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RNA interference technology as a potential control method for fruit and horticultural crops pathogens *Botrytis cinerea* and *Plasmopara viticola*

Presentata da: Daniel Endale Gebremichael

Coordinatore Dottorato

Prof. Massimiliano Petracci

Supervisore

Prof. Dr. Elena Baraldi

Co-supervisore

Dr. Zeraye Mehari Haile

Dr. Francesca Negrini

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I, Daniel Endale Gebremichael, hereby declare that this thesis is my own work with due acknowledgment of other materials used. I further state that the thesis has not been submitted for any award at any other university than the University of Bologna.

Daniel Endale Gebremichael

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List of acronyms and abbreviations

ANOVA	Analysis of Variance
BCA	Biological control agent
BcAlgl	alpha-1,3-glucan synthase
BcBeg	1,3-β-D-glucan synthase
BcCHSI	*Botrytis cinerea chitin* synthase 1
BcCHSIIIa	*Botrytis cinerea* chitin synthase 3a
BcCHSVI	*Botrytis cinerea chitin* synthase 6
B. cinerea	*Botrytis cinerea*
BLAST	Basic local alignment tool
cDNA	Complementary DNA
αCOP/Cop	Constitutive Phitomorphogenic1
CTAB	Cetyltrimethylammonium bromide
CWDEs	Cell wall degrading enzymes
DCL	Dicer-like
DNA	Deoxyribonucleic acid
DNTP	Deoxynucleoside triphosphate
dpi	Days post inoculation
ds	Double strand
dsRNA	Double-stranded RNA
ET	Ethylene
EU	European Union
FAOSTAT	Food and Agriculture Organization Corporate Statistical Database
G3PDH	Glyceraldehyde-3-phosphate dehydrogenase
HIGS	Host-induced gene silencing
hpRNA	Hairpin RNA
HSP60	Heat-shock protein 60
IPM	Integrated pest management
IPTG	Isopropyl β-D-1-thiogalactopyranoside
ISR	Induced systemic resistance
ITS	Internal transcribed spacer region
LB	Luria–Bertani
MAS	Marker-assisted selection

MCS	Multiple cloning site
mL	Milliliter
NaCl	Sodium chloride
NCBI	National Center for Biotechnology Information
NEP1	Necrosis and ethylene-inducing proteins 1
NEP2	Necrosis and ethylene-inducing proteins 2
nm	Nanometre
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
P. viticola	*Plasmopara viticola*
QTLs	Quantitative trait loci
rDNA	Recombinant DNA,
RFLP	Restriction fragment length polymorphism markers
RISC	RNA-induced silencing complex
RNAi	RNA interference
RPB2	DNA-dependent RNA polymerase subunit II
RT-qPCR	Reverse transcription quantitative PCR
SA	Salicylic acid
SAR	Systemically acquired resistance
SIGS	Spray-induced gene silencing
siRNA	Small interfering RNA
TAE	Tris-acetate-EDTA
UP-PCR	Universal-primed polymerase chain reaction
UV	Ultraviolet
µg	Microgram
%	Percent
<	Less than

Problem setting and thesis outline

Botrytis cinerea are among the most prevalent and widely distributed diseases of particularly fresh fruits and vegetables at both pre-and post -harvest stages. Contrary to other closely related *Botrytis* species that normally infects mainly specific host plants (Mansfield, 1980), *B. cinerea* has the ability to attack many hosts. The fungus causes a gray mold on more than 1400 different plant species covering nearly 600 genera (Elad et al., 2007; Jarvis, 1962; Dean et al., 2012; Rodríguez-García et al., 2013; Elad et al., 2016). Its success as a pathogen can be attributed to a variety of factors, including a wide host range, quick fungicide adaptability, infection of multiple host life stages, and numerous infection and overwintering techniques. These make *B. cinerea* an important disease of ripening fruits (Fillinger and Elad, 2016). Each year gray mold disease caused by *B. cinerea* has been estimated to cause annual losses of $10 billion to $100 billion of global agricultural losses (Dean et al., 2012; Boddy, 2016; Petrasch et al., 2019) and fungicides that specifically target *B. cinerea* represent about 10% of the global fungicide market (UIPP, 2002). *Botrytis* spp. can cause direct crop losses depending on the pathosystem when an agricultural product, such as berries, fruits, flowers, or bulbs, is impacted to the point of becoming unmarketable. *Botrytis* spp. also causes indirect losses with an estimated higher average control costs. Furthermore, the effective management of *B. cinerea* is a challenge, owing to the wide host range and its short life cycle, and the ability to produce abundant sporulation. Fungicide applications have been the primary component of *B. cinerea* management programs (Fillinger and Walker, 2016). However, some fungicides have a detrimental effect on non-target beneficial microorganisms (Yang et al., 2011; Karlsson et al., 2014) and human health (Pearson et al., 2016), and may also exhibit phytotoxicity, negatively affecting photosynthesis and plant biomass production (Dias, 2012). Furthermore, the excessive and repeated use of fungicides has resulted in the development of resistant strains (Chapeland et al., 1999; Rupp et al., 2017b; Shao et al., 2021). These repercussions evidence the urgent need for alternative controls that are effective and environmentally sustainable.

Downy mildew, a disease caused by the oomycete *Plasmopara viticola* is the most prevalent disease of grape vine and is native from North America where it is endemic in wild *Vitis* species (Gessler et al., 2011; Wilcox et al., 2015). *P. viticola* attacks leaves, shoots, inflorescences, and infructescences. All the vine cultivars used in Europe are susceptible to downy mildew, and this disease cause enormous economic damage both quantitatively and qualitatively. At present the downy mildew control in grape vine and other crops depends on synthetic fungicides (Armijo et al., 2016). Consequently, downy mildew control together with powdery mildew control, consumes around two-thirds of all synthetic fungicides sprayed for crop disease

management in the European Union (Eurostat., 2007). It is challenging to estimate the total costs sustained in the EU or in other member states for fungicide treatments against *Plasmopara viticola*. Even though few studies are addressing the economic aspects of downy mildew, a study conducted in Italy estimated the annual cost of controlling downy mildew between 8 and 16 million euros (Salinari et al., 2006). As mentioned above, *P. viticola* is mainly controlled by repeated applications of chemical fungicides to prevent substantial losses in vineyard. However, the ecological problems of pesticides and the rapid occurrence of resistant pathogen strains (Gisi and Sierotzki, 2008) triggered interests in sustainable management alternatives. The main goal of this thesis was to investigate the potential of RNA interference (RNAi) technology as a possible control method for both *Botrytis cinerea* and *Plasmopara viticola*.

In **Chapter 1**, a general introduction on *Botrytis cinerea* and its threat to crop production is presented. What *Botrytis* looks like, its life cycle, why it is a threat to agricultural production, its worldwide pest status, and its current state of management is further elaborated on. The application of a management method based on RNAi technology to *Botrytis cinerea* control is presented.

In **Chapter 2**, a general introduction on *Plasmopara viticola* and its threat to grape production is presented. Its life cycle, why it is a threat to grape production, its worldwide pest status, and its current state of management is further elaborated on. on. The application of a management method based on RNAi technology to *Plasmopara viticola* control is presented.

Chapter 3, titled " RNA Interference Strategies for Future Management of Plant Pathogenic Fungi: Prospects and Challenges ", presents the rapid improvement and extensive implementation of RNA interference (RNAi) technology for the management of fungal pathogens. In this chapter, we describe the application of exogenous RNAi involved in plant pathogenic fungi and discuss dsRNA production, formulation, and RNAi delivery methods. The potential challenges faced while developing a RNAi strategy for fungal pathogens, such as off-target and epigenetic effects, with their possible solutions are also discussed.

Chapter 4, titled " Exogenous dsRNAs against chitin synthase and glucan synthase genes suppress the growth of the pathogenic fungus *Botrytis cinerea* " addresses two important questions: Is RNAi technology functional for *B. cinerea* control? And which target genes can

be exploited for RNAi-based *B. cinerea* disease control? Upon target genes selections, an exogenous RNAi protocol was set up and we could effectively deliver a known dose of bacterially produced double stranded RNA (dsRNA) to induce RNAi in *B. cinerea*.

Chapter 5, titled " Double-Stranded RNA Targeting Dicer-Like Genes Compromises the Pathogenicity of *Plasmopara viticola* on Grapevine ", which deals mainly on RNAi induction against *Plasmopara viticola.* This chapter addresses two main questions: Is RNAi technology functional in contrasting *Plasmopara viticola*? And which target genes can be exploited for RNAi-based disease control in *Plasmopara viticola*? Upon target gene selection an exogenous RNAi protocol was set up, and we could effectively deliver a known dose of double stranded RNA (dsRNA) to induce RNAi in *Plasmopara viticola*.

In the last Chapter (**Chapter 6**) titled " General discussions and perspectives for future research", the major research findings from this thesis are discussed together with perspectives for future research.

