's medium. M – Analysis of sporulating bacteria, the samples were kept for 20 minutes in a water bath at 80 °C. N – Count of fungal colony forming units. O – Count of bacterial colony forming units.

2.5 Statistical analysis

The results of the population quantification for total fungi, total bacteria, sporulating bacteria and total actinomycete were analyzed by the non-parametric method, Kruskal Wallis test. All statistical analyzes and graphing were performed in R version 3.1.1 (R CORE TEAM, 2019).

3 RESULTS

The maximum soil temperature in all treatments was higher than the greenhouse air temperature in both experiments (Figure 2). However, at the end of experiment 2 – starting at 41 days after, the holes were punctured on the polyethylene mulch - both temperatures, air and maximum soil temperature, had similar measurements (Figure 2B). Throughout the experiment period, in both experiments, the maximum soil temperature did not exceed 41 °C and it didn't go below 32 ºC; except in the treatments: (C+M), (P+M), (C+M+CS), and (P+M+CS), which at seven days in the first experiment the maximum soil temperature reached 42 ºC (Figure 2). The relative air humidity was similar until day 41 in both experiments, then it became higher in experiment 1 in comparison to experiment 2 (Figure 3).

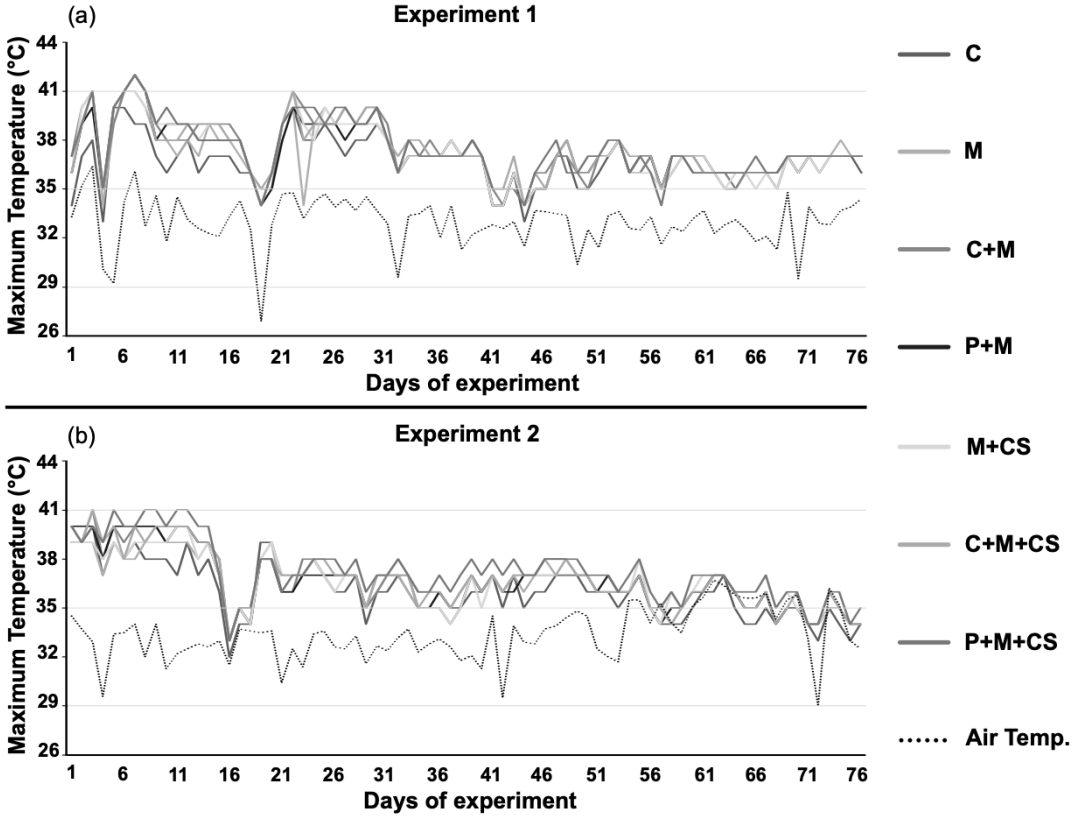

Figure 2. Maximum soil temperature measured in experiment 1 (A) and in experiment 2 (B).

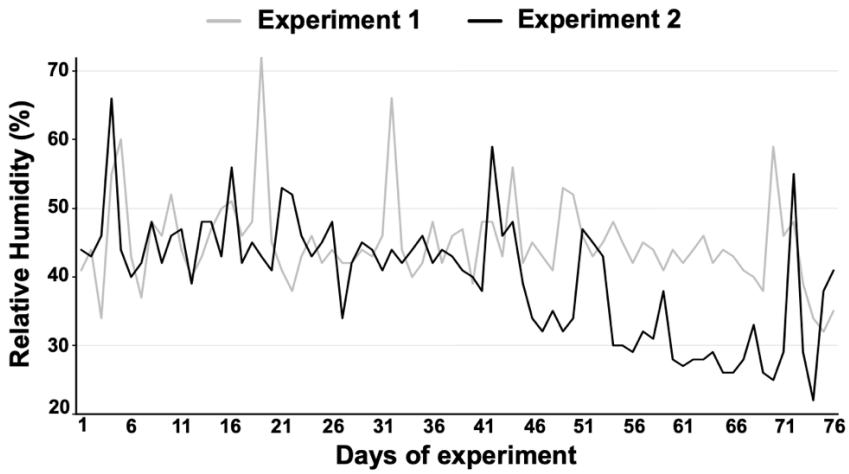

Figure 3. Relative humidity inside the greenhouse for the duration of experiments 1 and 2.

The initial fungal population was similar in the first and second experiments, $5.90x10^3$ and $5.10x10^3$ CFUs g^{-1}, respectively (Table 1). After drilling a hole in the polyethylene mulch (pre-planting), the (P+M) treatment in the first experiment had the largest total fungal population ($8.31x10^3$ CFUs g^{-1}) in comparison to all other treatments. Interestingly, at the end of the cycle (post-harvest), the treatment (C+M) in experiment 1, which had the largest total fungi population ($14.20x10^3$ CFUs g^{-1}), was precisely the one with the lowest population at pre-planting ($4.36\ x10^3$ CFUs g^{-1}). Additionally, the total fungi population was statistically higher in (C+M) than in (C) treatment; but the (C+M) total fungi population was not statistically different from the ones in (P+M) and (C+M+CS) treatments. In the second experiment after drilling a hole in the polyethylene mulch (pre-planting), the (C+M+CS) treatment had the largest total fungal population; however, it did not differ statistically from the other treatments: (C), (C+M), (P+M), and (M+CS). At harvest, the treatment (P+M+CS) was statistically higher than the control treatment (C), it had the highest fungal population increase among all treatments, reaching $26.10x10^3$ CFUs, which corresponds to over 86% increase of the total fungi population from the pre-planting period to the end of the cycle in that treatment. It also had the lowest population count at pre-planting ($3.44x10^3$ CFUs). In the first experiment, the treatment (P+M+CS) did not show statistical difference, but it increased 68% of the population in post-harvest compared to the treatment (C). In the second experiment, in addition to this result being statistically

different, this increase in the population was even greater, reaching an increase of 297% of the treatment (P+M+CS), compared to the treatment (C).

Table 1. Number of colonies forming units (CFUs) of total fungi in different treatments.

Treatments	Total fungi					
	Exp. 1			Exp. 2		
	Pre-mulch	Pre-planting	Post-harvest	Pre-mulch	Pre-planting	Post-harvest
	----------10^3 of the number of CFUs g^{-1}----------					
C	5.90 a	6.07 abc	5.95 a	5.10 a	6.41 c	6.58 a
M	5.90 a	6.48 bc	7.78 a	5.10 a	4.51 ab	21.80 ab
C+M	5.90 a	4.36 a	14.20 b	5.10 a	6.03 bc	7.36 ab
P+M	5.90 a	8.31 c	14.00 ab	5.10 a	5.85 abc	16.70 ab
M+CS	5.90 a	7.25 c	5.91 a	5.10 a	5.66 abc	13.10 ab
C+M+CS	5.90 a	4.60 ab	12.20 ab	5.10 a	6.70 c	15.20 ab
P+M+CS	5.90 a	6.23 abc	10.00 a	5.10 a	3.44 a	26.10 b

(C)- Control; (M)- polyethylene mulch; (C+M)- incorporation of *Crotalaria juncea* L. + polyethylene mulch; (P+M)- incorporation of *Pennisetum glaucum* L. + polyethylene mulch; (M+CS)- polyethylene mulch + (Compost-Aid® + Soil-Set®); (C+M+CS)- incorporation of *C. juncea* L. + polyethylene mulch + (Compost-Aid® + Soil-Set®); (P+M+CS)- incorporation of *P. glaucum* L. + polyethylene mulch + (Compost-Aid® + Soil-Set®). Means followed by the same letter in the same column do not differ by the same letter in the same column do not differ by Kruskal Wallis test ($p<0.05$).

The initial total bacteria population was $3.63x10^5$ CFUs g^{-1} in the first and $5.63x10^5$ CFUs g^{-1} in the second experiment (Table 2). During the first experiment, the treatment that reached the largest total bacteria population was (P+M) in pre-planting, which differed statistically from the (C), (M), and (C+M) treatments. Although no statistical significance was observed among the treatments in post-harvest evaluations, in the second experiment the largest increase of total bacteria population in pre-planting evaluations was observed in the (C+M+CS) treatment ($9.30x10^5$ CFUs g^{-1}), the population count was 67% superior to (C) treatment. In post-harvest, the (P+M+CS)

treatment had the highest total bacteria population among the treatments; however, it was only statistically different from the (C+M+CS) treatment. In post-harvest, the treatment (P+M+CS), when compared to control, showed a 10% drop in the population in the first experiment; on the other hand, in the second experiment the same treatment increased 119% of total bacteria.

Table 2. Number of colonies forming units (CFUs) of total bacteria in different treatments.

Treatments	Total bacteria					
	Exp. 1			Exp. 2		
	Pre-mulch	Pre-planting	Post-harvest	Pre-mulch	Pre-planting	Post-harvest
	----------10^5 of the number of CFUs g^{-1}----------					
C	3.63 a	3.71 a	7.64 a	5.63 a	3.10 a	6.95 ab
M	3.63 a	4.40 ab	6.79 a	5.63 a	5.36 ab	8.16 b
C+M	3.63 a	4.20 a	6.77 a	5.63 a	7.83 bc	7.92 b
P+M	3.63 a	22.50 c	9.78 a	5.63 a	6.70 bc	8.13 b
M+CS	3.63 a	6.69 bc	7.46 a	5.63 a	3.50 a	6.81 ab
C+M+CS	3.63 a	6.33 bc	7.34 a	5.63 a	9.30 c	5.29 a
P+M+CS	3.63 a	9.40 c	6.90 a	5.63 a	6.05 bc	15.20 b

(C)- Control; (M)- polyethylene mulch; (C+M)- incorporation of *Crotalaria juncea* L. + polyethylene mulch; (P+M)- incorporation of *Pennisetum glaucum* L. + polyethylene mulch; (M+CS)- polyethylene mulch + (Compost-Aid® + Soil-Set®); (C+M+CS)- incorporation of *Crotalaria juncea* L. + polyethylene mulch + (Compost-Aid® + Soil-Set®); (P+M+CS)- incorporation of *Pennisetum glaucum* L. + polyethylene mulch + (Compost-Aid® + Soil-Set®). Means followed by the same letter in the same column do not differ by Kruskal Wallis test ($p<0.05$).

The initial population of sporulating bacteria was $5.20x10^4$ CFUs g^{-1} in the first experiment, while in the second experiment it was much higher: the population started at $29.70x10^4$ CFUs g^{-1} (Table 3). There was no statistical difference between treatments in pre-planting and in post-harvest evaluations in the first experiment, the only statistical difference was between treatments (C) and (M) in post-harvest. In the second

experiment after drilling a hole in the polyethylene mulch (pre-planting), the (C+M+CS) treatment had the largest sporulating bacteria population, but it did not differ statically from (P+M+CS), (P+M), and (C+M) treatments. At harvest, the treatment with the largest population was (P+M+CS), with 57.10 x10^4 CFUs g^{-1}. Interestingly, (C+M+CS) had the smallest population count in post-harvest, but it had the highest population count in the pre-planting evaluations, a drastic reduction in the population count from 24.40 x10^4 to 8.45 x10^4 CFUs g^{-1}. The population of sporulant bacteria followed the same pattern as the total bacteria, where there was a drop in the population in the first experiment, on the treatment (P+M+CS) of 17%, but in the second experiment increased of 58%, both in comparison to treatment (C).

Table 3. Number of colonies forming units (CFUs) of sporulating bacteria in different treatments.

Treatments	Sporulating bacteria					
	Exp. 1			Exp. 2		
	Pre-mulch	Pre-planting	Post-harvest	Pre-mulch	Pre-planting	Post - harvest
	----------10^4 of the number of CFUs g^{-1}----------					
C	5.20 a	15.90 a	14.90 b	29.70 a	6.10 a	36.20 bc
M	5.20 a	15.90 a	7.25 a	29.70 a	8.26 ab	29.90 bc
C+M	5.20 a	9.81 a	12.10 ab	29.70 a	11.90 bc	20.00 ab
P+M	5.20 a	14.30 a	10.10 ab	29.70 a	14.70 c	32.00 bc
M+CS	5.20 a	13.40 a	9.78 ab	29.70 a	5.69 a	17.90 ab
C+M+CS	5.20 a	12.20 a	12.30 ab	29.70 a	24.40 c	8.45 a
P+M+CS	5.20 a	14.70 a	12.30 ab	29.70 a	15.10 c	57.10 c

(C)- Control; (M)- polyethylene mulch; (C+M)- incorporation of *Crotalaria juncea* L. + polyethylene mulch; (P+M)- incorporation of *Pennisetum glaucum* L. + polyethylene mulch; (M+CS)- polyethylene mulch + (Compost-Aid® + Soil-Set®); (C+M+CS)- incorporation of *Crotalaria juncea* L. + polyethylene mulch + (Compost-Aid® + Soil-Set®); (P+M+CS)- incorporation of *Pennisetum glaucum* L. + polyethylene mulch + (Compost-Aid® + Soil-Set®). Means followed by the same letter in the same column do not differ by Kruskal Wallis test ($p<0.05$).