Chapter 1: *Botrytis* spp. and current status of management strategies

Abstract

Botrytis spp. are effective pathogens, causing destructive diseases and considerable crop losses in a wide variety of plant species. The species in the genus *Botrytis* vary greatly in terms of their biology, ecology, morphological features, and host range. Progress in molecular genetics especially the development of phylogenetic markers has resulted in the establishment of several species. Species of *Botrytis* are responsible for heavy losses on more than 1400 species of cultivated plants covering nearly 600 genera, many of which are economically important horticultural and floral crops. Most species, except *Botrytis cinerea*, have a limited host range. Both the sexual and asexual stages are recognized for *B. cinerea*. The production of asexual spores (macroconidia, also known as conidia), which are transported by wind or water, is the typical way of dispersal for almost all species. Sclerotia production is typically how plants survive from one season to the next. Infections are most easily identified by the presence of distinctive grey conidial clusters on the surfaces of infected hosts. The disease caused by the pathogen can be controlled by implementing different management practices. *Botrytis* epidemics on many crops can still be controlled most easily with chemicals. However, there are real concerns about the environment, human health, and the emergence of resistance in pathogen populations. Many biological control agents and other biopesticides, including plant extracts, minerals, and organic compounds, have been developed in recent years to combat diseases caused by *Botrytis.* Different cultural methods aiming to decrease humidity can be combined with chemical and biological methods for improved *Botrytis* disease control. Breeding resistant plants is currently the most popular and sustainable strategy for fungal disease management including *Botrytis*. In contrast to chemical fungicide-based control approaches of *Botrytis*, RNAi-based control methods via host induced gene silencing and spray induced gene silencing offer eco-friendly strategies to combat *Botrytis diseases*.

Keywords : *Botrytis , Botrytis cinerea, RNAi, control method, life cycle*

1. Introduction

Diseases caused by *Botrytis,* a fungal genus, especially *Botrytis cinerea* (*B. cinerea*) are among the most prevalent and widely distributed postharvest decay of fresh fruits and vegetables (Droby and Lichter, 2004). *Botrytis* species have been found infecting their host species in all climatic zones and have been discovered in various environments, including fields, nurseries, greenhouses, storage rooms, and transit houses (Elad et al., 2007). In addition, the disease can be found inside stems, leaves, flowers, fruits, and seeds (Legard et al., 2000; Dean et al., 2012; Elad et al., 2016). *Botrytis* may trigger clear disease symptoms (Figure.1) before harvest or remain quiescent until the post-harvest period (Fillinger and Elad, 2016). Its success as a pathogen can be attributed to a variety of factors, including a wide host range, quick adaptability to fungicides, infection of multiple host life stages, and numerous infection and overwintering techniques (Williamson et al., 2007; Elad et al., 2016). *Botrytis* species are generally necrotrophic pathogens (Staats et al., 2005), which induce host-cell death resulting in progressive decay of infected plant tissue, and also have excellent saprotrophic capabilities. However, there are exceptions. The recently described species *Botrytis deweyae*, which infects cultivated *Hemerocallis* (daylily) and, causes a disease of emerging spring foliage was shown to have an endophytic lifestyle under appropriate conditions (Grant Downton et al., 2014). Similarly, even *B. cinerea* can adopt an endophytic behaviour in some situations. This has been demonstrated for lettuce where *B. cinerea* can penetrate through the flowers and grow systemically in the plant (Sowley et al., 2010). Additionally, *B. cinerea* has been reported to exhibit short phases of endophytic growth in undamaged plant tissues of strawberries (Bristow et al., 1986), blackcurrant (McNicol and Williamson, 1989), and raspberries (Williamson et al., 1987). Likewise, Zuccaro et al., 2008 demonstrated that *B. cinerea* may also be able to interact with tissues of the seaweed *Fucus serratus* in an endophytic manner (Zuccaro et al., 2008). The ability of endophytic development before turning to disease (necrotrophy) makes the infection cycle more complicated and renders *Botrytis* disease management even more difficult. Such infections may develop into aggressive conditions later, particularly during flowering or storage, or they may spread non-symptomatically by clonal or seed propagation of the host (Barnes and Shaw, 2003). In general, *B. cinerea* causes losses in both quality (taste, aroma, and oxydasic casse in wine) and quantity (reduced yields of fruit, vegetable crops, and ornamentals). The most common disease caused by *B. cinerea* is probably fruit rot or gray mold of grapes and tomatoes (Fillinger and Elad, 2016). However, *Botrytis spp*. also causes blossom blight, stem cankers, leaf spots, diebacks, damping-off, tuber, corm, bulb, and root rot (Fillinger

and Elad, 2016). Once fruits, berries, vegetables, and flowers have been harvested, *Botrytis* spp. may cause secondary soft rot during storage or transit or even in the marketplace (Fillinger and Elad, 2016).

Despite the importance of *Botrytis* diseases around the world, accurate estimates of crop losses are scarce in the scientific literature. According to Elmer and Michailides, 2007, the global economic impact of *Botrytis* bunch rot of grapes is projected to be up to US$2 billion. In addition, each year gray mold disease caused by *B. cinerea* has been estimated to cause annual losses of $10 billion to $100 billion in global agricultural losses (Boddy, 2016). *Botrytis* spp. can cause direct crop losses depending on the pathosystem when an agricultural product, such as berries, fruits, flowers, or bulbs, is affected to the point of becoming unmarketable. In a study conducted in Florida, on strawberries, losses from fruit rot can exceed 50%, fungicides are usually applied once a week and their cost was roughly 7% of pre-harvest variable costs (about $ 690 per acre) (IFAS, 2010). Shoemaker and Lorbeer, 1971 stated onion yield losses triggered by *B. Squamosa* range from 7 to 30% in unsprayed plots. Similarly, in the Netherlands, yield reductions of 26% were documented (De Visser, 1996). Direct crop losses caused by *Botrytis* bunch rot in New Zealand's wetter regions, for example, can reach NZ$5000/ha, while the cost of control is NZ$1500/ha (Hoksbergen, 2010). *Botrytis* spp. also causes indirect losses. Therefore, the economic damage attributed to *Botrytis* is enormous. It includes pre- and post-harvest losses in quantity and quality, expenses for plant protection measures in the fields and greenhouses, direct and indirect costs to retailers and consumers for cooling facilities, and losses suffered by rotten plants.

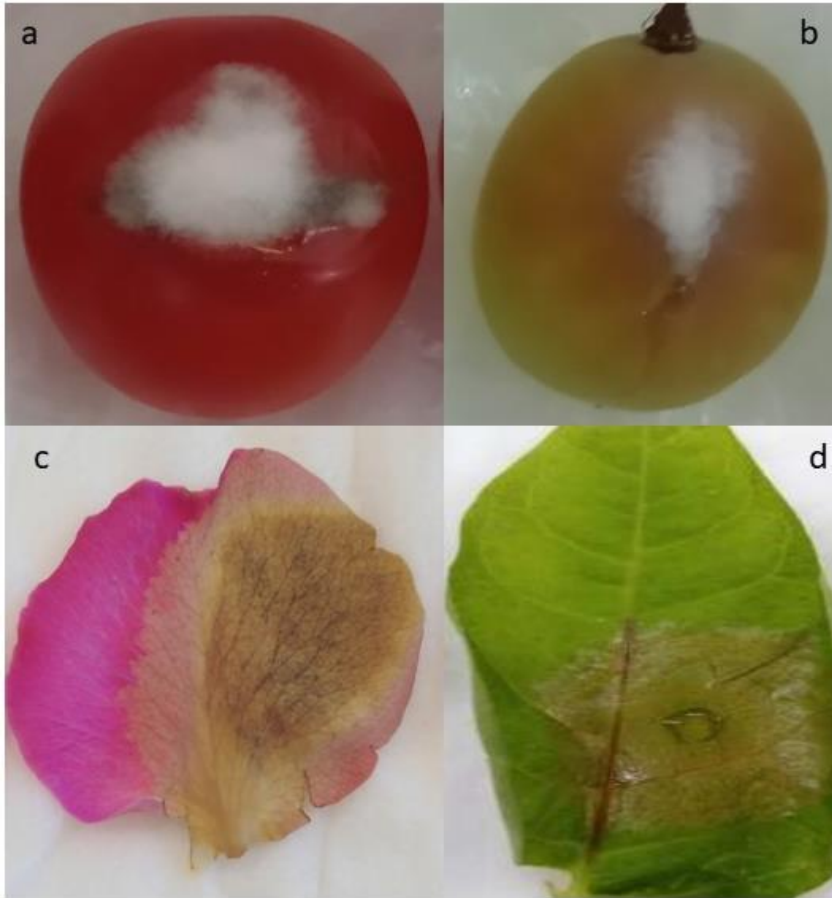

Figure 1. *B. cinerea* associated symptoms on a) tomato fruit (a), grape (b) rose petal (c), tomato leaf (d)

2.Taxonomy and species identification in *Botrytis*

Botrytis is a highly diverse genus, with various species that differ in biology, ecology, morphological traits, and host range (Elad et al., 2004). *Botrytis* belongs to the kingdom Eumycota, phylum Deuteromycotina, class Leotiomycetes, order Heliotiales, and family Sclerotiniaceae (Hennebert, 1973; Yohalem et al., 2003; Elad et al., 2016). The genus name "*Botrytis*" is derived from the classical Greek word 'botrus', meaning grapes. Grapes refer to the bunching of the conidia (spores) on their conidiophores (organs that produce spores). The genus name *Botrytis* was retained after the 2011 changes to the fungal naming system, known as one-fungus-one-name (Johnston et al., 2014). Pier Antonio Micheli described the genus for the first time in 1729 in his book *Nova Plantarum Genera* (Micheli, 1729). Buchwald (1949), Groves and Loveland (1953), Hennebert (1973), and Beever and Weeds (2004) were eventually able to establish the majority of the species. The term "*Botrytis cinerea*" first appeared in von Haller's " Synopsis Methodica Fungoruma," which was published in Zurich, Switzerland, in

1771. De Bary finally discovered the connection between *Botryotinia fuckeliana* (de Bary) Whetzel, the sexual stage, and *B. cinerea* in 1866. *Botrytis* is very closely linked to *Sclerotinia*, with the proteins encoded by the genomes of *B. cinerea* and *S. sclerotiorum* showing 83% identity (Amselem et al., 2011). Macroconidia (dry spores dispersed by wind over long distances), sclerotia for survival (female mating partner), microconidia (not infectious and play the role of spermatia as male gametes), and teleomorphic stage (Urbasch, 1983), with ascospores as sexual structures are all included in the life cycle of *B. cinerea* (Figure.2).