The initial population count of actinomycetes was $12.70x10^4$ CFUs g^{-1} in the first and $16.00x10^4$ CFUs g^{-1} in the second experiment (Table 4). Treatment (P+M) had the largest population count among all treatments in the pre-planting and post-harvest evaluations in experiment 1. In the second experiment, the (C+M+CS) treatment had the highest population in pre-planting evaluations, while in post-harvest evaluations (P+M+CS) treatment had the highest actinomycetes total population count. Even though, there was no significant difference in both experiments in comparison to treatment (C), in the first experiment treatments such as (P+M), (C+M), and (P+M+CS) showed an increase in population actinomycetes of 122, 68, and 49%, respectively. In the second experiment, in post-harvest evaluation, the treatment (P+M+CS) showed the greatest increase among all treatments, compared to treatment (C), there was an increase of 242% in the population of actinomycetes.

Table 4. Number of colonies forming units (CFUs) of total actinomycete in different treatments.

Treatments	Total actinomycete					
	Exp. 1			Exp. 2		
	Pre-mulch	Pre-planting	Post-harvest	Pre-mulch	Pre-planting	Post - harvest
	----------10^4 of the number of CFUs g^{-1}----------					
C	12.70 a	5.45 a	15.60 bc	16.00 a	19.10 a	17.10 ab
M	12.70 a	12.80 b	5.68 a	16.00 a	37.10 ab	13.40 ab
C+M	12.70 a	8.27 ab	26.20 bc	16.00 a	42.90 ab	7.07 a
P+M	12.70 a	48.00 c	34.70 c	16.00 a	48.90 ab	21.00 b
M+CS	12.70 a	10.10 ab	10.10 ab	16.00 a	42.00 ab	15.30 ab
C+M+CS	12.70 a	7.19 ab	9.37 ab	16.00 a	63.70 b	4.56 a
P+M+CS	12.70 a	11.10 b	23.20 bc	16.00 a	51.50 ab	58.40 b

(C)- Control; (M)- polyethylene mulch; (C+M)- incorporation of *Crotalaria juncea* L. + polyethylene mulch; (P+M)- incorporation of *Pennisetum glaucum* L. + polyethylene mulch; (M+CS)- polyethylene mulch + (Compost-Aid® + Soil-Set®); (C+M+CS)- incorporation of *Crotalaria juncea* L. + polyethylene mulch + (Compost-Aid® + Soil-Set®); (P+M+CS)- incorporation of *Pennisetum glaucum* L. + polyethylene mulch + (Compost-Aid® + Soil-Set®). Means followed by the same letter in the same column do not differ by Kruskal Wallis test ($p<0.05$).

4 DISCUSSION

Temperature was measured every day during the course of the experiments in the hottest hour of the day for the region, between 12 and 1 p.m., and it did not vary significantly among treatments in both experiments over time. It did follow the air temperature trend - when the air temperature dropped so did the maximum temperature in each treatment (Figure 2), unlike the results obtained by Nascimento et al. (2016a), which showed that in the period from 10 to 18 days after transplanting the melon seedlings the treatment with vegetable cover + polyethylene mulch had the highest soil temperature. However, the same did not occur in the period from 30 to 46 days after the transplant, the soil temperature dropped in that treatment. According to the authors, that was due to the shade generated by the plants that grew taller in the course of that study. We did not observe this effect in our experiments.

Humidity interferes with the soil microbiota population, because high soil humidity means low availability of oxygen for microbial development (SOUTO et al., 2008), and that depends on the characteristics of the soil and the metabolic requirements of each class of microorganisms. In our study, the largest populations of the soil microbes evaluated were identified in the post-harvest in the second experiment, in which the air humidity measured throughout the experiment reached the lowest levels after day 41 (Figure 3).

Vegetation cover has been shown to increase the abundance and species richness of soil microbiota (MORENO et al., 2009). Although we did not directely attempt to quantify the microbiota specie richness in our study, our results corroborate Moreno et al. (2009), the treatments where *C. juncea* L. and *P. glaucum* L. were incorporated into the soil significantly increased soil fungal abundance. When combined with Compost-Aid® and Soil-Set® products, the incorporation of vegetal material yielded even more expressive results, especially the treatment (P+M+CS), in which the population increased by 68% and 297% in relation to treatment (C) in the first and second experiments, respectively (Table 1). On the other hand, studies have shown that the population of fungi is not affected by the incorporation of green manure into the soil for cultivation of lettuce when compared with the chemical fertilization (crop recommended mineral fertilization) (DE OLIVEIRA et al., 2012). However, because the Compost-Aid® and Soil-Set® products significantly increased the fungal population count in our second experiment, it is plausible that they meet their objectives. The Compost-Aid® is

used to accelerate the decomposition of organic materials, promote microbiological activation and improve the balance of the soil microbiota. In other hand, Soil-Set® promotes greater balance and it hinders the appearance of effects caused by environmental stresses.

Changes in the microbial community interfere directly with biological and biochemical processes in the soil, in agricultural productivity, and in the sustainability of agro-ecosystems – acting as an indicator of soil degradation (MATSUOKA et al., 2003). Covering the soil with polyethylene mulch + spontaneous vegetation offers great water retention in the system, which increases humidity that in turn may favor the growth of bacteria in the soil, as low humidity is known to restrict the movement and replication of bacterium cells (BERNARDES; DOS SANTOS, 2007). This was exactly what we observed in this study. The treatment (P+M+CS) in the evaluation of sporulating bacteria, only in the second experiment, achieved an increase of 58% of the population in comparison to the control treatment. At the time of pre-planting to total bacteria, evaluations done right after drilling holes in the polyethylene mulch reviewed that most treatments involving polyethylene mulch and the incorporation of plant material showed significantly higher microbe population counts than the control treatment (Table 2). That did not happen in the post-harvest evaluations in our experiments.

Studies evaluating population and microbial activity in agroecological production system indicate that there is a significant effect of the crop developmental stage on the soil microbiota - higher actinomycetes counts were consistently found in post-harvest than before and during planting stage (FERREIRA et al., 2017). However, in our study, the period that showed the highest actinomycetes counts was pre-planting. It is possible that this stage presents greater plant material decomposition and better soil conditions for the development of actinomycetes, given the treatments carried out. That is a plausible hypothesis, since different cultivation systems cause distinct changes in soil microbiological attributes, prompting different effects on soil health and plant development (FERREIRA et al., 2017).

A correct management of the soil and cultural remains supports the soil microbial population, benefiting groups of specific microorganisms such as actinomycetes, bacteria and fungi (HUNGRIA et al., 1994). In fact, at 60 days after vegetal material incorporation in the soil, *C. juncea* L. and *P. glaucum* L. were shown to stimulate the highest number of fungi and actinomycetes propagules, significantly

higher than the control (no incorporation of plant material) (BOTELHO et al., 2007). We observed the same trend in the treatments where *P. glaucum* L. was incorporated. Noteworthy, this effect was amplified when Compost-Aid® and Soil-Set® products were applied concomitantly with *P. glaucum* L., there was an increase in the population count of the microbes evaluated. This enhancement effect may occur because these products are known for stimulating the decomposition of organic matter.

5 CONCLUDING REMARKS

The incorporation of vegetal materials, *P. glaucum* or *C. juncea*, accompanying with polyethylene mulch when added the soil amendment composts, Compost-Aid® and Soil-Set® increased the population of all soil microbes evaluated in this study (actinomycetes, sporulating bacteria, total bacteria and total fungi), more expressive in the second experiment. The treatment that showed the best results was (P+M+CS), in average of the results obtained from the two experiments in comparison to the control treatment (C), increased in the total fungi population in 183%, total bacteria in 55%, sporulating bacteria in 21%, and actinomycetes in 146%. Therefore, the use of those composts combined is recommended for increase of soil beneficial microbes that will potentially alleviate the detrimental effects of the intensive melon production activities. This was the first step of a promising study, and it should be repeated in field conditions for the direct application of the results. By fostering the establishment and conservation of soil-dweller communities, we can harness great benefits for all crops and food supply.

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CHAPTER III - DETECTION OF MULTIPLE GRAPEVINE VIRUSES IN NEW ENGLAND VINEYARDS

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ABSTRACT

Vineyards in the New England, a region of the USA, were surveyed for the occurrence of grapevine viruses. A total of ten vineyards were visited and 62 composite samples of leaves with the petioles were collected from symptomatic grapevines (*Vitis* spp.). All samples were assayed by double-antibody sandwich enzyme-linked immuno-sorbent assays (DAS-ELISA) using antibodies specific for four major grapevine leafroll-associated viruses (GLRaV-1, GLRaV-2, GLRaV-3, and GLRaV-4), grapevine fanleaf virus (GFLV), tobacco ringspot virus (TRSV) and tomato ringspot virus (ToRSV). Positive ELISA samples were further tested by reverse transcription polymerase chain reaction (RT-PCR) with primers specific for each of the viruses to confirm the ELISA results. Twenty-two samples were infected with at least one of the viruses tested. GLRaV-3 (24.19%) was the most prevalent virus detected followed by GLRaV-1 (12.90%), ToRSV (3.23%), GLRaV-2 and TRSV (1.61%). This is the first study reporting on the presence of grapevine viruses in New England. Extensive surveys need to be conducted to evaluate the prevalence and economic impact of these viruses on New England vineyards.

Keywords: Grapevine viruses. New England. Vineyards.

1 INTRODUCTION

Grapevine (*Vitis* spp.) is the most cultivated fruit crop in the world, approximately 74 million tons are produced each year in an estimated planted area of over 7.5 million hectares. The total crop value is close to $70 billion yearly and on average, 39% of the world grape production is concentrated in Europe, 32% in Asia, and 20% in the Americas (FAO & OIV, 2016). In 2018, the total crop value in the USA reached $6.6 billion. Currently, in New England (NE), over 100 wineries are maintained on more than 950 acres with grapes planted for commercial wine production valued over $3.6 million, with fast expansion (USDA-NASS, 2018). Most of the NE wineries are situated in the New York City-Boston metropolitan area, making that region a hub for tourists and wine enthusiasts from around the world. Grapevine diseases have the potential to jeopardize the regional wine industry.

Recent studies reported that nearly 70 viruses and virus-like agents are capable of infecting grapevines (MARTELLI, 2017). Among the diseases caused, grapevine leafroll disease (GLD) poses a significant threat to grape production worldwide. It is caused by several serologically and genetically distinct þssRNA viruses including grapevine leafroll-associated virus (GLRaV-1, GLRaV-2, GLRaV-3 and GLRaV-4). All of the viruses are graft-transmissible and most of them are vectored by insects such as mealybugs (Pseudococcidae) and scale (Coccidae). In red grape cultivars, GLRaVs induces red or reddish-purple discolorations in the interveinal areas, but a narrow strip of leaf tissue on either side of the main veins remain green, giving the appearance of “green veins”. In contrast, white-berried cultivars show mild yellowing or chlorotic mottling of interveinal areas of leaves (NAIDU et al., 2015). Another major threat to grape production is the recently described virus, *Grapevine red blotch virus*, a DNA virus that causes symptoms similar to GLD (SUDARSHANA et al., 2015). The disease grapevine fanleaf degeneration/decline caused by some nepoviruses - e.g. *Grapevine fanleaf virus*, *Tobacco ringspot virus*, and *Tomato ringspot virus* – is considered one of the oldest grape viral diseases that cause a significant economic loss in vineyards (MALIOGKA et al., 2015). The disease causes leaf distortion with toothed margins (fan-like) and bright yellow discolorations. Although grapevine viruses have been identified and are under constant surveillance in all major grape-growing states in the USA, there is no current effort in NE to determine the occurrence of major virus species in regional vineyards. Here, we report for the first time the occurrence of GLRaVs and

other grape viruses in NE vineyards, discussing the impact that those viruses may cause on the local grape and wine industry sustainability.

2 MATERIALS AND METHODS

2.1 Sample collection

On October 18th, 2019 a total of 62 symptomatic grapevine plants displaying yellowing, leaf distortion, redness, and chlorotic mottling of interveinal areas in the leaves were randomly sampled from ten different vineyards in the New England (NE) region of the United States (USA). Four leaves with petioles were collected from each plant and combined, making a total of one composite sample per plant. Samples were placed in sealed plastic bags and transported to the plant virology lab at The Connecticut Agricultural Experiment Station (CAES), New Haven - CT, for testing.

2.2 DAS-ELISA assay

Composite samples were individually transferred to Bioreba extraction bags (12 x 15 cm) (Bioreba AG, Kanton Reinach, Switzerland) and ground in 5 mL of a filter-sterilized grapevine extraction buffer (2.40 g L^{-1} of TRIS, 8.00 g L^{-1} of NaCl, 20.00 g L^{-1} of PVP K25 (MW 24000), 10.00 g L^{-1} of PEG, 0.20 g L^{-1} of NaN_3, and 0.50 g L^{-1} of Tween 20) using a tissue homogenizer hand model (Bioreba AG, Kanton Reinach, Switzerland). Leaf extracts were tested by double-antibody sandwich-enzyme-linked immunosorbent assay (DAS-ELISA) with specific antibodies for the following viruses: grapevine fanleaf virus (GFLV), tobacco ringspot virus (TRSV), tomato ringspot virus (ToRSV), grapevine leafroll-associated virus 1 (GLRaV-1), grapevine leafroll-associated virus 2 (GLRaV-2), grapevine leafroll-associated virus 3 (GLRaV-3), and grapevine leafroll-associated virus genetic 4 strains (GLRaV-4 strains) (Bioreba AG, Kanton Reinach, Switzerland), following manufacturing instructions. Each ELISA antibody came with a corresponding positive control and a negative control for each virus, which were used as positive and negative controls on ELISA and reverse transcription polymerase chain reaction (RT-PCR) assays. ELISA plates (96 wells) were analyzed using an Epoch Microplate Spectrophotometer (BioTek, Winooski, VT, USA) for the substrate *para*-Nitrophenylphosphate (pNPP) hydrolysis at 405 nm absorbance after a 60min incubation period at room temperature. Positive samples presented optical density (OD405nm) readings at least twice of healthy controls.

2.3 RT-PCR, PCR, and sequencing

Positive DAS-ELISA samples were subsequently tested by Immunocapture-Reverse-Transcriptase-PCR (IC-RT-PCR) (MULHOLLAND, 2009), using the virus-specific antibodies described above. An ELISA plate (96 wells) was first coated with the specific antibody (Bioreba AG, Kanton Reinach, Switzerland), following manufacturer instruction, and incubated for 2 h at 37 °C. Then, the plate was washed three times with phosphate-buffered saline (PBS) + Tween buffer (8.00 g L^{-1} of NaCl, 0.20g L^{-1} of KH_2PO_4, 1.15 g $L^{-1}$of Na_2HPO_4, 0.20 g L^{-1} of KCl, and 0.50 mL L^{-1} of Tween 20) and 100 µL of plant crude extract were added to each well and incubated overnight at 4 °C. After incubation, the plate was washed three times with PBS + Tween buffer and twice with distilled water. 50 µL of half-strength TE buffer - 25 mL of TE buffer (10 mM of Tris-HCl, 1 mM of disodium EDTA, pH 8.0) and 25 mL of Invitrogen ultraPure DNase⁄RNase-free distilled water (Thermo Fisher Scientific, Waltham, MA, USA) - were added in each well, the plate was sealed with an adhesive PCR plate seal (Thermo Fisher Scientific, Waltham, MA, USA) and incubated at 95 °C for 5 min in a water bath. A reverse transcriptase (RT) reaction was set up using 5 µL of the ELISA solution described above with the SuperScriptTM IV First-Strand Syn- thesis System (Thermo Fisher Scientific, Waltham, MA, USA) using random primers in a final volume of 20 µL, following the manufacturer's protocol. cDNA was generated by incubating the RT reaction tube in a thermocycler for 10 min at 50 °C followed by an enzyme inactivation step at 80 °C for 10min. The cDNA was subjected to PCR using specific primer pairs for each of the following ELISA positive virus, one primer pair per reaction (primer information is available in Supplemental Table 1). PCR reactions were carried out using Phusion Green Hot Start II High-Fidelity PCR Master Mix (Thermo Fisher Scientific, Waltham, MA, USA) with 5 µL of cDNA and 50pmoles of each virus-specific primer in a 50 µL final volume, according to the manufacturer's protocol. The reactions were conducted in a thermocycler using the following protocol: initial denaturation at 95 °C for 30s followed by 30 cycles of 10s denaturation at 95 °C, 30 s at each primer set specific annealing temperature as determine by Tm Calculator (Thermo Fisher Scientific, Waltham, MA, USA), 1 min elongation, with a final extension at 72 °C for 10min. PCR amplicons were checked by gel electrophoresis in 2% agarose gel and visualized under UV light after 30 minutes staining in a gel red solution (Biotium, Fremont, CA, USA). DNA was extracted from the leaf petioles of each composite sample using a DNA kit (Omega Bio-Tek Inc., Norcross, GA, USA) and subjected to

PCR using specific primer sets to detect grapevine red blotch virus (GRBV) (CPfor/CPrev and Repfor/Reprev) (KRENZ et al., 2014). Grapevine leaves infected with GRBV were generously provided by Mark Fuchs from Cornell University and they were used as GRBV positive control in our assays. A 105-bp fragment of the 16S rDNA (primers 16Sfor and 16Srev) was used as an internal control for each PCR and RTR-PCR. Samples containing the expected band size were purified using the ChargeSwitch® kit (Thermo Fisher Scientific, Waltham, MA, USA) and sent to the Keck Lab at Yale University, New Haven - CT, for Sanger sequencing.

2.4 Phylogenetic analysis

The acquired sequences were edited in BioEdit v7.0.5 (HALL, 2011) and submitted to a BLAST search in the NCBI database. For each virus, the sequences from this study and sequences retrieved from GenBank were aligned using MUSCLE with default settings in MEGA 7 (KUMAR et al., 2016). We selected ten of the most closely related sequences from GenBank for each virus species and chose sequences representing different grape grown regions in the world to strength the phylogenetic analysis. A phylogenetic inference was performed using the maximum likelihood method and the general time-reversible model (GTRþGþI) in the model selection test in MEGA 7 with 1000 bootstrap values (KUMAR et al., 2016; NEI; KUMAR, 2000).

3 RESULTS AND DISCUSSION

Twenty-two out of the 62 samples collected, representing seven of the ten vineyards sampled across New England (NE), were infected by at least one of the viruses tested by DAS-ELISA (Table 1). The vineyards' names and locations were omitted to maintain the privacy of the businesses. GLRaV-3 had the highest occurrence, it was found in five vineyards and in 15 samples (24.19%), followed by GLRaV-1, which was found in four vineyards and eight samples (12.90%), and by ToRSV, which was detected in two vineyards and two samples (3.23%). TRSV and GLRaV-2 were detected in only one sample in one vineyard (1.61%). Four samples were infected with multiple viruses and GLRaV-3 was present in all mixed infection samples. A Merlot plant from Rhode Island was infected with four virus species, TRSV, TORSV, GLRaV-1 and GLRaV-3. GFLV and GLRaV-4 were not detected in any of the ELISA-tested samples. All samples tested negative for GRBV by PCR (Table 1).

Table 1. Number of grapevine samples tested by ELISA for several viruses.

Vineyard	State	# Collected Samples	Cultivar	Viruses Tested								Negative
				TRSV	TORSV	GLRaV-1	GLRaV-2	GLRaV-3	GLRaV-4 strains	GFLV	GRBV	
1	CT	3	Cabernet Frank	0	0	0	0	1	0	0	0	2
		1	Cabernet Sauvignon	0	0	0	0	1	0	0	0	0
2	CT	4	Cabernet Sauvignon	0	0	2	0	2	0	0	0	2
		2	Cabernet Frank	0	0	0	0	2	0	0	0	0
		1	Cayuga	0	0	0	0	0	0	0	0	1
		2	Riesling	0	0	0	0	0	0	0	0	2
3	RI	3	Merlot	1	1	1	0	3	0	0	0	0
		1	Cabernet Frank	0	0	0	0	0	0	0	0	1
		1	Riesling	0	0	0	0	0	0	0	0	1
		3	Cabernet Sauvignon	0	0	0	0	0	0	0	0	3
4	CT	1	Cabernet Frank	0	0	0	0	1	0	0	0	0
		1	Merlot	0	0	0	0	1	0	0	0	0
		1	Chambourcin	0	0	0	1	0	0	0	0	0
		3	Marquette	0	0	0	0	0	0	0	0	3
5	CT	1	Traminette	0	0	0	0	0	0	0	0	1
		1	Vidal Blanc	0	0	0	0	0	0	0	0	1
		1	Cabernet Frank	0	0	1	0	0	0	0	0	0
		1	Lemberger	0	0	0	0	0	0	0	0	1
		1	St. Croix	0	0	1	0	0	0	0	0	0
		1	Riesling	0	0	0	0	0	0	0	0	1

6	**CT**	2	Cabernet Frank	0	0	2	0	0	0	0	0	0
		1	St. Croix	0	0	1	0	0	0	0	0	0
7	**CT**	1	Cabernet Frank	0	0	0	0	1	0	0	0	0
		2	Chambourcin	0	0	0	0	2	0	0	0	0
		5	Vidal Blanc	0	0	0	0	1	0	0	0	4
		4	Chardonnay	0	1	0	0	0	0	0	0	3
8	**MA**	1	Cayuga	0	0	0	0	0	0	0	0	1
		1	Riesling	0	0	0	0	0	0	0	0	1
		1	Itasca	0	0	0	0	0	0	0	0	1
9	**MA**	1	Marquette	0	0	0	0	0	0	0	0	1
		1	Tru Disi	0	0	0	0	0	0	0	0	1
		1	Corot Noir	0	0	0	0	0	0	0	0	1
		1	Malvasia Bianca	0	0	0	0	0	0	0	0	1
		1	Noiret	0	0	0	0	0	0	0	0	1
10	**RI**	2	Cabernet Sauvignon	0	0	0	0	0	0	0	0	2
		1	Cayuga	0	0	0	0	0	0	0	0	1
		2	Merlot	0	0	0	0	0	0	0	0	2
		1	Brianna	0	0	0	0	0	0	0	0	1
Total		62		1 (1.61%)	2 (3.23%)	8 (12.90%)	1 (1.61%)	15 (24.19%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	40

CT = Connecticut, RI = Rhode Island, and MA = Massachussetts.

All positive ToRSV, GLRaV-2, and GLRaV-3 DAS-ELISA samples also tested positive by RT-PCR. However, one TRSV and three GLRaV-1 DAS- ELISA samples were not RT-PCR positive. We hypothesize that the discrepancy between ELISA and IC-RT-PCR may be due to mutations on the primer's binding sites that interfere with PCR amplification as observed for GLRaV-3 (THOMPSON et al., 2018). TRSV and GLRaV-1 are RNA viruses that are part of a group referred to as the "masters of mutations" for their high mutation rate. This contributes to great genetic diversity among their populations, which in turn hinders their accurate diagnosis (STERN; ANDINO, 2016). One way to confirm our speculation would be to deep sequence those samples and perform a *de novo* assembly analysis to see if those viruses' genomes could be retrieved from the sequencing data (MASSART et al., 2018); however, such analyses can be cost prohibited and were out of the scope of this research. All RT-PCR amplicons were sequenced (accession numbers are available in Supplemental Table 2) and BLAST searches in the NCBI databases (all sequences were over 99% identical to the corresponding virus sequence on GenBank) corroborated the virus identities found in the DAS-ELISA. Phylogenetic analyses reveal that GLRaV-3 isolates from this study belong to groups I and II (Fig. 1), previously reported by Thompson et al. (2018). Although all samples were collected from symptomatic plants, 64% of the samples tested negative for all viruses analyzed. This indicates that the symptoms observed in those plants may be elicited by other causes such as other viruses or even by physiological issues such as nutritional deficiency.

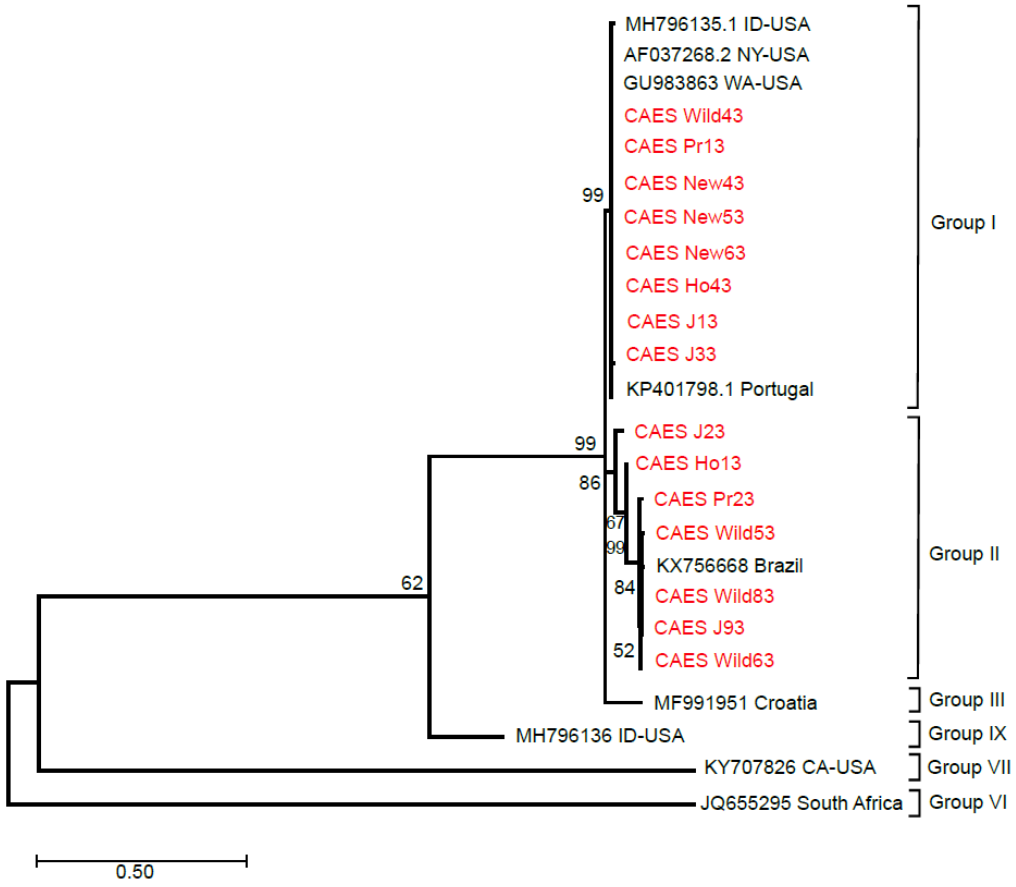

Figure 1. Phylogenetic analysis of the grapevine leafroll associated virus-3 (GLRaV-3) partial coat protein (CP) nucleotide sequences. The tree is unrooted and the maximum likelihood method using the GTR+G+I model was applied. Values at the nodes represent percent bootstrap support at 1000 replications. Isolates sequenced in this study are depicted in red.

The virus isolates detected in this study likely originated from New York (NY) state. According to the growers, all plant material was acquired from nurseries in NY. Furthermore, it appears that this is a case of primary infection due to infected plant material as these viruses, especially GLRaVs, may take up to five years to show symptoms (WILCOX et al., 2015). We did not observe any sign of mealybugs or scale insects, known vectors of GLRaVs, on the bark of the sampled plants after thorough visual inspections. This may be due to the heavy insecticide application regime in NE vineyards. Application occurs at least once a week throughout the high production season to mitigate pest damage on the berries.

4 CONCLUSION

This is the first incidence report of grapevine viruses in the New England region of the USA. These results enforce the need to instate a grapevine testing program in NE to improve the sanitary status of planting material. This will reduce the threat of grapevine viruses on the livelihood of the local grape and wine industry. Meanwhile, growers are advised to continue to monitor and control the GLRaVs' insect vectors, mealybugs, and scale as it is an important disease management strategy to limit virus spread within a vineyard. We strongly encourage growers to test symptomatic plants for known grapevine viruses before their removal. Finally, studies aimed to investigate the extension of the spread, genetic diversity and economic impact of grapevine viruses need to be conducted to provide growers with information for proper management of these viruses in the New England vineyards.