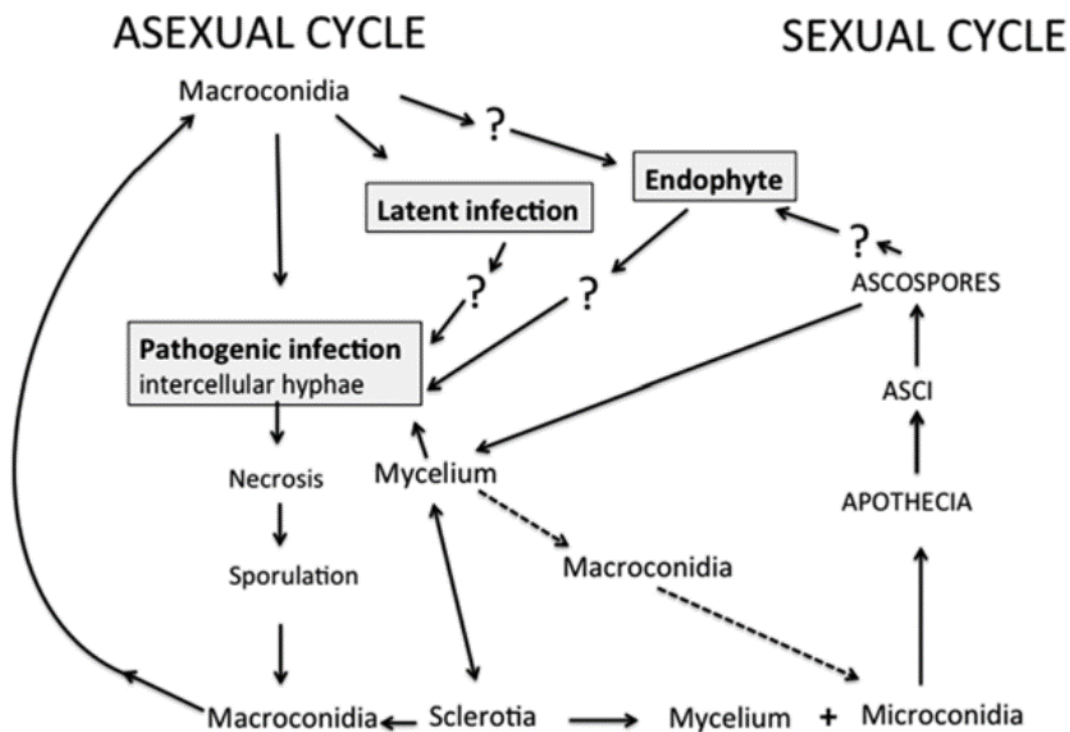

Figure. 2. Diagram of the life cycle of *Botrytis cinerea* showing known and possible interactions between different phases of growth. Note: Macroconidia common name is conidia (Source: Molly and Grant-Downton, 2016).

Until the validation of the genus by Persoon in 1801, *Botrytis* was thought to have only five species (Persoon, 1801) and by 1822, Persoon had included 27 species. The genus was then updated by Saccardo in 1886 to include 128 species (Saccardo, 1886), and over time expanded to include up to 380 species, the majority of which are published in separate, challenging-to-access articles (Hyde et al., 2014) written in a variety of languages. Buchwald (1949) revised the description limited the genus to 23 species. Therefore, it is important to proceed with

caution when citing works published before 1949. Buchwald in 1949 suggested three new subgenera: A) *Eubotrytis,* comprising two sections the Macrosclerotiophorae comprising *B. allii (B. aclada), B. anthophila, B. byssoidea, B. cinerea* sensu stricto, and *B. cinerea* sensu lato*, B.convoluta*, *B.porri, B. squamosa, B.trifolii,* and the section Microsclerotiophorae with the species *B. croci, B. elliptica, B. fabae, B. galanthina, B. gladioli, B. hyacinthi, B. narcissicola, B. paeoniae,* and *B. tulipae;* B) *Sphaerobotrytis* comprising the globose-spored species *B. ricini, B. globosa, B. polyblastis, B. ricini and B. sphaerosperma;* C) *Verrubotrytis, comprising the only species B. geranii.* The numbers before 1949 reflected a vast overestimation of the number of species because of the liberal morphological parameters used to identify the genus, the lack of agreement over the real lectotype specimen, and later concurrent mycological study using different types of material (Groves and Loveland, 1953; Jarvis, 1980). For instance, Saccardo (1886) divided *Botrytis* into 4 subgenera based on conidiophore structure: *Phymatotrichum* (Bon.) Sacc., *Eubotrytis* Sacc., *Polyactis* (Link) Sacc., and *Cristulina* Sacc.; *B. cinerea* was referred to as *Polyactis* in this system. In addition to *Beauveria Vuill*., *Hyphelia* Fr., *Chromelosporium Corda*, and *Haplaria* Link, several genera are mistaken for *Botrytis*. Early *Botrytis* taxonomy was also troubled by the absence of a solid link between the two contrasting morphological states seen in several species of fungus. The asexual morph (the anamorph, imperfect, or conidial stage) and the sexual morph (the teleomorph, perfect, or apothecial stage) are the two distinct morphological phases of several species of *Botrytis.* Both of these states have different morphologies. As a result, historically, the sexual morph was called *Botryotina,* and the asexual morph *Botrytis* (Jarvis, 1980). *Botrytis* species share morphological similarities with other *Sclerotiniacea* family members that are not *Botrytis* fungi. The asexual and sexual stages of *Botrytis* species strongly mimic those of *Amphobotrys* and *Streptobotrys* species, and the sexual stage of these latter two closely matches that of *Sclerotinia* species. These genera have morphological similarities as well as many of the same disease symptoms that are brought on by each of these groups. Numerous species in the *Sclerotiniaceae* family were mistakenly attributed to *Botrytis* due to liberal interpretations of genus boundaries, the difficulties of pleomorphism and confusing taxonomy, and the likeness of *Botrytis* to other genera of fungi (Groves and Loveland, 1953; Hennebert, 1973; Jarvis, 1977). Ultimately, despite numerous attempts to remove species from the genus (Buchwald, 1949; Buchwald, 1953; Groves and Loveland, 1953; Hughes, 1958), 22 *Botrytis/Botryotinia* species persevered and served as the basis for modern *Botrytis* taxonomy; this culminated in the re-evaluation of the genus performed in 1973 by Hennebert (Hennebert, 1973). These 22 species are related to inoperculate *Discomycetes* of the family *Sclerotiniaceae*

Whetzel, in the genus *Botryotinia* Whetzel. Within the *Botrytidaceae*, three new genera were erected by Hennebert (1973), namely : *Amphobotrys* n.g., associated *with Botryotinia* and containing one species*, A. ricini* (*Botrytis ricini Buchw*)*; Streptobtrys* n.g., associated with *Streptotinia* Whetzel; and *Verrucobotrys* n.g., associated with *Seaverinia* Whetzel and having one species, *V. geranii* (*Botrytis geranii* Seaver). Following Hennebert revisions, the taxonomy of *Botrytis* entered a period of relative calm for the following several decades. In the past 25 years, at least 10 new species of *Botrytis* anamorphs have been described and published (Table 1). Although *B. cinerea* is the name of the asexual stage (anamorph) and *Botryotinia fuckeliana* is the name of the sexual stage (teleomorph), the *Botrytis* community agreed to use *B. cinerea* as the generic name in 2013 at the *Botrytis* Symposium in Bari, Italy (Elad et al., 2016). As a result, the teleomorph name should no longer be utilized. The species prefix *Botrytis* is used for all other species of the same genus. Apothecia of *B. cinerea* is unusual in the field but they are found in abundance in other *Botrytis* spp.

Distinguishing various *Botrytis* species in sympatry and defining their contributions to disease could support adapting management strategies (Walker, 2016). *Botrytis* species can be distinguished by morphological features (morphology of colonies, shape, and size of macroconidia, length and width of conidiophores, the size, number, shape, and production of sclerotia, and the morphology of the mycelium on artificial media), biological characteristics, ecological method (focusing on adaptation to a particular ecological niche), and phylogenetic techniques (Hennebert, 1973; Jarvis, 1977, 1980; Taylor et al., 2000; Staats et al., 2005; Li et al., 2012; Lorenzini and Zapparoli, 2014). According to studies conducted numerous *B. cinerea* isolates showed morphological variations in terms of the number, size, and arrangement of conidiophores and sclerotia (Paul, 1929). Some *B. cinerea* cultures were completely unable to produce conidia and/or sclerotia and instead remained fully vegetative, only producing mycelium (Paul, 1929). Other scientists have noticed significant variations in conidial sizes among isolates of *B. cinerea* from a single host, leading to the classification of these isolates as separate races of the pathogen (Jarvis, 1980). Conidial measures have historically been a key criterion in determining species. Contrary to some isolates of *B. cinerea*, several novel species appear reluctant to develop conidia on culture media and/or only sporulate under extremely precise cultural circumstances. This is true for the newly discovered species *B. deweyae*, which only consistently produces macroconidia after being exposed to near-ultraviolet light for seven days without the presence of any other light sources (Grant-Downton et al., 2014). According to Li et al., 2012, conidia and conidiophore measurements were