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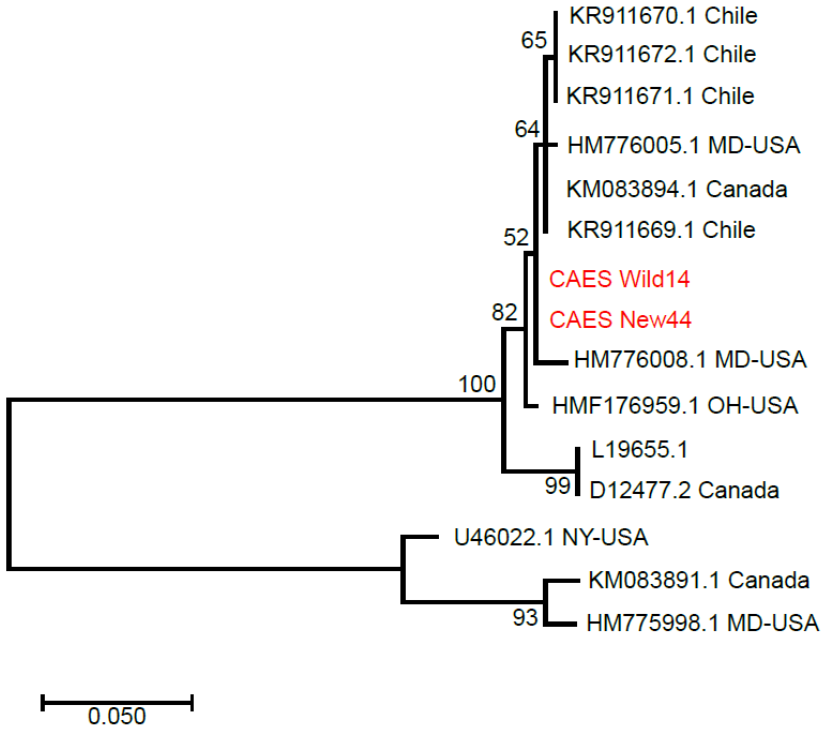
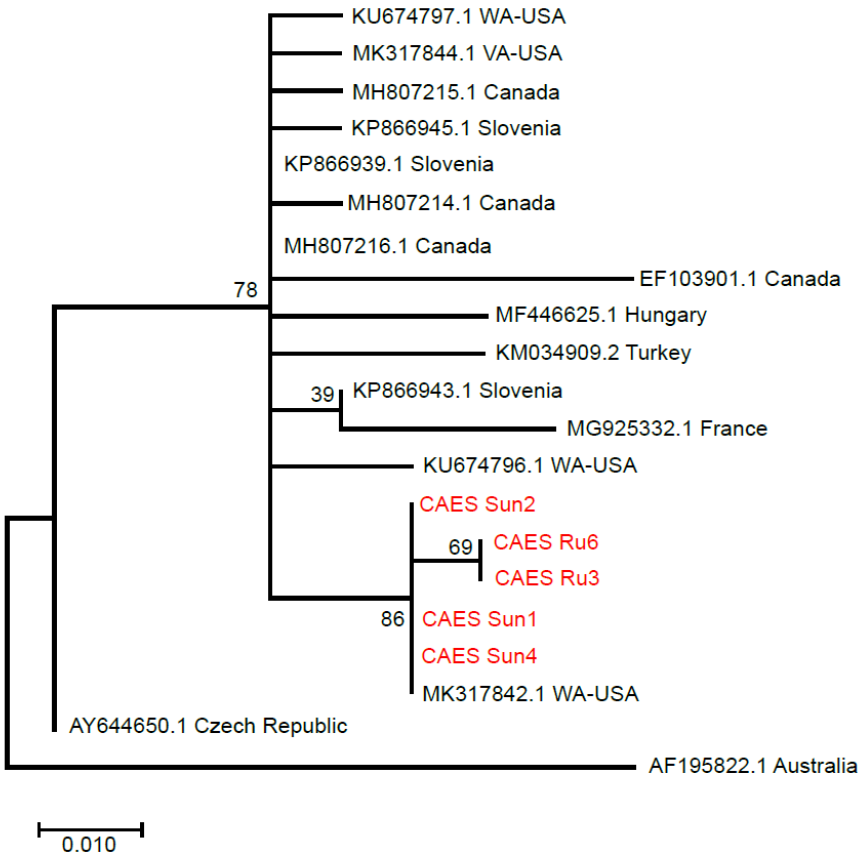
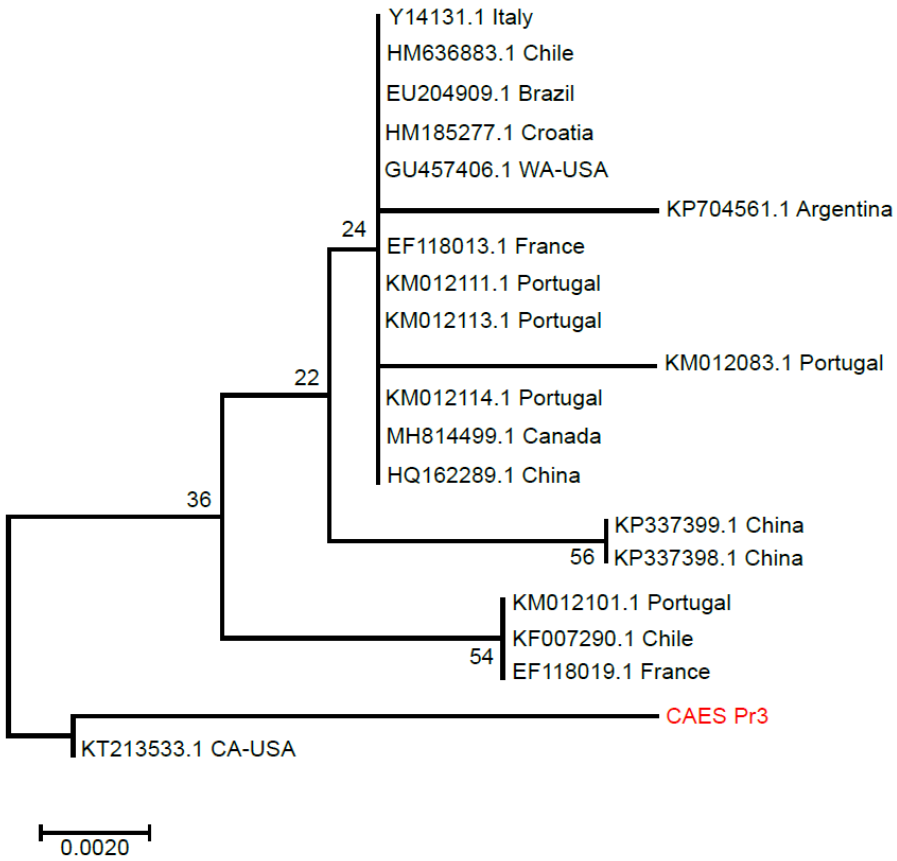
6 APPENDIX



Supplemental Figure 1. Phylogenetic analysis of the tomato ringspot virus (ToRSV) partial 3' untranslated nucleotide sequences. The tree is unrooted and the maximum likelihood method using the GTR+G+I model was applied. Values at the nodes represent percent bootstrap support at 1000 replications. Isolates sequenced in this study are depicted in red.



Supplemental Figure 2. Phylogenetic analysis of the partial heat shock 70-like protein nucleotide sequences (HSP70) of grapevine leafroll associated virus-1 (GLRaV-1). The tree is unrooted and the maximum likelihood method using the GTR+G+I model was applied. Values at the nodes represent percent bootstrap support at 1000 replications. Isolates sequenced in this study are depicted in red.



Supplemental Figure 3. Phylogenetic analysis of the partial coat protein nucleotide sequences of Grapevine leafroll associated virus-2 (GLRaV-2). The tree is unrooted and the maximum likelihood method using the GTR+G+I model was applied. Values at the nodes represent percent bootstrap support at 1000 replications. Isolates sequenced in this study are depicted in red.

Supplemental Table 1. The primers used for RT-PCR or PCR.

Virus	Primer	Sequence	Target	Reference
GLRaV-1	GLRaV-1F GLRaV-1R	5’ - GAGCGACTTGCGACTTATCGA - 3’ 5’ - GGTAAACGGGTGTTCTTCAATTCT - 3’	HSP70	Osman et al., 2006. J. Virol. Methods 133, 130–136
GLRaV-2	L2 F U2 R	5’ - GCCCTCCGCGCAACTAATGACAG - 3’ 5’ - ATAATTCGGCGTACATCCCCACTT - 3’	CP	Bertazzon et al., 2004. Plant Pathol. 86:283, 2004
GLRaV-3	CP-111 F CP-722 R	5’ - AAAGTAGGTTAAGGACGGGACACA - 3’ 5’ - AGGGTCGCCGTGATGAAG - 3’	CP	Osman et al., 2006. J. Virol. Methods 133, 130–136
TRSV	TRSVR-F TRSVR-R	5’ - GAGTGTTGTGCAATTATCTGCATA - 3’ 5’ - CAAAGATGCCAAGAAAAGTTGCAAG - 3’	Polypro tein	J. Han et al. 2014. Plant Dis. 2014 98:2, 284-284
ToRSV	ToRSV5 ToRSV6	5’ - AGGTAGGACGCYATTGTTCCAGG - 3’ 5’ - AGTCTCAACTTAACATACCACTAC - 3’	3’ UTR	Li et al. 2011. Can. J. Plant Pathol., 33:1, 94-99
GRBV	CPfor CPrev	5’ - AGCGGAAGCATGATTGAGACATTGACG - 3’ 5’ – AACGTATGTCCACTTGCAGAAGCCGC - 3’	CP	Krenz et al., 2014. Phytopathology 104, 1232–1240
Plant 16S	16Sfor 16Srev	5’ - TGCTTAACACATGCAAGTCGGA - 3’ 5’ - AGCCGTTTCCAGCTGTTGTTC - 3’	16S	Krenz et al., 2014. Phytopathology 104, 1232–1240

Supplemental Table 2. GenBank Accession Numbers of the partial virus sequences.

Virus	Sequence name	GenBank accession #
ToRSV	CAES_Wild14	MN067889
ToRSV	CAES_New44	MN067890
GLRaV-1	CAES_Ru6	MK643136
GLRaV-1	CAES_Sun1	MK643138
GLRaV-1	CAES_Sun2	MK643139
GLRaV-1	CAES_Sun4	MK643137
GLRaV-2	CAES_Pr3	MK651095
GLRaV-3	CAES_J13	MK651096
GLRaV-3	CAES_J23	MK651097
GLRaV-3	CAES_J33	MK651098
GLRaV-3	CAES_J93	MK651099
GLRaV-3	CAES_New13	MK651100
GLRaV-3	CAES_New23	MK651101
GLRaV-3	CAES_New43	MK651102
GLRaV-3	CAES_Pr13	MK651103
GLRaV-3	CAES_Pr23	MK651104
GLRaV-3	CAES_Wild43	MK651105
GLRaV-3	CAES_Wild53	MK651106
GLRaV-3	CAES_Wild63	MK651110
GLRaV-3	CAES_Wild83	MK651107
GLRaV-3	CAES_Ho13	MK651108
GLRaV-3	CAES_Ho43	MK651109

CHAPTER IV - A FIG DEAL: A GLOBAL LOOK AT FIG MOSAIC DISEASE AND ITS PUTATIVE ASSOCIATES

(Published in Plant Disease: https://doi.org/10.1094/PDIS-06-20-1352-FE)

ABSTRACT

Fig mosaic disease (FMD) is a complex viral disease with which 12 viruses, including a confirmed causal agent - fig mosaic emaravirus (FMV) - and three viroids are associated worldwide. FMD was first described in California in the early 1930s. Symptoms include foliar chlorosis, deformation, and mosaic patterns. FMD is disseminated by vegetative propagation, seed transmission and vectors, including a mite, *Aceria ficus.* Management of the disease in fig orchards relies on scouting and elimination of infected trees. In this review, we focus on the distribution of the FMD-associated viruses and viroids by summarizing worldwide surveys and their genome structure. We also determined the full-length sequence of FMV and fig badnavirus 1 (FBV-1) isolates from Connecticut and compared the virus and viroid sequences from fig isolates. We suggest important areas of research including determining the potential synergistic effect of multiple viruses, elucidating the full-length genome sequence of each associated virus, and relating virus titer to phenotypic changes in *Ficus carica.*

Keywords: Fig. Virus. Viroids. Fig mosaic disease. High throughput sequencing.

1 DESCRIPTION OF FIG MOSAIC DISEASE AND ITS ASSOCIATED CULPRITS

The common fig (*Ficus carica* L.) is native to the Middle East and western Asia. Figs were first domesticated around 11,400 years ago in the lower Jordan Valley (KISLEV et al., 2006). Fig fruits are widely consumed, and fig trees are often used as ornamental plants. Figs are grown worldwide with production surpassing 1 billion tons. The five largest fig fruit producers in the world are Turkey (305,689 tons), Egypt (177,135 tons), Morocco (137,934 tons), Algeria (128,684 tons) and Iran (70,730 tons) (FOOD AND AGRICULTURE ORGANIZATION - FAO 2019). The United States (US) rank as the eighth largest fig producer in the world, approximately 28,300 tons; the majority of U.S. production occurs in California (Fig. 1). Figs are commercially propagated by grafting or self-rooted cuttings; these methods favor the dissemination of various pests and diseases, including the viruses and viroids associated with fig mosaic disease (FMD).

Figure 1. Fig plantations. A) A fig plantation in California in the early 1900s. B) A modern fig plantation in California.

FMD is a major disease affecting fig trees throughout the world. It was first described in California in the early 1930s (CONDIT, 1933). Symptoms include chlorotic and yellowish spots, discoloration, deformation, and mosaic patterns on the leaves and fruit (Figs 2 and 3). Although symptoms have been observed in fig trees for almost a century, the etiological agents associated with FMD have been investigated only within the past decade (ELBEAINO et al., 2006, 2007b, 2009a, 2010). Twelve

viruses and three viroids have been identified and found to be associated with FMD in various fig producing areas of the world (Table 1).

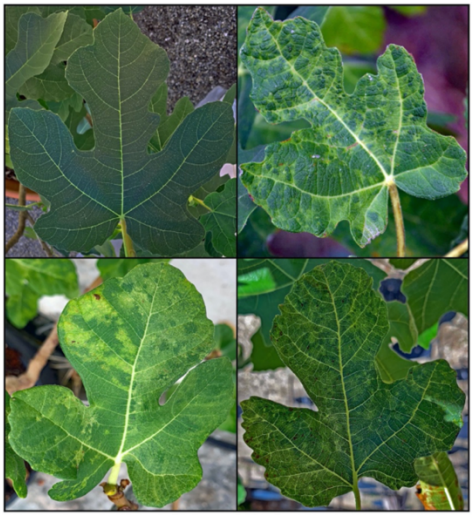


Figure 2. An asymptomatic leaf of Ficus carica (top left), and leaves displaying symptoms of fig mosaic disease (top right, bottom left and right).

Figure 3. Fruits collected from a fig plant displaying fig mosaic disease symptoms.

Table 1. Viruses and viroids associated with fig mosaic disease in fig trees.

Virus/Viroid Species	Genus	Countries	References
Fig leaf mottle-associated virus 1 (FLMaV-1)	*Closterovirus*	Syria, Saudi Arabi, Egypt, Montenegro, Lebanon, Tunisia, Italy, Albania, China	(ALHUDAIB, 2012; ELBEAINO et al., 2012; ELBEAINO et al., 2006; ELBEAINO et al., 2007b; ELBEAINO et al., 2009b; ELBESHEHY; ELBEAINO, 2011; MIJIT et al., 2017; NAHDI et al., 2006; PEROVIĆ et al., 2016).
Fig leaf mottle-associated virus 2 (FLMaV-2)	*Ampelovirus*	Syria, Egypt, Lebanon, Tunisia, Albania, Algeria, Turkey	(CAGLAR et al., 2011; ELBEAINO et al., 2007a; ELBEAINO et al., 2009b; ELBEAINO et al., 2012; ELBESHEHY; ELBEAINO, 2011).
Fig leaf mottle-associated virus 3 (FLMaV-3)	*Closterovirus*	Iran	(NOROZIAN et al., 2014).
Arkansas fig closterovirus-1 (AFCV-1)	*Closterovirus*	United States	(TZANETAKIS et al., 2010).
Arkansas fig closterovirus-2 (AFCV-2)	*Closterovirus*	United States	(TZANETAKIS et al., 2010).
Fig mosaic virus (FMV)	*Emaravirus*	Syria, Iran, Saudi Arabi, Egypt, Montenegro, Japan, China, Turkey	(ALE-AGHA; RAKHSHANDEHROO, 2014; ALHUDAIB, 2012; CAGLAR et al., 201; ELBEAINO et al., 2012; ELBESHEHY; ELBEAINO, 2011; ISHIKAWA et al., 2012; MIJIT et al., 2015; MIJIT et al., 2017; PEROVIĆ et al., 2016; TZANETAKIS et al., 2010).
Fig latent virus 1 (FLV-1)	*Trichovirus*	United States Syria, Tunisia, Saudi Arabia	(EL-AIR et al., 2013; ELBEAINO et al., 2012; ELBESHEHY et al., 2017).

Fig mild mottle-associated virus (FMMaV)	*Closterovirus*	Syria, Egypt, Tunisia, Montenegro, Iran, China	(ALISHIRI et al., 2018a; EL-AIR et al., 2013; ELBEAINO et al., 2012; ELBESHEHY; ELBEAINO, 2011; MIJIT et al., 2017).
Fig cryptic virus (FCV)	*Alphacryptovirus*	Albania, Algeria, Italy, Lebanon, Syria, Tunisia, Iran, Turkey	(ALE-AGHA; RAKHSHANDEHROO, 2014; ELBEAINO et al., 2011b; ELCI et al., 2017).
Fig fleck-associated virus (FFKaV)	*Maculavirus*	Albania, Algeria, Italy, China Lebanon, Syria, Tunisia, Iran, Turkey	(ALE-AGHA; RAKHSHANDEHROO, 2014; ELBEAINO et al., 2011a; ELBEAINO et al., 2012; ELCI et al., 2017; MIJIT et al., 2017).
Fig badnavirus 1 (FBV-1)	*Badnavirus*	United States, Iran, Croatia, China	(ALIMORADIAN et al., 2014; ALISHIRI et al., 2018b; LANEY et al., 2012; MIJIT et al., 2017; TZANETAKIS et al., 2010; VONČINA et al., 2015).
Apple dimple fruit viroid (ADFVd)	*Apscaviroid*	Italy	(CHIUMENTI et al., 2014).
Citrus exocortis viroid (CEVd)	*Pospiviroid*	Tunisia	(YAKOUBI et al., 2007).
Hop stunt viroid (HSVd)	*Hostuviroid*	Tunisia, Syria	(ELBEAINO et al., 2012; YAKOUBI et al., 2007).

Most viruses associated with FMD are transmitted via vegetative propagation of infected plant material. Exceptions are fig latent virus-1 (FLV) and fig cryptic virus (FCV), which are seed transmissible (CASTELLANO et al., 2009; FAUQUET et al., 2005). Vectors of the viruses and viroids associated with FMD include a mite, *Aceria ficus* (FLOCK, 1955), which has been confirmed to transmit FMV - the causative agent

of FMD (ELBEAINO et al., 2009a). The transmission rate of FMV can be as high as 70% (CAGLAYAN et al., 2012).

FMD's spatial dispersion is largely due to the use of infected propagative plant material (MINAFRA et al., 2017). The main form of disease control is prevention, which relies on the production of confirmed pathogen-free plant material. Fig seedlings should be tested to ensure that the plants are virus-free at the time of purchase, before transplantation.

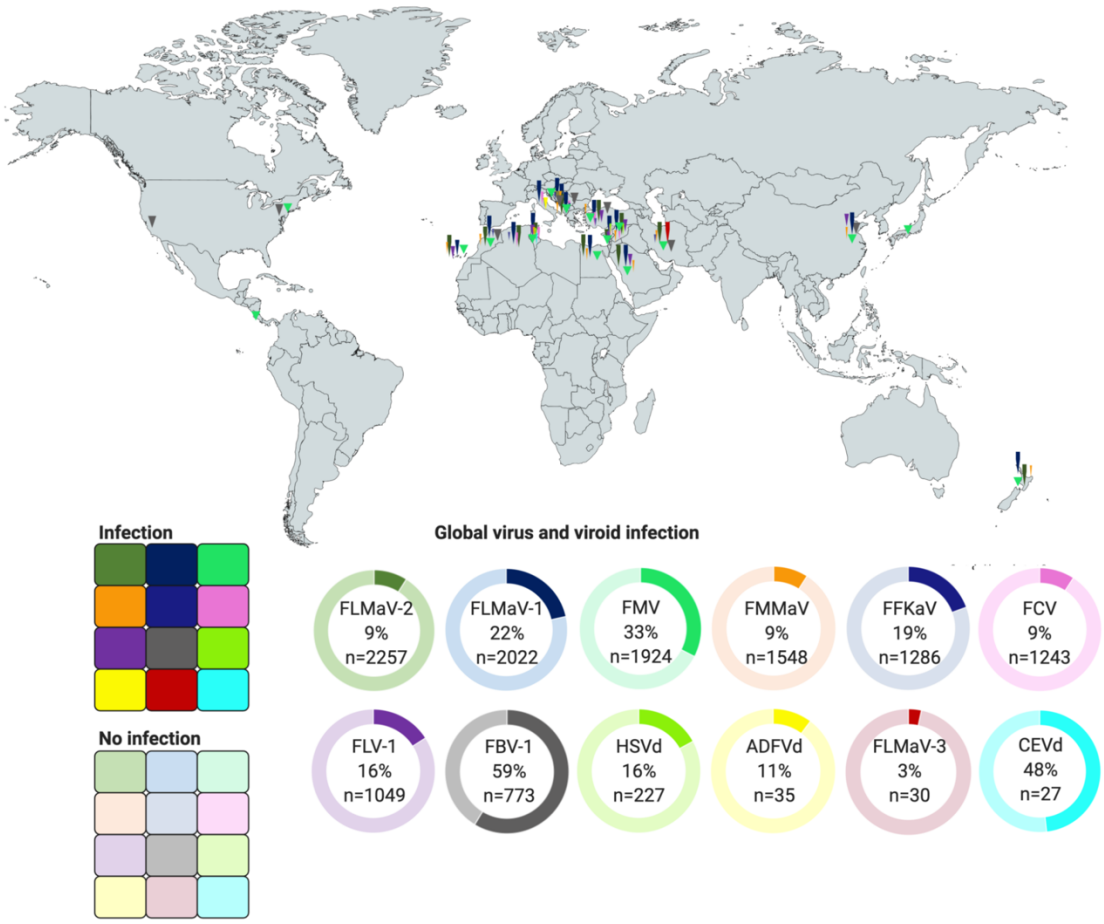


Figure 4. Global distribution of the viruses and viroids associated with fig mosaic disease. Each circular chart depicts the associated pathogen with its percentage of infected samples from the total number of samples (n) tested.

2 VIRUSES AND VIROIDS ASSOCIATED WITH FIG MOSAIC DISEASE

2.1 Fig leaf mottle-associated virus 1 (FLMaV-1)

Fig leaf mottle-associated virus 1 (FLMaV-1) is a (+)ssRNA virus belonging to the genus *Closterovirus*, in the family *Closteroviridae*. It has filamentous particles of 1,800 nm in length, a genome that is approximately 19 kb in length, and it is associated with FMD (ELBEAINO et al., 2006) (Fig. 5). The whole genome of FLMaV-1 has not been sequenced yet, which makes phylogenetic studies difficult to conduct. Two open reading frames (ORFs) have been described on the FLMaV-1 genome. One is a partial ORF of a putative polyprotein, which contains a viral helicase1 domain (pfam01443) (accession number AM279676.1) involved in viral RNA replication. The second ORF is the 70 kilodalton heat shock protein (HSP70) that contains the conserved protein domain family called the nucleotide-binding domain of the sugar kinase/HSP70/actin superfamily (accession number cl17037) (Fig. 5).

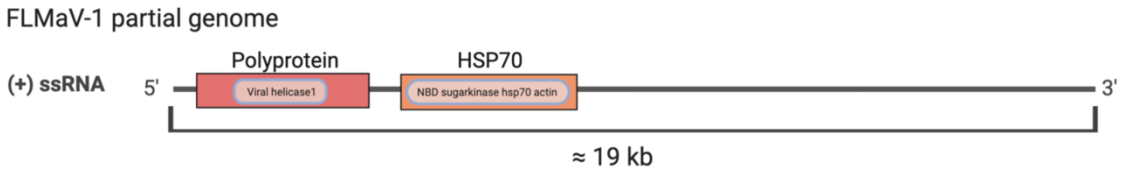


Figure 5. The partial FLMaV-1 genome map from the sequences available in NCBI with the predicted conserved functional units.

The molecular marker for identification of FLMaV-1 is the HSP70 ORF for which a 351 bp amplicon is amplified by RT-PCR. The amplified region contains the phosphate motifs 1 and 2. A high amino acid residue conservation was found in this genomic region when the sequence of multiple isolates of FLMaV-1 were compared (ELBEAINO et al., 2006) (Fig. 6).

Figure 6. The amino acid sequence logo of the FLMaV-1 identification molecular marker, a 351 bp region of the HSP70 gene. The sequence logo displays high conservation among the 10 FLMaV-1 isolates with 100% query over from NCBI used in the analysis (accession numbers: ANB45579.1, ANB45584.1, ANB45588.1, ANB45587.1, ANB45581.1, ANB45583.1, CTQ47930.1, ANB45585.1, ANB45582.1, and ANB45580.1).

FLMaV-1 is vectored by the wax scale insect (*Ceroplastes rusci* L.) (YORGANCI; AÇIKGÖZ, 2019). Several epidemiological studies conducted in various locations where figs are planted commercially confirmed that plants displaying FMD symptoms have a high rate of FLMaV-1 infection. In Apulia (Southern Italy) and in Tunisia, 64.9% and 28.8% of the samples tested positive for FLMaV-1, respectively (ELBEAINO et al., 2006; NAHDI et al., 2006). A survey conducted during the spring of 2016 found that 51% (n= 49) of the samples collected in Bosnia and Herzegovina were positive for FLMaV-1, while in Montenegro 57% (n= 35) of tested samples were positive (DELIĆ et al., 2017). In China, 5.6% (n = 252) of the samples tested were positive for FLMaV-1 (MIJIT et al., 2017). Globally, a literature search concluded that 22% (n = 435) of the 2,022 samples of symptomatic and asymptomatic fig plants tested positive for FLMaV-1 (Fig. 1). However, studies need to be conducted to investigate the genetic makeup of the virus, sequence its whole genome, elucidate its impact on fig production worldwide, and establish efficient virus control strategies.

2.2 Fig leaf mottle-associated virus 2 (FLMaV-2)

Fig leaf mottle-associated virus 2 (FLMaV-2) belongs to the genus *Ampelovirus* in the family *Closteroviridae.* Its virions are approximately 2,100 nm in length and 12

nm in diameter with a (+)ssRNA genome of approximately 19 kb in size (ELBEAINO et al., 2007a). Six ORFs have been described in the FLMaV-2 genome, but the full genome has yet to be sequenced (Fig. 7). The first ORF codes for a putative coat protein duplicate containing a conserved domain from a Closter Coat superfamily (accession number cl03354), which is present in several members of the family Closteroviridae. BLAST analysis of the first ORF sequence revealed no FLMAV-2 homologs, with the closest alignment having 41% identity with the coat protein of little cherry virus 2. The second ORF has no putative function or conserved domains. The third ORF codes for a HSP70 protein with an HSP70_NBD domain (accession number cd10170). The fourth ORF contains a P55 protein and a viral_Hsp90 domain (accession number cl20248). The fifth ORF, p22, has no putative function or domain discovered yet. The sixth ORF is predicted to be the coat protein, with the conserved domain Closter coat (accession number cl03354). There are currently sequences from 28 isolates available in NCBI and the majority of the sequences are from the HSP70 gene, the molecular marker for the identification of FLMaV-2. The sequence of isolate FAR-1 (MG4075556.1) was submitted as a variant of FLMaV-2; however, based on a 97% sequence similarity to FLMaV-1 isolates, it is most likely a member of the genus *Closterovirus* rather than the genus *Ampelovirus*.