reported from artificially inoculated blackberries because the novel species *B. caroliniana* does not develop conidia on potato dextrose agar (PDA) medium, a typical growth medium for *Botrytis*. For the novel species, sporulation is also infrequent or nonexistent on PDA, *B. euroamericana* (Garfinkel et al., 2017), *B. polyphyllae* (Zhong et al., 2019), *B. sinoallii* (Zhang et al., 2010a), and *B. prunorum* (Ferrada et al., 2016). But for other novel specie *Botrytis sinoviticola* morphologically differentiated from *B. cinerea, B. californica,* and *B. pseudocinerea* mainly through the presence of villiform appendages on the conidial surface (Zhou et al., 2014). In the same way, *Botrytis californica* is morphologically differentiated from *B. cinerea, B. pseudocinerea, and B. sinoviticola* by the development of long conidiophores (Saito et al., 2016). Therefore, species classification by morphological method alone is challenging in *Botrytis.* Several species are morphologically similar (e.g., *B. cinerea and B. pseudocinerea* (Walker et al., 2011); *B. aclada* and *Botrytis sp. B83* (Lorenzini and Zapparoli, 2014). Variation may also be strongly influenced by growing conditions (Grindle, 1979; Martinez et al., 2003). Moreover, a single species may exhibit great degrees of morphological diversity (Lorenz, 1983; Martinez et al., 2003). Biological characterization depends on interbreeding to determine fertility among individuals. Sexual crosses between species have been used to differentiate some species (Bergquist and Lorbeer, 1972). However, in *Botrytis*, homothallism (i.e.self-fertilization) is prevalent, making it difficult to determine whether progeny had two parents (Buchwald, 1953; Elliott, 1964). Other *Botrytis* species appear to be completely devoid of sexuality, limiting the use of the biological species concept for species differentiation. However, biological characterization has been successfully used to distinguish between *B. squamosa* and *B. cinerea* as well as between *B. cinerea* and *B. pseudocinerea* (Bergquist and Lorbeer, 1972; Walker et al., 2011). According to reports, some species, like *B. porri* and *B. globosa*, are homothallic (i.e., self-fertile) and can produce sexual offspring in a single culture (Buchwald, 1953; Elliott, 1964). Others require strains with compatible mating types to produce their progeny since they are heterothallic (i.e., self-sterile). Meanwhile, the use of biological methods to define a new *Botrytis* taxon is time-consuming, laborious, and sometimes requires special knowledge. For instance, inducing the production of ascospores in *Botrytis* often takes a lengthy period (Fareta and Antonacci, 1987). *Botrytis* sp. B83, a novel species, rarely develops sclerotia, making it impossible to validate its existence using biological criteria (Lorenzini and Zapparoli, 2014). Morphological characters, in combination with DNA sequence data from various protein-coding genes, can be used to identify *Botrytis* species and open the way to a better understanding of the genetic diversity within the genus. Different genotypes of *Botrytis* vary in their ecological behavior and in their

ability to accumulate fungicide resistance (Leroch et al., 2013). Thus, knowledge of the genetic diversity in *Botrytis* can provide important information for the evaluation of the resistance situation or management methods. Therefore, rather than on morphological features, species can be delimited by phylogenetic analyses of variable nucleic acid sequences (Taylor et al., 2000). A work by Giraud et al., 1997 as the first to apply genetic methods for *Botrytis* species delineation. The work of Girauid et al., 1997 is notable since it marked the beginning of species recognition based on genetic markers in *Botrytis*, even though this particular study did not use phylogenetics but rather restriction fragment length polymorphism markers (RFLP) of the intergenic spacer rDNA region. The groundwork for a novel species that was not formally described until 2011 along with genus-wide phylogenetic comparisons was laid by the Girauid et al., 1997 research and later work describing a *B. cinerea* complex using additional genetic data (Fournier et al., 2005). In the phylogenetic method, an evolutionary tree is used to model the relationships of a group of individuals. Terminal monophyletic clades can be used to identify phylogenetic species. Consequently, the phylogenetic analysis should ideally be based on molecular data from several different gene areas. Holst-Jensen et al. (1998) reported that internal transcribed spacer region (ITS) sequences are useful in distinguishing *Botrytis/Botryotinia* from the other genera in *Sclerotiniaceae* but are not useful in differentiating species within *Botrytis/Botryotinia* due to lack of informative loci. Phylogenetic analysis was also used in other early attempts to study *Botrytis*, with some degree of success. For example, *B. cinerea*, *B. squamosa*, *B. byssoidea, B. aclada,* and *B. allii* (a hybrid between *B. aclada* and *B. byssoidea*) (Nielsen et al., 2001), were identified using universal-primed polymerase chain reaction (UP-PCR) fingerprinting and the restriction of ITS rDNA regions (Nielsen and Yohalem, 2001; Yohalem et al., 2003). Phylogenetic analysis of *Botrytis* species using three nuclear housekeeping gene sequences, glyceraldehyde-3-phosphate dehydrogenase (*G3PDH*), heat-shock protein 60 (*HSP60*), DNA-dependent RNA polymerase subunit II (*RPB2*), and necrosis and ethylene-inducing proteins 1 and 2 (NEP1 and NEP2) (Staats et al., 2005; Staats et al., 2007; Walker, 2016), was a turning point in *Botrytis* taxonomy and supported the morphological delimitation of *Botrytis* species by Hennebert, 1973 and Yohalem et al., 2003. The sequences of two *NEP* genes are unique at the species level as some species are not easy to distinguish based on the housekeeping gene sequences (Staats et al., 2007). Staats et al., 2005 stated that the variations in the DNA sequences of the genes for *G3PDH, HSP60,* and *RPB2* supported the conventional *Botrytis* species delineation by Hennebert, 1973. Staats et al., 2005 employed PCR-amplified products from three single-copy nuclear genes, *G3PDH, RPB2, and HSP60*. The sequences from each gene were utilized to create trees, and a

combined analysis of all three genes identified two phylogenetic clades that were sharply divided apart. Clade I include plant pathogens that infect mostly or exclusively dicotyledonous as well as host-specific species such as *B. cinerea, B. pelargonii, B. calthae, and B. fabae.* Clade II is phylogenetically more diverse and could be subdivided into five smaller clades, and it mainly consisted of *Botrytis* species that infect predominantly monocots while also including a few species that are specialized dicot pathogens. Clade I of *Botrytis* currently contains about 10 species and Clade II contains more than 25 species (Shaw et al., 2016; Garfinkel et al., 2019). Moreover, these five phylogenetically informative genes have had much value in identifying new species and their evolutionary relationships, such as *B. caroliniana*, *B. deweyae, B. fabiopsis, B. sinoviticola,* and *B. sinoallii*. Additionally, the *HSP60* sequence has also been used to detect endophytic isolates as a probable novel, undescribed species in Centaurea (Shipunov et al., 2008). The resurgence of the long-lost species *B. mali* was also facilitated by the phylogenetic study of genes *G3PDH* and β-tubulins (O'Gorman et al., 2008). The *G3PDH, HSP60*, and calmodulin genes were combined by Andrew et al., 2012 to better comprehend the taxonomic relationships within the *Sclerotiniaceae* family. Likewise, Khan et al., 2013 used ITS, IGS, and *G3PDH* in conjunction to identify the *Botrytis* species infecting onions. The formal descriptions of two new Chinese *Botrytis* species, *for B. fabiopsis* (using *G3PDH, RPB2, and HSP60*) and for *B. sinoallii* (using *NEP 1 and NEP2*), from the broad bean and *Allium* crops, respectively, in 2010 marked the beginning of a new decade of taxonomic transformation. According to Leroch et al., 2013, a novel clade of *Botrytis* that is common in German strawberry-growing regions but uncommon in vineyards has been discovered. This clade is closely related to *B. cinerea* and *B. fabae*. These isolates, known as group S, exhibit high levels of multi-drug resistance. Combined evaluation of sequence data from several genes, *HSP60* and *NEP2* but also the zinc finger transcription factor mrr1, ms547, and fg1020, proved the distinction of these isolates. Furthermore, a few species-specific PCR primers have been successfully developed based on related target DNA sequences for the detection and differentiation of *Botrytis* species, including *B. aclada, B. allii, B. byssoidea, B. cinerea, B. fabae, and B. fabiopsis* (Nielsen et al., 2002; Saito et al., 2009; Fan et al., 2015). Since the genus was described in 1729, over 35 species (Table 1) of polyphagous and host-specific *Botrytis* have been described, with a significant increase in the number of species described due to the advancement of molecular methods (Ferrada et al., 2016; Garfinkel et al., 2017). In general, the major technical improvements that have been made in the past decade in molecular tools have helped significantly in the classification of the genus, identification of new species,

broadening our knowledge of host ranges, and advanced our understanding of the biology of *Botrytis* species.