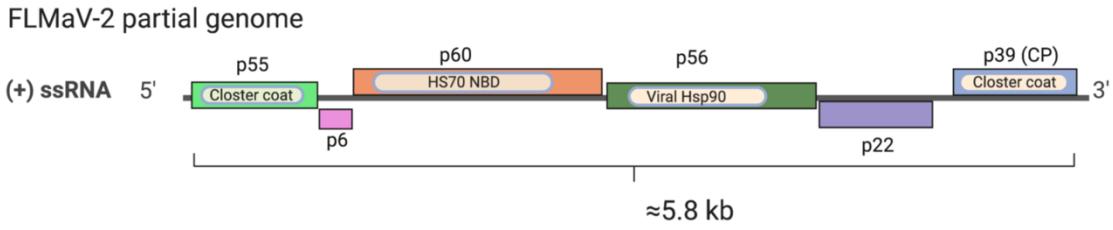


Figure 7. Partial FLMaV-2 genome map from the most complete partial sequence in NCBI (FJ473383.1) with the predicted conserved functional units.

Surveys of FLMaV-2 indicate geographic variation in the occurrence and spread of this virus. In Montenegro, no positive samples (n= 35) were observed, whereas in Bosnia and Herzegovina only 2% of the samples (n= 49) tested positive for FLMaV-2 (DELIĆ et al., 2017). In Lebanon, the occurrence of this virus was up to 24.9% (n= 102) in fig plantations (ELBEAINO et al., 2007a), and 31.1% in Syria (ELBEAINO et al., 2012). In Turkey, only 4.5% of the samples (n= 132) tested were positive for FLMaV-2 (CAGLAR et al., 2011). Globally, approximately 2,257 samples were tested for the

presence of FLMaV-2 with 202 positive samples (9%) (Fig. 4). The occurrence and distribution of FLMaV-2 have been studied primarily in the Mediterranean region, which begs the question of the presence of this virus in other fig producing areas of the world.

2.3 Fig leaf mottle-associated virus 3 (FLMaV-3)

Fig leaf mottle-associated virus 3 (FLMaV-3) is a putative member of the genus *Closterovirus*, in the family *Closteroviridae,* and is associated with FMD (ELÇI et al., 2012; NOROZIAN et al., 2014). FLMaV-3 has a linear (+)ssRNA genome; however, its whole genome has not been sequenced yet (Fig. 8), and there is currently no prediction on genome size. Only four partial sequences are available in GenBank from the heat shock protein 70-like gene (HSP70h): one sequence from the USA (EF654103.1) (TZANETAKIS et al., 2010) and three sequences from Iran (MG407557.1, KM516761.1 and KM516760.1) (NOROZIAN et al., 2014). Each of the HSP70h proteins contains an NBD sugar kinase HSP70 actin domain, which is highly conserved among the four isolates (Fig. 9).

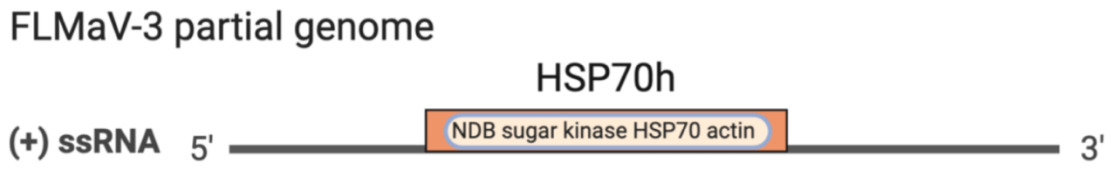


Figure 8. Partial FLMaV-3 genome map drawn from the sequences available in NCBI with the predicted conserved functional unit.

Figure 9. A sequence logo of the partial HSP70h protein from the four FLMaV-3 isolates available in GenBank (accession numbers: EF654103.1, MG407557.1, KM516761.1, and KM516760.1). The sequence logo shows high amino acid conservation among the isolates.

A survey conducted in Iran found only one positive sample out of 20 tested (NOROZIAN et al., 2014). Globally, testing for FLMaV-3 has been extremely limited (n= 30) with only one positive sample (3%). Further studies are essential to sequence and annotate the full genome of FLMaV-3, identify vectors, determine global spread, and gauge the severity of damage caused by FLMaV-3.

2.4 Arkansas fig closterovirus-1 (AFCV-1) and Arkansas fig closterovirus-2 (AFCV-2)

Two new viruses, Arkansas fig closterovirus-1 (AFCV-1) and Arkansas fig closterovirus-2 (AFCV-2), were found to be associated with FMD. They were first reported in 2008 from the state of Arkansas, USA (TZANETAKIS et al., 2010). AFCV-1 and AFCV-2 are (+)ssRNA viruses that have tentatively been proposed to belong to the genus *Closterovirus* and their occurrence has been reported only in the USA. The isolates of AFCV-1 remain unverified in GenBank and there are currently no sequences available for AFCV-2, although diagnostic primers are available (TZANETAKIS et al., 2010).

BLAST analysis of the sequence of AFCV-1 isolate YN4 (JN882590.1) shows 97.39%, 95.42%, 95.42% and 90.01% sequence identity to four FLMaV-3 isolates (accession numbers: EF654103.1, MG407557.1, KM516761.1, and KM516760.1) (Fig. 10). There are no conserved domain calls for any of the AFCV-1 isolates, or for the aligned region of FLMaV-3 isolates (Fig. 10). Furthermore, after carefully checking the detection primers (AFCV-1F: 5'- CTGTAATCTGTCACCTTCGGG-3' and AFCV1R: 5'-ATGCTTCCTCGGCTGC-3') for the putative AFCV- 1, we found that they align on the HSP70h region of the four FLMaV-3 isolate sequences available in NCBI. One wonders if AFCV-1 is actually FLMaV-3 and if AFCV-2 could be a putative variant of FLMaV-3 instead of AFCV-1 although the latter hypothesis is difficult to confirm since no sequences of AFCV-2 are available in NCBI. Without more quantitative and qualitative data, it is difficult to be sure of the true nature of AFCV-1 and AFCV-2; however, we hypothesize that they are members of FLMaV-3. Therefore, we omitted AFCV-1 and AFCV-2 from the global study due to a lack of information on these two viruses.

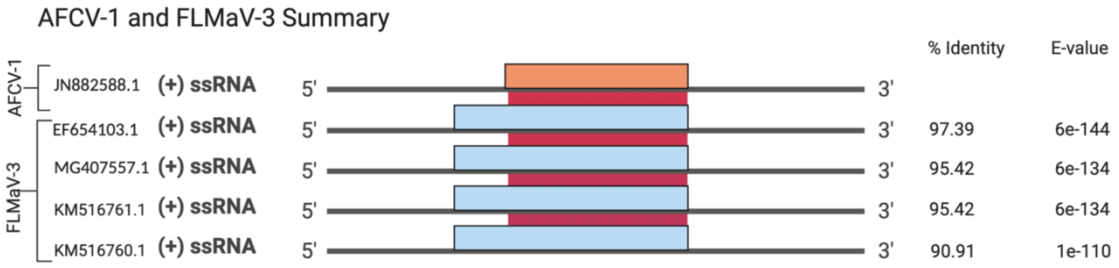


Figure 10. Depiction of the partial genome of AFCV-1 compared to the genome of FLMaV-3 isolates available in NCBI.

More studies need to be conducted to corroborate the hypothesis that AFCV-1 is actually FLMaV-3. Also, if AFCV-1 and AFCV-2 are proved to be independent species, it is unlikely that these viruses occur only in the United States. Currently, only one study has identified AFCV-1 and AFCV-2 (TZANETAKIS et al., 2010) and it is imperative to perform surveys in other fig producing countries to acquire more epidemiological and biological information on these viruses. This information would contribute to the development of possible control methods, document the geographic range of the viruses, confirm pathogenicity and quantify the economic losses they cause.

2.5 Fig mosaic emaravirus virus (FMV)

Fig mosaic emaravirus virus (FMV), also known as fig mosaic virus, is a (-)ssRNA virus and a member of the *Emaravirus* genus, in the family *Fimoviridae*. The genome is multipartite (contains six RNA segments). Each RNA segment has a single ORF with a 5' and a 3' end, and each is individually encapsulated in a separate virus capsid (WALIA et al., 2014) (Fig. 11). RNA1 is 7,039 bp long, and codes for the RNA dependent RNA polymerase (RdRp). This ORF is used as a molecular marker for diagnoses by RT-PCR and for phylogenetic studies. There are five conserved motifs (A-E) used for virus identification toward the 3' end of the genome. RNA2 is 2,252 bp in length and codes for a putative glycoprotein. RNA3 is approximately 1,490 bp long that putatively codes for the nucleocapsid protein (NP). RNA4 is approximately 1,452 bp long that presumably codes for a movement protein as it contains an emaravirus P4 movement domain (accession number cl24917). No information is available on the functions or the proteins coded by RNA5 (1,752 bp) and RNA6 (1,212 bp).

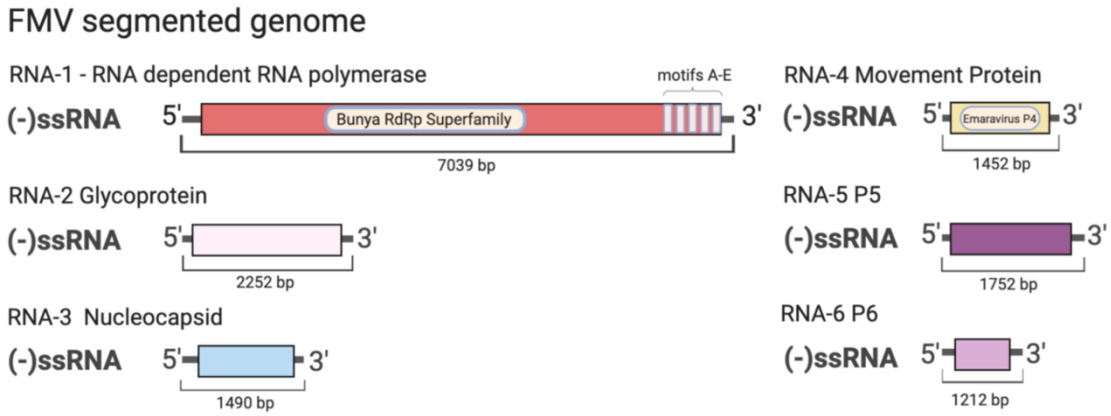


Figure 11. The complete FMV genome map containing six RNA segments. The predicted conserved functional units for RNA1 and RNA4 are highlighted inside the boxes depicting the segments.

We used Illumina high throughput technology (HTS) to sequence the total RNA from an FMD symptomatic fig leaf sample isolated from a Connecticut orchard (Fig. 2). The ribosome RNA depletion method was used for library preparation (NEB, Ipswich, MA-USA). A consensus whole genome sequence of FMV isolate CAES was extracted from the HTS dataset (150 bp reads) using established bioinformatics tools (VILLAMOR et al., 2019). The CAES isolate contains the six (-)ssRNA segments. BLAST analysis of the RNA-dependent RNA-polymerase CAES sequence showed

>97%, 94.92%, and 92.17% nucleotide identity with 100% query cover to other FMV isolates deposited in GenBank.

FMV is transmitted by grafting and vectored by an eriophyid mite, *Aceria ficus* (CAGLAYAN et al., 2012; FLOCK, 1955). Among all viruses associated with fig mosaic disease, FMV is the only virus identified to be an etiological agent of FMD (ELBEAINO et al., 2009a). Recently, antibodies were developed against the nucleocapsid protein (NP) of FMV (SHAHMIRZAIE et al., 2019), an important step on the development of an immunoassay for a rapid and cost-efficient diagnosis of this virus. Furthermore, FMV was found naturally infecting Persian cyclamen (*Cyclamen persicum* Mill.) (ELBEAINO et al., 2018), which is the only non-fig host found for this virus - adding more complexity to the management of this important fig virus.

Surveys indicated that FMV is one of the most commonly found viruses infecting fig plants in the world. However, the percentage of FMV infected plants varies drastically by region: 43% (n= 21) in Montenegro (PEROVIĆ et al., 2016), 56.7% (n= 90) in Syria (ELBEAINO et al., 2012), 87% (n= 30) in Croatia (VONČINA et al., 2015), 7.6% (n= 132) in Turkey (CAGLAR et al., 2011). In China, 44.4% (n= 252) plants tested positive for FMV (MIJIT et al., 2017). Globally, approximately 1,924 samples have been tested for FMV and 627 (33%) tested positive for FMV. Future studies should investigate global genome diversity of FMV isolates, vector transmission and possible management strategies.

2.6 Fig latent virus 1 (FLV-1)

Fig latent virus 1 (FLV-1) is a (+)ssRNA virus with 700 nm particles in size and an 8 kb genome composed of four ORFs. It is a member of the genus *Trichovirus*, in the family *Betaflexiviridae* (ELBESHEHY et al., 2017; GATTONI et al., 2009) (Fig. 12). The whole genome of FLV- 1 is yet to be sequenced, but there are four partial genome sequences available in NCBI (accession numbers: KM156763.1, KM156762.1, FN377573.1 and MG407553.1). ORF1 is approximately 4,264 bp in length and it harbors three domains: a 20 G Fe II Oxygenase (accession number pfam03171), a viral helicase 1 with NTPase activity (accession number cl26263), and an RdRp 2 superfamily (accession number cl03049). ORF2 is approximately 1,144 bp and codes for a putative movement protein as it contains a viral movement protein domain (accession number cl03100). According to NCBI's conserved domain search tool, ORF 2's protein domain is critical in the early stages of plant virus infection. ORF3 is

approximately 1,236 bp in length and codes for the coat protein, which is the molecular marker used for FLV-1 identification. It contains the domain superfamily Tricho coat (accession number cl05455). ORF4 is approximately 348 bp in length and codes for a putative nucleic acid-binding protein. It harbors the domain Carla C4 that includes a motif for a C-4 zinc finger with four conserved cysteine residues (accession number cl03285).

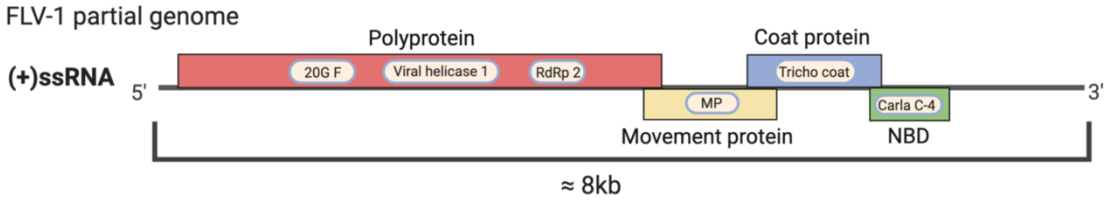


Figure 12. Partial FLV-1 genome depicting the four gene sequences available in NCBI (accession numbers: KM156763.1, KM156762.1, FN377573.1, and MG407553.1).

FLV-1 has an unusually high seed transmission rate (92%) in comparison with other seed-transmitted plant viruses, which usually have a transmission rate of less than 10% (CASTELLANO et al., 2009). Surveys in fig plantations in Syria revealed that 11.1% (n= 90) of tested plants were infected by FLV-1 (ELBEAINO et al., 2012). In Tunisia, 44% (n= 85) of the samples tested positive for FLV-1 (EL-AIR et al., 2013); while in Saudi Arabia the percentage was >33% (n= 60) (ELBESHEHY et al., 2017). Globally, approximately 1,049 samples have been tested for FLV-1 with 168 positive samples (16%). It is important to note that this virus has also been detected in FMD-asymptomatic plants. Disease management should consider testing of asymptomatic plants as they could be overlooked as source of inoculum for this virus.

2.7 Fig mild mottle-associated virus (FMMaV)

Fig mild mottle-associated virus (FMMaV) is a (+)ssRNA virus and a putative member of the genus *Closterovirus*, in the Family *Closteroviridae* (ELBEAINO et al., 2010). Only 26 partial sequences of FMMaV have been deposited in GenBank, encompassing seven ORFs (accession number FJ611959) (Fig. 13). The full-length genome size has yet to the deciphered and the 5' and the 3' ends have not been sequenced yet. ORF1 codes for a putative RdRp, with a domain for an RdRp 2 superfamily (pfam cl03049). No putative function or domain calls have been found for ORF2 and ORF3. ORF4 codes for an HSP70 protein with an NDB sugar kinase domain (pfam cd10170). ORF5 codes for a p64 protein with a viral Hsp90 domain (accession

number cl20248). ORF6 codes for a putative minor coat protein (CPm), and ORF7 codes for a putative coat protein (CP); they both contain the Clostercoat coat domain (pfam cl03354).

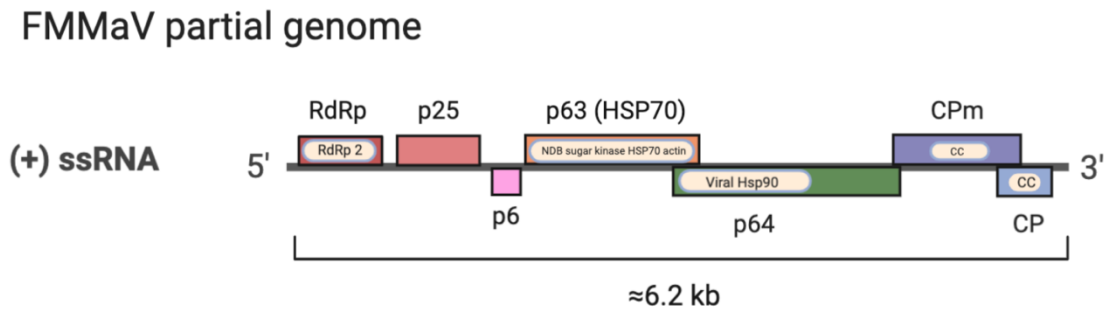


Figure 13. FMMaV genome map containing the largest partial sequence available in NCBI (FJ611959).

Surveys of the fig germplasm collection from the High Agronomic Institute (I.S.A) in Tunisia revealed that 17.25% (n= 29) of the tested plants were infected with FMMaV (BAYOUDH et al., 2017). In Iran, 11% of the samples tested (n= 44) were positive for FFMaV (ALISHIRI et al., 2018a); in Syria, 12.2% of the samples tested (n= 90) were positive (ELBEAINO et al., 2012); and 10% in Montenegro (n= 21) (PEROVIĆ et al., 2016). The incidence of FMMaV was relatively low in China, where only 0.4% of the samples were positive (n= 252) (MIJIT et al., 2017). Globally, approximately 1,548 samples have been tested and 140 of them (9%) were positive for FMMaV. Furthermore, studies on the occurrence of FMMaV have been limited to only areas where FMD has been reported.

2.8 Fig cryptic virus (FCV)

Fig cryptic virus (FCV) is a dsRNA bipartite genome virus. The RNA1 segment is approximately 1,696 bp and codes for the RdRp protein with an RT-like domain (accession number cl02808). The RNA2 segment is approximately 1,415 bp and codes for the coat protein (CP) (ELCI et al., 2017) (Fig. 14). FCV is the first member of the genus *Alphacryptovirus*, in the family *Partitiviridae,* to be detected in fig trees, it was first reported in fig trees in Italy in 2011. Although FCV is associated with FMD, it does not induce symptoms in fig trees, and it is neither graft nor mechanically transmitted (ELBEAINO et al., 2011b).

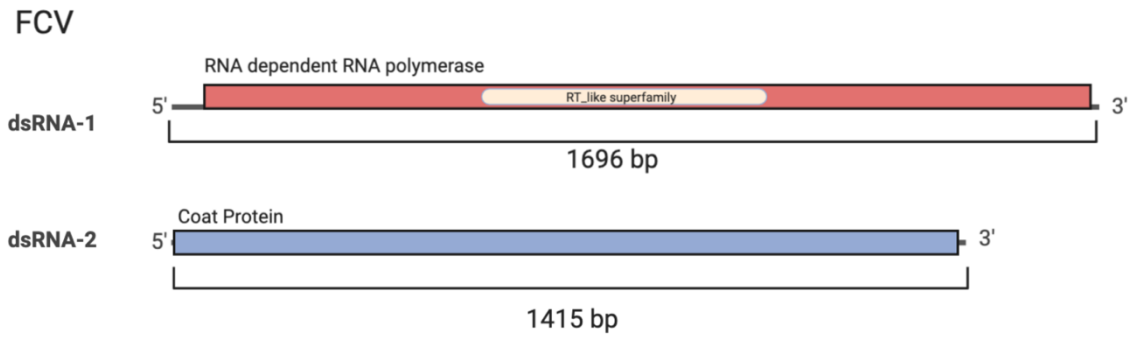


Figure 14. Complete FCV genome map containing the reference sequences NC_015494 and NC_015495, dsRNA-1 and dsRNA-2, respectively.

A survey conducted in the Mediterranean region revealed that 18.5% of the samples tested (n= 210) were positive for FCV (ELBEAINO et al., 2011b). The highest occurrence of FCV was reported in Lebanon at 23.3% (n= 60). In Turkey, 20% of the tested plants (n= 65) were positive for FCV (ELCI et al., 2017); while in Iran, the incidence was only 4.5% (n= 197) (ALE-AGHA; RAKHSHANDEHROO, 2014). FCV does not appear to induce noticeable symptoms in fig plants. However, all plant material should be tested for FCV before planting to avoid the introduction of this virus in newly planted areas.

2.9 Fig fleck-associated virus (FFKaV)

Fig fleck-associated virus (FFKaV) is a (+)ssRNA virus, and is a member of the genus *Maculavirus* in the family *Tymoviridae* (Fig. 15). It was first reported in Apulia (southern Italy) in 2011 (ELBEAINO et al., 2011a).

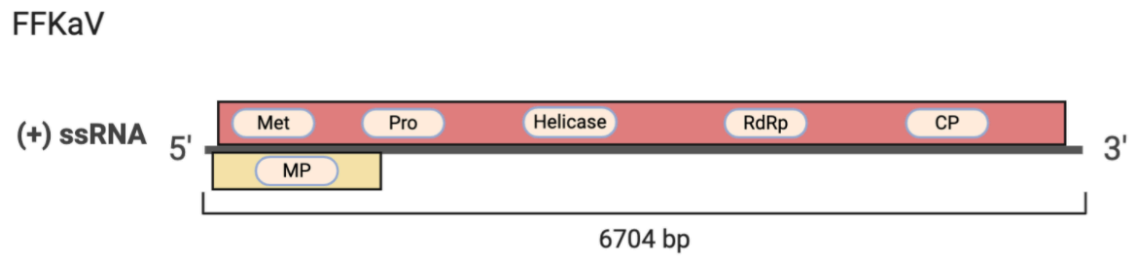


Figure 15. An FFKaV genome map from NCBI sequences and from Elbeaino et al. (2011a).

Surveys conducted in Albania, Algeria, Italy, Lebanon, Syria, and Tunisia revealed that 20% of the samples (n= 210) tested positive for FFKaV (ELBEAINO et al., 2011a). In Syria, 36.7% (n= 90) collected in nine different cities were positive for

FFKaV (ELBEAINO et al., 2012). The occurrence of this virus was much lower in Turkey, 9.2% (n= 65) of the samples were positive for FFKaV (ELCI et al., 2017). Similarly in Iran, 8.6% tested positive (n= 197) (ALE-AGHA; RAKHSHANDEHROO, 2014). In contrast, a relatively high rate of infection was found in China, 44% positive samples (n= 252) for FFKaV (MIJIT et al., 2017). Globally, approximately 1,286 published samples have been tested for FFKaV, with 250 (19%) positive samples detected.

Blast searches of the NCBI database revealed that a variant of this virus was discovered in China, named fig fleck-associated virus 2 (accession numbers: KT438719.1, KT438721.1, KT438722.1, KT438723.1, and KT438724.1); however, no description of this virus was found in the literature. Therefore, more research is needed to describe this virus further and determine if it is indeed a new species or a new strain of FFkaV. Furthermore, there is a need to verify if these viruses are associated with FMD and to what extent, if any, they can be detrimental to fig production.

2.10 Fig badnavirus 1 (FBV-1)

Fig badnavirus 1 (FBV-1) belongs to the genus *Badnavirus,* family *Caulimoviridae*; it is a dsDNA virus with a genome of 7,141 bp. The genome is monopartite, open circular, and harbors three ORFs with a 3 bp overlap on each reading frame (Fig. 16). Eight complete genome sequences for different FBV-1 isolates are available in GenBank. One of these isolates (accession number MK348055.1) was called to have a putative ORF4 (ALISHIRI et al., 2018b). Accordingly, the putative ORF4 was found in the one of the FBV-1 isolates in GenBank.

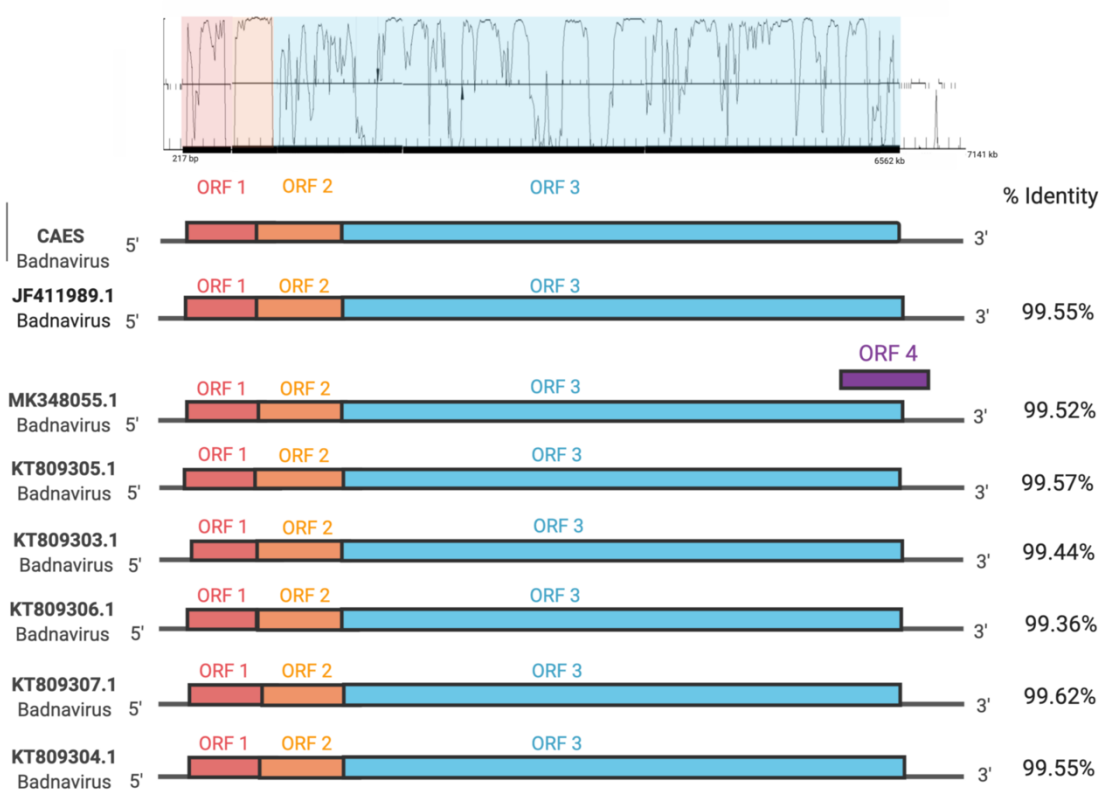


Figure 16. A comparison of the genomes of the CAES_FBV-1 isolate with the full genome FBV-1 isolates available in NCBI. The purple box depicts the ORF4 called for isolate MK348055.1, which our evidence suggests does not exist but was rather an annotation error.