Table 1. A list of *Botrytis* species with importance in agriculture and horticulture

Botrytis sp. (anamorph)	*Botryotinia sp*. (teleomorph)	Disease	Mating system	Major plant hosts	Date of description	References
B. aclada Fresen	-	Gray-mold neck rot	-	*Allium*	1850	Hennebert, 1973; Jarvis, 1980; Farr et al., 1989; Yohalem et al., 2003
B. acladiopsis	-	-	-		1996	Wang et al., 1996
B. anthophila Bondartsev	-	Gray-mold	-	*Trifolium*	1914	Noble, 1948; Farr et al., 1989; Jarvis, 1980
B. byssoidea Walker	-	Mycelial neck rot	-	*Allium*	1925	Hennebert, 1973; Farr et al., 1989; Jarvis, 1980; Yohalem et al., 2003
B. calthae Hennebert	*Bt. calthae* Hennebert & Elliott	Lesions	-	*Caltha*	1963	Hennebert and Groves, 1963; Hennebert, 1973; Jarvis, 1980; Plesken et al., 2015
B. caroliniana	-	Gray mold	-	*Rubus*	2012	Li et al., 2012
B. californica	-				2016	Saito et al., 2016
B. cinerea Pers.: Fr	*Bt. fuckeliana (de Bary) Whetzel*	Gray mold	Heterothallic	*Polyphagous/ Multiple host genera*	1801	Hennebert, 1973; Kohn, 1979; Jarvis, 1980; Farr et al., 1989
B. convallariae (Kleb.) Ondřej	-		-	*Convallaria*	1972	Jarvis, 1980

Table 1. Cont.

Botrytis sp. (anamorph)	*Botryotinia* sp. (teleomorph)	Disease	Mating system	Major plant hosts	Date of description	References
B. convoluta Whetzel & Drayton	*Bt. convoluta (Drayton) Whetzel*	*Botrytis* rhizome rot	-	*Iris*	1932	Whetzel and Drayton, 1932; Hennebert, 1973; Kohn, 1979; Jarvis, 1980; Farr et al., 1989
B. croci Cooke and Massee	-	Crocus blight	-	*Crocus*	1887	Hennebert, 1973 ; Jarvis, 1980
B. deweyae	-	Spring sickness	Heterothallic	*Hemerocallis*	2014	Grant Downton et al., 2014
B. elliptica (Berk.) Cooke	*? Botryotinia sp*	Lilyblight, fire blight	Heterothallic	*Lilium*	1901	Hennebert, 1973; Jarvis, 1980; Farr et al., 1989; Van den Ende and Pennock, 1996 ; Van den Ende and Pennock-Vos, 1997
B. eucalypti	-	Gray mold	-	*Eucalyptus*	2016	Liu et.al., 2016
B. euroamericana	-			*Viti, paeonia, Cicer*	2017	Garfinkel et al., 2017; Moparthi et al., 2020
B. fabae Sardiña	*Bt. fabae Lu & Wu*	Chocolate spot	-	*Vicia*	1929	Hennebert, 1973; Jarvis, 1980; Wu and Lu, 1991
B. fabiopsis	-	Chocolate spot		*Vicia faba*	2010	Zhang et al., 2010b
B. ficariarum Hennebert	*Bt. ficariarum Hennebert*	-	-	*Ficaria verna*	1963	Hennebert and Groves, 1963; Hennebert, 1973; Kohn, 1979; Jarvis, 1980

Table 1. Cont.

Botrytis sp. (anamorph)	*Botryotinia* sp. (teleomorph)	Disease	Mating system	Major plant hosts	Date of description	References
B. fragariae	-	Gray mold	-	*Fragaria*	2017	Rupp et al., 2017a
B. fritillarii pallidiflori (Chen &Li) Seifert & Kohn	*Bt. fritillarii-pallidiflori Chen & Li*	-	-	-	1987	Li and Chen, 1987
B. galanthina (Berk.& Broome) Sacc.	-	Blight	-	*Galanthus*	1886	Hennebert,1973; Jarvis, 1980; Farr et al., 1989
B. gladiolorum Timmerm.	*Bt. draytonii (Buddin & Wakef.) Seaver*	Gladiolus blight	-	*Gladiolus*	1941	Hennebert,1973; Kohn, 1979; Jarvis, 1980 ; Farr et al., 1989
B. globosa Raabe	*Bt. globosa Buchw.*	Neck rot	Homothallic	*Allium*	1938	Buchwald, 1953; Hennebert, 1973; Kohn, 1979; Jarvis, 1980
B. hyacinthi Westerd. & Beyma	-	Hyacinth fire	-	*Hyacinthus*	1928	Hennebert, 1973; Jarvis, 1980; Farr et al., 1989
B. mali	-	Gray mold	-	Malus	2008	O'Gorman et al., 2008
B. medusae	-	Gray mold		*Vitis*	2019	Harper et al., 2019

Table 1. Cont.

***Botrytis* sp. (anamorph)**	***Botryotinia* sp. (teleomorph)**	**Disease**	**Mating system**	**Major plant hosts**	**Date of description**	**References**
B. narcissicola Kleb. Ex Westerd. & Beyma	*Bt. narcissicola (Greg.) Buchw.*	Smoulder mold	-	*Narcissus*	1928	Buchwald, 1949, Hennebert, 1973; Kohn, 1979; Jarvis, 1980; Farr et al., 1989
B. paeoniae Oudem	-	Peony blight	-	*Paeonia, Allium*	1897	Jarvis, 1980
B. pelargonii Røed	*Bt. pelargonii Røed*	-	-	*Pelargonium*	1949	Røed, 1949; Hennebert, 1973 ; Jarvis, 1980 ; Kohn, 1979
B. polyblastis Dowson	*Bt. polyplastis (Greg.) Buchw.*	Narcissus fire	-	*Narcissus*	1928	Buchwald, 1949; Hennebert, 1973; Kohn, 1979; Jarvis, 1980; Farr et al., 1989
Botrytis polygoni	-	Brown leaf spots	-	*Polygonaceae*	2020	He et al., 2020
B. porri Buchw.	*Bt. porri (Beyma) Whetzel*	-	Homothallic	Allium	1949	Seaver, 1951; Elliott, 1964; Hennebert, 1973; Kohn, 1979; Jarvis, 1980
B. prunorum	-	Blossom Blight	-	*Polyphagous*	2016	Ferrada et al., 2016

Table 1. Cont.

***Botrytis* sp.** (anamorph)	***Botryotinia* sp.** (teleomorph)	**Disease**	**Mating system**	**Major plant hosts**	**Date of description**	**References**
B. pseudocinerea	*Bt. pseudofuckeliana*	-	Heterothallic	*Polyphagous*	2011	Walker et al., 2011
B. pyiformis	-		-	*Saprotroph*	2016	Zhang et al., 2016
Botrytis sp.	*Sclerotinia spermophila Noble*	-	Homothallic	*Trifolium*	-	Noble, 1948; Kohn, 1979; Jarvis, 1980; Farr et al., 1989
Botrytis sp. B83	-	Gray mold	-	*Polyphagous*	2014	Lorenzini and Zapparoli, 2014
B. ranunculi Hennebert	*Bt. ranunculi Hennebert & Grove*	-	Heterothallic	*Ranunculus*	1963	Hennebert and Groves, 1963 ; Hennebert, 1973; Kohn, 1979; Jarvis, 1980
B. ricini Buchw	*Bt. ricini (Godfrey) Whetzel*	Gray mold	Homothallic	*Ricinus*	-	Godfrey, 1923; Hennebert, 1973 ;Jarvis, 1980; Farr et al., 1989
B. sinoallii	-	leaf blight	-	*Allium*	2010	Zhang et al., 2010a
B. sinoviticola	-	Gray mold	-	*Vitis*	2014	Zhou et al., 2014
B. sphaerosperma Buchw.	*Bt. Sphaerosperma (Greg.) Buchw.*	Blight		*Allium*	1949	Buchwald, 1949; Hennebert, 1973; Kohn, 1979; Jarvis, 1980
B. squamosa Walker	*Bt. squamosa Vienn.-Bourg.*	Onion leaf blight	Heterothallic	*Allium*	1925	Viennot-Bourgin, 1953; Bergquist and Lorbeer, 1972; Hennebert, 1973; Kohn, 1979; Jarvis, 1980 ; Farr et al., 1989
B. tulipae Lind	-	*Tulip fire*	-	*Tulipa, Allium, Lilium*	1923	Hennebert, 1973; Kohn, 1979; Jarvis, 1980 ; Farr et al., 1989
Botrytis sp. Group S				*Fragaria, Vitis*	2013	Leroch et al., 2013

3. *Botrytis* host plant

The majority of *Botrytis* species have a global distribution and usually occur wherever their host crops are grown. *Botrytis* species those are considered specialists are with a narrow host range (Mansfield, 1980) confronting either monocotyledonous or dicotyledonous plants (Table 1) but *B. cinerea* is a generalist and a model plant pathogen, which causes a gray mold on more than 1400 different plant species covering nearly 600 genera (Jarvis, 1962; Elad et al., 2007; Dean et al., 2012; Rodríguez-García et al., 2013; Elad et al., 2016). However, the actual number of hosts is likely to be substantially higher, and it will continue to rise as more reports are generated. *B. pseudocinerea* has also been recognized in a variety of plant hosts and may have a vast host range as well (Walker et al., 2011; Plesken et al., 2015a). Recently described species appeared to be polyphagous, for example, *B. prunorum* has been reported as pathogen of plum (Ferrada et al., 2016), kiwi (Elfar et al., 2017), grape (Esterio et al., 2020), pear (Ferrada et al., 2020) and penoy (Garfinkel et al., 2019). Likewise, *B. euroamericana,* has been found in chickpea, grapes and penoy (Moparthi et al., 2020) and *B. caroliniana* has also been reported as a pathogen of blackberry (Li et al., 2012), strawberry (Fernandez-Ortuno et al., 2012), apple, lemon, pear, orange, grape and raspberry (Walker, 2016). On the other hand, other species of *Botrytis* have a narrow host range or are even host-specific (Table 1), such as *B. fabae* (broad bean) (Wu and Lu, 1991), *B. galanthina* (snowdrop) (Beever and Weeds, 2004), and *B. calthae* (marsh marigold) (Plesken et al., 2015b). However, it is now apparent that such strict host specificity may not be always the true case, especially in man-made environments. For instance, in addition to its traditional host *Lilium*, *B. elliptica* has been reported from various genera of distantly related dicot- for example *Stephanotis* (Tompkins and Hansen, 1950), as well as the monocots daylily, *Hemerocallis* (Chang et al., 2001), tuberose, *Polianthes* (Horst, 2013), and toad lily, *Tricyrtis* (Furukawa et al., 2005). In some cases, multiple *Botrytis* species can infect the same host plant; e.g., *B. squamosa*, *B. allii*, and *B. aclada* which poses significant economic threats to commercial onion production. *B.squamosa* is family-specific and pathogenic on onion, garlic, and leek (*Allium* spp.), whereas the closely related sister species are restricted to the lily (*B. elliptica*) and daylily (*B. elliptica*) (Farr et al., 1989; Yohalem et al., 2003). According to Droby and Lichter (2004), *Botrytis* host plants consist of crop species that produce in a variety of climate regions spanning from tropical to temperate regions, in humid as well as in dry locations, in open fields, in greenhouses, in closed environments, and even during cold storage. Host plants affected by *Botrytis* spp. are native to most continents. Vegetables and small fruit crops for example tomato, raspberry, grape, strawberry, blueberry,