However, in GenBank, the genomes of all the other seven isolates cited in the paper were published without the putative ORF4. To shed light on this discrepancy, we extracted total RNA from an FMD symptomatic fig plant leaf collected in a Connecticut vineyard in October 2019 and processed it for high throughput sequencing (HTS) via Illumina platform using the ribosome RNA depletion library preparation method (NEB, Ipswich, MA-USA). A consensus whole genome sequence of FBV-1 was extracted from the HTS dataset (150 bp reads) using well-established bioinformatics tools (VILLAMOR et al., 2019). The CAES isolate was 7,141 bp long, containing a short 5' end of 217 bp, and a longer 3' end of 579 bp. BLAST analysis of the CAES sequence showed over 99% nucleotide identity to the other eight isolate sequences available in the NCBI database (Fig. 16). Only three ORFs were detected on the FBV-1_CAES genome after running Genemark S, a gene prediction algorithm. The same analyses were also run on the genomes from the other eight FBV-1 isolates, and only 3 ORFs were detected on those genomes. Based on all this evidence and the extremely

conserved nature of the FBV-1 genome (Fig. 16), it is likely that ORF4 does not exist, but it may be part of the large ORF3 polyprotein. We conclude that the ORF4 calling was an error caused by mis-annotation and that the FBV-1 genome contains only 3 ORFs, comparable to most badnaviruses species (BHAT et al., 2016).

FBV-1 was first reported in 2008 in the USA (TZANETAKIS et al., 2010). It was later demonstrated that, besides replicating, FBV-1 integrates into the fig genome. It is found infecting both asymptomatic and symptomatic fig trees (LANEY et al., 2012). FBV-1 is widespread in fig producing regions and it was found to be prevalent in the National Germplasm Repository (NCGR) in Davis, California, USA (LANEY et al., 2012). In Croatia, 30 samples were tested and all were positive for FBV-1 (VONČINA et al., 2015). In Iran, 66.6% of samples tested positive (n= 92) (ALIMORADIAN et al., 2016). In China, 48% fig samples (n= 525) assayed were positive for FBV-1 (MIJIT et al., 2017). Although widespread, globally only 773 fig samples have been tested for FBV-1 with 454 (59%) positive for the virus.

FBV-1 is one of the most common viruses found in fig trees because it integrates the fig genome and it can be detected in asymptomatic fig trees. This poses the question of whether FBV-1 causes symptoms of FMD or if it is just coincidentally found in plants displaying FMD. More research needs to be conducted to address this point and also to develop ways of generating fig planting material that is free of FBV-1, as it was detected in every commercially available fig plant, meristem tip cultured plants, and fig seedlings tested in the USA (LANEY et al., 2012).

2.11 Apple dimple fruit viroid (ADFVd)

Apple dimple fruit viroid (ADFVd), genus *Apscaviroid*, family Pospiviroidae, is a viroid. Viroids are the smallest plant disease-causing agents. They have a single-stranded, circular and non-protein-coding RNA pathogen of only 246-401 nucleotides in size (NAVARRO et al., 2012). ADFVd is 306 nucleotides long; it was first identified in the “Starking Delicious” apple cultivar in Italy in 1996 (DI SERIO et al., 1996). Then in 2012, also in Italy, it was found in FMD symptomatic fig trees (CHIUMENTI et al., 2014). Viroids are known to occur at extremely low titer (number of infectious units per amount of sample) in their hosts (NAVARRO et al., 2012), which often results in false-negative RT-PCR results. Therefore, high throughput sequencing (HTS) to characterize viroid-derived small RNAs of 21-24 nt (vd-sRNAs) has been established as a powerful tool for the detection and characterization of viroids. Even though ADFVd was detected

in fig trees displaying FMD symptoms (CHIUMENTI et al., 2014), it accumulates at low levels in figs, and more research is needed to elucidate the role of this viroid in causing disease in fig plants. Other FMD associated viruses (FMV, FLV-1, and FBV-1) were also detected in the same samples that were positive for ADFVd (CHIUMENTI et al., 2014).

2.12 Citrus exocortis viroid (CEVd)

Citrus exocortis viroid (CEVd) is a member of the genus *Pospiviroid*, in the family *Pospiviroidae*. It was identified as the causal agent of bark shelling symptoms in citrus in the 1940s (BENTON et al., 1950). Since then it has been isolated from several plant hosts (*e.g.*, members of the Solanaceae family, and a range of other vegetable crops) (SZYCHOWSKI et al., 2005). In 2007, a study in Tunisia reported the first occurrence of CEVd associated with FMD (YAKOUBI et al., 2007) - CEVd was detected in every FMD-symptomatic plant tested (n= 13), but it was not detected in any of 14 asymptomatic plants tested. The occurrence of CEVd in FMD- symptomatic plants does not guarantee that this viroid causes FMD. Additional studies are necessary to dissect the role of CEVd in the occurrence of FMD.

2.13 Hop stunt viroid (HSVd)

Hop stunt viroid (HSVd) is a member of the genus *Hostuviroid* in the family *Pospiviroidae* (ASTRUC et al., 1996; DI SERIO et al., 2014). HSVd has the broadest host range know for a viroid - infecting plants such as hop, cucumber, grapevine, citrus, plum, peach, pear and apricot. In 2007, it was detected in fig trees displaying FMD symptoms in Tunisia (YAKOUBI et al., 2007). As for CEVd, all 13 FMD-symptomatic plants tested were positive for HSVd and none of the 14 asymptomatic plants tested positive for this viroid. Furthermore, in Syria, HSVd was identified in 13.3% of sampled trees (n= 90) (ELBEAINO et al., 2012). Of the three viroids detected in FMD-symptomatic plants, HSVd is the most consistently associated with this disease. Globally, approximately 227 samples have been tested for HSVd, with 37 (16%) testing positive for this viroid. However, there are no studies on pathogenicity or effects that this viroid may have on fig orchards.

3 CONCLUDING REMARKS AND FUTURE PROSPECTS

Fig mosaic disease (FMD) affects fig trees worldwide, causing economic loss and posing a serious threat to fig production. Since its discovery, 12 viruses, including a confirmed causal agent (fig mosaic virus - FMV), and three viroids have been detected

in fig trees display FMD symptoms (ELBEAINO et al., 2009a). More research into the epidemiology of these viruses and viroids is needed to determine whether they are truly associated with FMD, are synergistic with FMV, or are just part of the plant's virome without association of this disease complex. As high throughput sequencing continues to generate viral metagenomic data sets, new viruses and viroids that may not have an etiological association with figs will be uncovered. It is important to perform thorough testing with all the viruses associated with FMD to elucidate disease-causing viruses and target efforts and resources of FMD management toward the viruses and viroids that are causing crop damage.

The genomes of most of the associated viruses/viroids remain to be fully sequenced, with some isolates containing only partial ORFs published in GenBank. While the sequences of most viruses have highly conserved regions that help in making inferences on the genus and species, whole genome sequencing of several isolates of the same species in different areas of the world would be highly beneficial for phylogenetic and mutational analysis studies. Those analyses would help to pinpoint the geographic origin and evolutionary dynamics of those pathogens, thereby supporting and validating epidemiological and surveillance methods designed to mitigate the spread of these viruses and viroids in fig plantations.

Combining the data from published FMD studies begins to paint a global picture of the distribution of the viruses and viroids associated with FMD over the last 15 years. From the data collected, FLMaV-2, FLMaV-1, and FMV have been tested most frequently (n>1,900). FLV- 1, FMMaV, FFKaV, FCV and FBV-1 have had between 700-1,500 samples tested. However, FLMaV-3, ADFVd, HSVd, and CEVd have had only 30-227 samples tested globally. AFCV-1 and AFCV-2 were not included in this portion of the review because AFCV-1 is most likely a variant of FLMaV-3 and not a novel virus infecting fig. Although primers for RT-PCR assays exist, AFCV-2 sequences are not available in the NCBI database. Properly designed epidemiology studies will provide a new understanding of FMD and aim detection efforts in the direction of the viruses and viroids that cause this disease complex.

This review will facilitate future studies of FMD encompassing all viruses and viroids associated with this disease complex. We highlighted herein important areas of future research and emphasized the importance of continued surveys of fig trees and germplasm, as well as the importance of each virus and viroid in disease development, isolate aggressiveness alone or in combination with other FMD-associated and vectors.

Geographic studies of the disease will help to determine where the viruses and viroids associated with FMD are located. The development of isolation protocols to study these viruses and viroids *in vivo* would be beneficial for characterization and symptom development studies with a reproducible protocol. This information would serve to properly conduct disease resistance testing, diagnostic development, and phylogenetic studies. Data from characterization studies will aid the development of control methods, plant breeding, and phylogenetic studies on each virus and viroid associated with FMD. For now, an important step forward in the organization and fine-tuning of FMD is to standardize the names of each associated virus. Plants with FMD in both, the Old and New World, may play a significant role in the expression of the disease. It is possible that some of the newly identified viruses may share vectors that may account, in addition to clonal propagation, for the large number of viruses and viroids that fig harbors.

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6 APPENDIX

Supplemental Table 1. RT-PCR primers used to identify viruses and viroids associated with FMD

Virus/ Viroid	Primer	Sequence	Gene	Reference
FLMaV-1	CPtr-a N17-s	5 '-CGTGGCTGATGCAAAGTTTA-3' 5 '-GTTAACGCATGCTTCCATGA-3'	HSP70h	(ELBEAINO et al., 2007)
FLMaV-2	F3-s F3-a	5 '-GAACAGTGCCTATCAGTTTGATTTG-3' 5 '-TCCCACCTCCTGCGAAGCTAGAGAA-3'	HSP70h	(ELBEAINO et al., 2007)
FLMaV-3	FLMaV-3sF FLMaV-3sR	5'-CTGTATCTGTCATTACCTCTTCGGG-3' 5'-CTGTATCTGTCATTACCTCTTCGGG-3'	HSP70h	(NOROZIAN et al., 2014)
FMV	EMARV-GP-s EMARV-GP-a	5'-GGGTACATATGCGTCATTCTTG-3' 5'-CGTTTGTCTTGGATCACAGCAA -3'	RdRp	(WALIA et al., 2009)
FLV-1	FLV1-s FLV1-a	5'-CCATCTTCACCACACAAATGTC-3' 5'-CAATCTTCTTGGCCTCCATAAG-3'	Coat Protein	(EL-AIR et al., 2013)
AFCV-1	AFCV1F AFCV1R	5'-CTGTATCTGTCATTACCTCTTCGGG-3' 5'-ATGCTTCCTCGGCTGC-3'	---	(TZANETAKIS et al., 2010)
AFCV-2	AFCV2F AFCV2R	5'-GTTCGGAATTAGTTAATAVTA-3 5'-ACCCGCTAGAGTAATCAGTCAAAGTT-3'	---	(TZANETAKIS et al., 2010)
FMMaV	LM3s LM3a	5′-AAGGGGAATCTACAAGGGTCG-3′ 5′-TATTACGCGCTTGAGGATTGC-3'	HSP70h	(ELBEAINO et al., 2010)
FCV	FCV-R2s FCV-R2a	5'-TTGGCCGACTACTCAAGTCA-3' 5'-TGCGAGGTAGCATGTGTAGC-3'	CP	(ELBEAINO et al., 2011b)
FFKaV	D8-s D8-a	5'-TCAATCCCAAGGAGGTGAAG-3' 5'-ACACGGTCAATGAGGGAGTC-3'	RdRp	(ELBEAINO et al., 2012)
FBV-1	1094F 1567R	5'-ACCAGACGGAGGGAAGAAAT-3' 5'-TCCTTGCCATCGGTTATCTC-3'	Poly-protein	(LANEY et al., 2012; TZANETAKIS et al., 2010)
ADFVd	ADFVd- for ADFVd- rev	5'-CCCCCCTGCGCTACTGACTAAAAG-3' 5'-GTGTTTTACCCTGGAGGCTCCACTC-3'	---	(CHIUMENTI et al., 2014)
CEVd	CEVd-F CEVd-R	5-GGAAACCTGGAGGAAGTCGAGG-3 5-CCGCCTCTTTTTTCTTTTCCTGCCTGC-3	---	(YAKOUBI et al., 2007)
HSVd	78P 83M	5'-AACCCGGGGCAACTCTTCTC-3' 5'-AACCCGGGGCTCCTTTCTCA-3'	---	(ELBEAINO et al., 2012)

FINAL CONSIDERATIONS

The cultivation of melon three times in the same year, and in the same area, using polyethylene mulch, has been causing an increase in the population of soilborne pathogens. In addition, it is causing damage to the beneficial community of microorganisms, which can be exacerbated by the indiscriminate application of chemicals aim to reduce soilborne pathogen problems in the cultivated area.

According to the conditions that the treatments were submitted to the incorporation of plant material (crotalaria or millet) associated with the use of polyethylene mulch and commercial products reduced the incidence and severity of rot in the melon root system and the occurrence of pathogens, even in a short-term observation. In addition to increasing the population of microorganism communities (actinomycetes, sporulating bacteria, total bacteria, and total fungi), which leads us to believe that the repeatability in the use of these green fertilizers, with soil conditioners, can considerably the factors that influence the life of microorganisms in the soil - bringing great benefits for the cultivation of melon in the main production region of Brazil.

The present thesis brings together promising results, under controlled conditions. This work must be repeated under field conditions, so that we can have more accurate information, regarding the use of green manure, and commercial products based on microorganisms and nutrients, combined with the application of polyethylene mulch, a practice already adopted by producers, for bringing notorious benefits for melon production.

In our studies in Connecticut – USA, on viruses in fruit crop, we detected the first incidence of grapevine viruses in the New England region. Where growers were advised to continue to monitor and control the insect vectors of these viruses as it is an important disease management strategy to limit virus spread within a vineyard. We encouraged all growers to test symptomatic plants for known grapevine viruses before their removal. Then, we need to study the extension of the spread, genetic diversity, and economic impact of grapevine viruses for better management of these viruses in the New England vineyards.

Fig mosaic disease (FMD) affects fig trees worldwide, causing economic loss and posing a serious threat to fig production. Our review called for more studies on FMD encompassing all viruses and viroids associated with this disease complex, 12

viruses and three viroids. Also, we proposed that the next step in the organization and fine-tuning of FMD is to standardize the names of each associated virus. Then, we recommended performing geographic studies of the disease, the development of isolation protocols to study these viruses and viroids *in vivo* that can be beneficial for characterization and symptom development studies with a reproducible protocol. With this information in hand, we can properly conduct disease resistance testing, diagnostic development, and phylogenetic studies. Data from characterization studies will aid the development of control methods, plant breeding and phylogenetic studies on each virus and viroid associated with FMD, minimizing the numerous problems caused by them.