apple, and pear are among the most severely affected by *Botrytis*. All plant parts, including seeds and other planting materials, seedlings, stems, leaves, flowers, and fruits at pre-harvest and post-harvest stages are infected by fungi belonging to the *Botrytis* genus. The bulk of the 600 genera infected by *B. cinerea* fall under the category of seed plants, while just a small number fall under the category of flowerless plants and only one under the category of spore-bearing vascular plants (Elad et al., 2016).

The grapevine (*Vitis vinifera* L.) is one of the most significant fruit crops in the world. Worldwide grape production is estimated to be about 73, 524,196.23 tonnes, and harvested area is nearly 6, 729,198 ha (FAOSTAT, 2021). A significant number of pathogens cause diseases in grapevine during the pre-and post-harvest phases, affecting production, processing, and export, as well as fruit quality. Gray mold, powdery mildew, and downy mildew are three of the most common diseases in *V. vinifera*, caused by *B. cinerea*, *Erysiphe necator*, and *Plasmopara viticola*, respectively. Infection of *V. vinifera* with *B. cinerea* causes a post-harvest disease known as " gray mold " which affects entire berry clusters during packaging, transport, and commercialization, making it one of the most serious infections harming export wine and table grapes (Dean et al., 2012). In grapevine, *B. cinerea* is feared by vine-growers because of its qualitative and quantitative impacts on vine production (Bulit and Dubos, 1988).

According to statistics from the Food and Agriculture Organization, tomato (*Solanum Lycopersicum* L.), family Solanaceae, which originated in the Andean area of South America, is the second most widely grown vegetable crop globally after potato, with 189,133,955.04 tonnes from 5, 167, 388 ha (FAOSTAT, 2021). In Southern Europe, it ranks as the highest-yielding vegetable with 213,499 ha. Depending on the growing areas, it is used as a fresh vegetable, in a salad, as ketchup, as a puree, as a pickle, and in many other forms. (http://faostat.fao.org/). In addition to being an important vegetable crop worldwide, the tomato is also used as a model plant species for genetic studies related to fruit quality, stress tolerance (biotic and abiotic), and other physiological traits. It is usually adapted to a variety of climates spanning the tropics to temperate regions. To meet the need for tomatoes, it is also cultivated in greenhouses. Because of its economic role in the agriculture industry, there is abundant interest in using genomic tools to improve tomatoes and develop new varieties. Despite decades of conventional breeding and selection, there are still a large number of fungal diseases that make tomato production challenging in various parts of the world. On tomato plants, more than 50 diseases caused by fungi, prokaryotes, viruses, and nematodes have been documented

(Blancard, 1997; Gleason and Edmunds, 2006). More than 20 fungal infections have been reported, including gray mold caused by *B. cinerea*, late blight caused by *Phytophthora infestans*, early blight caused by *Alternaria solani*, septoria leaf spot caused by *Septoria lycopersici* Speg, fusarium wilt caused by *Fusarium oxysporium fsp. oxysporium.*) and *verticillium* wilt caused by *Verticillium dahliae*. Other fungal diseases of tomato include powdery mildew caused by *Oidium lycopersicum* and leaf mold caused by *Cladosporium fulvum.* Under favourable conditions, *B. cinerea* causes the devastating gray mold disease on tomatoes and the disease can be seen in any cultivated tomato, both in open fields and greenhouses, but variation for resistance to *B. cinerea* among cultivars is small. Gray mold is most common on stems after side shoot trimming in commercial tomato cultivation (Verhoeff, 1970). The relationship between *B. cinerea* and tomato has been carefully investigated (Benito et al., 1998; ten Have et al., 1998; Diaz et al., 2002). According to different research, the species *S. lycopersicum* may have a low level of quantitative resistance to *B. cinerea.* Droplet inoculation of tomato leaves causes rapid development (16–24 hpi) of necrotic patches at the inoculation site, however, infection is temporarily halted for about 48 hours. From that point on, a part of the initial lesions expands, resulting in a rise in fungal biomass and colonization of the entire leaflet over the next 48 hours (Benito et al., 1998). Infection experiments utilizing leaves infected with mycelium grown on agar plugs revealed quantitative resistance to *B. cinerea* in wild relatives of *S. lycopersicum* (Urbasch, 1986). Urbasch, 1986 documented the differences in susceptibility between accessions qualitatively, although the resistance levels may have been overestimated due to the exceptionally favorable conditions for fungal growth created by the abundant nutrients in the agar. Several *Solanum* accessions have been investigated for resistance to *B. cinerea* in quantitative disease assays on inoculated leaves (Nicot et al., 2002; Guimaraes et al., 2004) or stem (Nicot et al., 2002).

4. Management Strategies for *Botrytis* spp.

4.1. Chemical control of *Botrytis* spp.

Current *Botrytis* disease management practices involve the use of chemical control, biological control, cultural practices, and the cultivation of genetically resistant plant varieties. In agricultural production, fungicides are predominantly used on fruits and vegetables and account for more than 35% of the global pesticide market share (Research and Markets, 2014). Chemical control which largely relies on the use of chemical pesticides has been the primary component of most *Botrytis* management programs (Fillinger and Walker, 2016). Without the

use of synthetic fungicides, crop losses to gray mold at pre- and post-harvest stages can reach 40 to 50% (Pedras et al., 2011; Villa-Rojas et al., 2012). Although *Botrytis* control through chemical pesticides has been largely successful, the effectiveness of the treatment depends on the types of pesticides used, when and how often it is applied, and other abiotic factors (Bandara et al., 2020; Carmona et al., 2020). Major fungicides to control *B. cinerea* can be categorized into five groups based on how they affect different processes, including respiration, microtubule assembly, osmoregulation, sterol biosynthesis, and those whose effects can be undone by methionine (Leroux, 2007). Some fungicides have a detrimental effect on non-target beneficial microorganisms (Yang et al., 2011; Karlsson et al., 2014), human health (Pearson et al., 2016), and may also exhibit phytotoxicity, negatively affect photosynthesis and plant biomass production (Dias, 2012). Furthermore, the excessive and repeated use of fungicides has resulted in the development of resistance strains to various fungicides with different modes of action (Chapeland et al., 1999; Rupp et al., 2017b; Shao et al., 2021). The fungicide resistance ability was first encountered in the 1970s when the more effective single-site modes of action started to substitute multi-site fungicides such as copper, sulfur, captan, and thiram. This is the case of two groups of site-specific chemicals, the benzimidazoles, inhibiting β-tubulin polymerization, and the dicarboximides, preventing triglyceride biosynthesis, which were initially highly effective against *B. cinerea* and have been used for many years for its management. However, the resistance to benzimidazoles has early been reported (Bollen and Scholten, 1971) and, for this reason, in the late 1970s and early 1980s, this class of phytosanitary products was replaced with dicarboximides. Once more, their excessive use led to high resistance frequencies and protection failures (Katan, 1982; Beever and Brien, 1983). A well-known example comes from the Champagne wine-growing region, where dicarboximides were initially sprayed four to five times per season until widespread resistance in the populations of gray mold caused a consistent decrease in their protective efficacy. As a consequence, the use of dicarboximides was halted, then later reinstated with only one treatment per season and lengthy rotations. This allowed a decrease in the resistance frequencies together with a raised dicarboximide activity (Leroux and Clerjeau, 1985; Leroux et al., 2002). In the years, some studies allowed a better understanding of the biological mechanisms underlying the resistance development by *B. cinerea*. In this regard, it has been pointed out that the gene responsible for resistance to benzimidazole was *Mbcl* (Yourman et al., 2000) while the *Dafl* gene was discovered to be involved in dicarboximide resistance (Faretra and Pollastro, 1991). The incidence of dicarboximide-resistant isolates generally decreased after the interruption of the fungicide administration (Lorenz, 1988). Another

example of acquired fungicide resistance by this pathogen is represented by that developed against diethofencarb, one of the new chemicals that have hit the market which is registered in Israel and is known to be effective against benzimidazole-resistant strains of *B. cinerea*. Indeed, its use has resulted in the emergence of insensitive isolates as reported by Elad et al. 1992.

Multi-site fungicides, such as dichlofluanid, thiram, captan, and chlorothalonil, have been used against gray mold for a long time. Their preventive activity is primarily due to the inhibition of spore germination, which is related to the block of several thiol-containing enzymes participating in spore germination. These compounds exert a lower efficacy compared to site-specific fungicides. On the other hand, the risk of resistance development is minor precisely because of their non-specific mode of action. However, there are reports of the decreased sensitivity of *B. cinerea* to these multisite fungicides (Barak and Edgington, 1984; Rewal et al., 1991; Pollastro et al., 1996). In general, the development of resistant isolates can be reduced by the applications of mixtures of fungicides, or a rotation of their use (Gullino et al., 1989). On the other hand, to counteract *B. cinerea* using synthetic fungicides is consistently expensive since its management normally requires higher dose rates than other fungal pathogens. In particular, the cost of phytosanitary products against *Botrytis* and associated species accounted for about 8% of the fungicide market worldwide (Fillinger and Elad, 2016). Fungicide investment can vary among crops corresponding to their economic value, their sensitivity to *Botrytis* infection, and their storage time. Additionally, chemical fungicides are harmful to the environment and human health, with particular reference to their toxicological residues. Consequently, botryticide applications are subject to a slew of regulatory restrictions (Droby et al., 2009; Fenner et al., 2013). According to Loomis and Durst, 1992, boron is a crucial element for plants and is useful in the management of *B. cinerea* (Qin et al., 2010). Boron can damage the cell membrane and result in the leakage of cytoplasmic materials from the pathogen (Qin et al., 2010). At present, there are no equivalent alternatives for chemical protection against gray mold; therefore, efforts should be made to find alternative antifungal products against *B. cinerea*. While fungicides have been the focus to provide growers with immediate chemical options, other management practices are critical to developing a sustainable *B. cinerea* integrated pest management program. A summary of knowledge and practices used for the integrated pest management of *Botrytis* around the world, including chemical, cultural, and biological controls is provided by different researchers (Fillinger and Walker, 2016). Furthermore, longer-term research is underway on the use of other methods, including,

biological control, post-harvest treatment, and biotechnological tools such as RNAi technology.

4.2. Biological Control of *Botrytis* spp.

The term "biocontrol" has been expanded in recent years to encompass a variety of different control methods. A wide range of plant and microbe groups have been reported to have inhibitory activity against *Botrytis*-incited diseases, both in laboratory and greenhouse trials but only a few have shown consistent field efficacy (Nicot et al., 2011) and successful protection of vegetable crops with applications of microbial preparations was already documented in the 1950s (Wood, 1951; Newhook, 1957; Dubos, 1992). Alternative methods and integrated protection strategies have drawn more attention globally as a result of the challenges posed by chemical control and mounting social demand to limit pesticide use. Biological control of plant diseases was frequently viewed as a weak link in integrated management strategies in certain crops (Nicot and Bardin, 2012), and anti-*Botrytis* biocontrol products long remained rare despite extensive research efforts (Nicot et al., 2011). However, recent years have seen an increase in the development and use of biopesticides against gray mold. In many cases, it can be believed that biocontrol is an outcome of numerous combined modes of action of the biocontrol agents. This section gives examples of different types of commercially available biopesticides named: plant extracts, living microorganisms, mineral oils, and organic acids. *Melaleuca alternifolia* (tea tree) is registered in over 25 countries, including the United States, Canada, most Central and South American countries, and several European and Southeast Asian countries (Antonov et al., 1997; Nguyen et al., 2013). In field experiments on grapes in California *M. alternifolia* application caused more than 90% suppression of gray mold incidence, which was comparable to the best fungicide treatments (Nguyen et al., 2013). *B. cinerea* conidial germination, germ tube growth, and mycelial growth are all inhibited by *M. alternifolia*, which has multi-target fungicidal properties (Antonov et al., 1997). Moreover, *Reynoutria sachalinensis* (an extract of the giant knotweed) treatment boosts the production and concentration of specific proteins and other anti-*Botrytis* substances (Marrone, 2002).

Biopesticides from living microorganisms comprise the largest number of commercial botryticide products with active ingredients from a wide range of microbial groups (Elad and Stewart, 2004) including bacteria (e.g. species belonging to genera *Bacillus, Brevibacillus,*

Pseudomonas, and *Serratia*) (Redmond et al., 1987; Edwards and Seddon, 1992; Elad et al., 1994; Leifert et al., 1995; Graber et al., 2010; Ajouz et al., 2011; Thakur et al., 2020; Booth et al., 2022), actinomycetes (e.g. *Streptomyces*) (Thakur et al., 2020), yeasts (e.g. species belonging to genera *Acremonium, Aerobasidium, Candida, Cryptococcus, Debaryomyces, Hanseniaspora, Issatchenkia, Pichia, Saccharomyces, Schizosaccharomyces, and Torulaspora*) (Redmond et al., 1987; Edwards and Seddon, 1992; Elad et al., 1994: Santos et al., 2004) and fungi (e.g. *Trichoderma, Ulocladium, Epicoccum, Chlonostachys, Pythium, and Gliocladium*) (Tronsmo and Dennis, 1977; Vinale et al., 2008; Rossi and Pattori, 2009; Vos et al., 2015). Blakeman and Fraser, 1971 and Blakeman, 1972 described the antagonistic effect of bacteria against *B. cinerea* on *chrysanthemum* and beetroot leaves. Blakeman and Brodie, 1976 later documented the suppression of *B. cinerea* and other pathogens by epiphytic bacteria as a general phenomenon. *Bacillus* species such as *B. circulans*, *B. pumilus*, *B. amyloliquefaciens*, *B. brevis*, and *B. subtilis* have been used to control *B. cinerea* (Elad et al., 1994; Mari et al., 1996; Elmer and Reglinski, 2006; Ben Maachia et al., 2015), under field conditions, *B. subtilis* strain QST-713, formulated as Serenade (Agra Quest, USA), offered good gray mold control (Esterio et al., 2000). Control of *B. cinerea* by *Bacillus brevis* which secretes gramicidin S was very effective (Edwards and Seddon, 1992), similarly to *B. cinerea* control by *Bacillus subtilis* and *Bacillus pumilus* as related to antibiotic production (Leifert et al., 1995) *B. subtilis* mode of action combines direct antifungal activity, site exclusion, nutritional competition, and induction of the plant's inherent systemic resistance. According to reports, the bacterium produces three separate kinds of lipopeptides that break down the fungal cells' membranes, killing the pathogen (Ongena, et al., 2010). Additionally, *B.subtilis* stimulates the plant's physiological reactions and internal defenses upon contact. In contrast to systemically acquired resistance (SAR) and induced systemic resistance (ISR), the action is systemic and appears to activate a pathway (associated with induction of PR1) (Ongena et al., 2010). In addition to *B. cinerea*, *Streptomyces* sp. Strain K61 can inhibit or suppress a variety of root rot and wilt fungi. It is approved for use on a variety of crops, including ornamentals, vegetables, and herbs, and is ideal for organic farming. Following application, the microbe develops on the plant's surface, building a biological barrier against plant pathogenic fungi. *S. lydicus* strain WYEC108, another actinomycete, is a commercially available product used to treat a variety of foliar diseases, including gray mold. The pathogen is combated by the actinomycetes, which develop on the foliage's surface, using a combination of competitive exclusion and the release of antifungal substances, including lytic enzymes like chitinases, glucanases, and peroxidases (Crawford et al., 2005; Lichatowich, 2007). There are various

species of *Pseudomonas* described as biological control agents and other bacteria like *Serratia plymuthica* that are known to produce pyrrolnitrin, an antibiotic that prevents mycelia growth of *B. cinerea* (Ajouz et al., 2011). Oxalate-degrading bacteria that protect *Arabidopsis thaliana*, cucumber, grapevine, and tomato leaves from *B. cinerea* were discovered by Schoonbeek et al. in 2007.

Biological control *via* antagonistic yeasts has been employed as another alternative to chemical control to prevent grey infections. Yeasts such as *Pichia* and *Rhodotorula* spp. are effective in the control of *B. cinerea* (Redmond et al., 1987; Edwards and Seddon, 1992; Elad et al., 1994). Santos et al., 2004 reported that some yeasts or their toxins such as *P. membranifaciens* CYC 1106 killer toxin, might have the potential as novel agents to control *B. cinerea*. Furthermore, different researchers reported that a variety of antagonistic yeasts can effectively prevent post-harvest decay caused by *B. cinerea* in different fruits (Piano et al., 1997; Fan and Tian, 200; Tian et al., 2002; Qin et al., 2004). Antagonistic yeasts modes of action against fungal pathogens include competing for space and nutrients as shown by yeasts such as *Candida oleophila* applied on apple fruits (Mercier and Wilson, 1994) or *Aureobasidium pullulans* on grapes (Schilder, 2013), *Penicillium expansum* on apple (Janisiewicz et al., 2000), *Pichia membranefaciens* on apple (Chan and Tian, 2005), inducing host resistance (Navazio et al., 2007; Tian et al., 2007; Hermosa et al., 2012). Two strains of *Aureobasidium pullulans* (14940 and 14941), registered for grape protection in the USA and several countries in Europe, were applied before harvest with no alteration of the colour or taste of the treated grapes. *A. pullulans* works through natural competition for space and nutrients in the berries. Because of the high proliferation rate of *A. pullulans* after application, the pathogen cannot infect the plant (Schilder, 2013). However, antagonistic yeasts do not have the same effect as fungicides, and to achieve a satisfactory result, a combination of fungicides and exogenous compounds is usually required (Droby et al., 2009). The postharvest pathogens *B. cinerea* and *Penicillium expansum* were investigated for this purpose, and two yeasts with higher (*Cryptococcus laurentii* LS-28) or lower (*Rhodotorula glutinis*) antagonistic activity were examined. LS-28 was more tolerant of oxidative stress brought on by ROS. According to Castoria et al., 2003, biocontrol yeasts' ability to fight off infections that cause postharvest wounds may be mediated by their resilience to oxidative stress.

Newhook, 1951 and Wood, 1951 research work contains early examples of biocontrol with common species of microorganisms. The researchers used antagonistic *Fusarium* spp. and

Penicillium claviforme that originated from the same crop to inoculate senescent lettuce leaves with successful prevention of *B. cinerea* primary establishment. Wood (1951) concluded that saprophytic activity on dead lettuce tissue was responsible for disease control to a great extent, under natural conditions. Later, Newhook (1957) used a spore suspension of *Cladosporium herbarum* and *Penicillium* spp. to counteract gray mold on glasshouse tomatoes by spraying it on the floral debris attached to the fruit. *C. herbarum*. The approach also efficiently controlled gray mold in strawberries by safeguarding the flowers under field conditions (Bhatt and Vaughan, 1962). Since the 1970s, even *Trichoderma* spp. have been employed to suppress this pathogen on strawberry (Tronsmo and Dennis, 1977), snap bean blossoms (Nelson and Powelson, 1988), grapes (Gullino, 1991; Dubos, 1992; O'Neill et al., 1996), and greenhouse crops (Elad et al., 1995). *Trichoderma* spp. strains are described by multiple mechanisms of action (induction of plant resistance, mycoparasitism, antibiosis, and competition for space and nutrients), which may all contribute to the reduction of plant diseases (Vinale et al., 2008; Rossi and Pattori, 2009; Vos et al., 2015). *T. harzianum* isolate T39 (Makhteshim-Agan, Israel) was the first to be developed as Trichodex (Elmer and Reglinski, 2006). The use of isolate T39 resulted in partial control of gray mold in table grape, which was significantly different ($p < 0.05$) from untreated controls and equal to or less than the control achieved with vinclozolin (Ronilan 50 WP, 1.5 kg ha1), even if similar to the control achieved with Captan (Captan 80 WP, 4 kg ha1) (Harman et al., 1996; Latorre et al., 1997). Similarly, research has revealed that *T.harzianum* T39 is implicated in a complex transcriptional reprogramming in grapevines (Palmieri et al., 2012) that affects proteins involved in stress responses, photosynthesis, redox signaling, and energy metabolism (Perazzolli et al., 2012). The *Trichoderma* population on table grape blossoms and clusters declined quickly, indicating that it was only present for a short time in the grape canopy (Latorre et al., 1997). Salicylic acid (SA) and ethylene (ET)-related genes are primed in strawberries by the *Trichoderma* that causes systemic resistance to *B. cinerea* (Meller et al., 2014). According to these and other studies, disease control in the field appears to be adequate only when disease pressure is low to moderate (Latorre, 2013; Montealegre and Perez, 2014). In addition, *Botrytis* conidiation has also been suppressed using *Ulocladium* species (Köhl and Fokkema, 1993). An extensive study was done on the effects of microbial compounds on *Botrytis* species (Elad and Stewart, 2004). For instance, *Penicillium chrysogenum* produced inhibitory compounds that inhibited the development of *faba bean* lesions on faba bean and *B. fabae* conidia germination (Jackson et al., 1994), and *B. cinerea* conidia were suppressed by peptaibol antibiotics from *T. harzianum* and gliotoxin from *G. virens* (Schirmböck et al., 1994). *T. hamatum*, an inhibitory volatile-producing fungus, reduced

snap bean gray mold (Nelson and Powelson, 1988). It should be emphasized that the antibacterial substances may affect both non-target organisms and the plant pathogen. Some of the most well-known mycoparasites capable of attacking the mycelium of *B. cinerea* include *Trichoderma*, *Gliocladium,* and *Pythium* spp (Elad, 1996). Sclerotia parasitism has also been documented (Dubos et al., 1982; Köhl and Schlösser, 1989). Cell wall degrading enzymes such as proteinases, mannanases, laminarinases, and chitinases have been linked to mycoparasitism (Labudova and Gogorova, 1988), and genes coding for some of these enzymes have been identified (Geremia et al., 1993; Viterbo et al., 2001; Kamensky et al., 2003). Further progress has been accomplished lately in the understanding of the *Botrytis* -biological control agent (BCA) interactions with the analysis of secreted protein patterns of *T. harzianum* ETS 323 in laboratory conditions (Yang et al., 2009). Two BCA endochitinases and one L-amino acid oxidase (LAAO) were specifically induced in the media that included only deactivated *B. cinerea* mycelium as a carbon source. β-1,3-glucanases, β-1,6-glucanases, chitinases, proteases, and xylanases activities were higher in media containing deactivated *B. cinerea* mycelium than in other media, implying that the cell wall of *B. cinerea* is indeed the principal target of the biological control agent in the biocontrol mechanism (Yang et al., 2009). The impact of *T. harzianum* ETS 323 on *B. cinerea* during the mycoparasitic process was assessed in culture using a biexponential equation (Cheng et al., 2012). When the BCA was grown with deactivated hyphae of *B. cinerea*, the secretion of LAAO by *T. harzianum* ETS 323 increased, and this oxidase inhibited *B. cinerea* growth in vitro and on apple fruit and tobacco leaves. Additionally, after being treated with LAAO, *B. cinerea* showed an apoptosis-like reaction, including the production of reactive oxygen species, which suggests that it causes *B. cinerea* to undergo programmed cell death. A two-step antagonism of the BCA against *B. cinerea* was proposed by Cheng et al. in 2012. However, in reality, a BCA's capacity to function as a mycoparasite or to produce cell wall-degrading enzymes does not necessarily ensure effective biocontrol under field circumstances, as the activity of mycoparasites is typically thought to be too slow to effectively reduce the fast *Botrytis* penetration process into the host tissue. As a result of interference with the pathogenicity processes, the *T. harzianum* strain T39 stops *B. cinerea* from penetrating the host tissue (Zimand et al., 1996). T39 reduced the activities of exo- and endo-polygalacturonase, pectin methyl esterase, and pectate lyase (Zimand et al., 1996), chitinase, β-1,3-glucanase and cutinase produced by *B. cinerea* (Kapat et al., 1998). T39 decreased the activities of pectin methyl esterase, pectate lyase, exo- and endo-polygalacturonase, chitinase, β-1,3-glucanase, and cutinase produced by *B. cinerea* (Zimand et al., 1996; Kapat et al., 1998). It was discovered that the *T.harzianum* T39 strain produced a

cysteine protease that inhibited pathogenicity-related enzymes of *B. cinerea* and subsequent disease development. As part of its biocontrol mechanism, *T.harzianum* T39 was found to produce a cysteine protease that inhibited the pathogenicity-related enzymes of *B. cinerea* and the subsequent development of disease (Elad and Kapat, 1999). This was further supported by the observation that the biocontrol activity was abolished by a specific inhibitor of the T39 protease (Elad et al., 1998). As epidemics produced by *Botrytis* spp. are usually polycyclic, a reduction in inoculum production could have a cumulative effect over numerous disease cycles (Köhl and Fokkema, 1993). *Ulocladium atrum* inhibited the sporulation of *B. cinerea* on the dead leaves of lily and onion subjected to field conditions (Köhl et al., 1995). The colonisation of necrotic tissue by *U. atrum* inhibits saprophytic colonization of those leaves by *B. cinerea*. The systemic resistance to *B. cinerea* was observed in leaves harvested from plants growing in the treated soils and drenching with a T39 suspension stimulated salicylate and ethylene-related gene expression in a way proportionate to the concentration of *Trichoderma* (Meller et al., 2014). For a biological control agent to activate the ISR pathway, the priming effect of T39 on defense and microbial recognition-related gene expression upon infection with *B. cinerea* has been documented (Shoresh et al., 2005; Ahn et al., 2007; Malmierca et al., 2012; Palmieri et al., 2012). Tucci et al. 2011 revealed that *Trichoderma* strain T22-induced gene expression resulted in the up-regulation of SA-marker genes before infection with *B. cinerea* and the downregulation of the same genes after infection. T39 suppressed the SA-responsive genes before inoculation. T39 induced substantial priming of the SA-responsive gene expression after *B. cinerea* inoculation (Perazzolli et al., 2011; Tucci et al., 2011).

A variety of oil-based products claim to be effective against *Botrytis*-related diseases. Paraffinic oil and neem oil are the most frequent ones. Paraffinic oil is used to treat fungal infections such as *Botrytis* bunch rot. The oil may smother fungal growth and inhibit conidia germination on treated surfaces, and it is largely fungistatic, stops fungal growth instead of killing the pathogen. To combat fungal pathogens, the oil must be administered prophylactically before infection. To attain desirable levels of control, the oil may need to be applied several times. The commercial product Neem Oil Trilogy ® 90EC is based on a purified hydrophobic extract of neem oil. This product is most commonly used to treat powdery mildew, although it has also been reported to work against *B. cinerea* on citrus, cucurbits, bulbs, vegetables, small fruit, and nuts. Thorough coverage of the plant is required, and caution should be exercised when applying the product to avoid the possibility of leaf burn. The product has a four-day re-entry interval and can be applied up until harvest day. It is suitable for organic

production, but bee toxicity means it can't be used during the bloom season. The product works by preventing fungal attacks on the plant tissue. Generally, extensive knowledge has been increased on biological control of plant diseases of fruit, vegetable, and flower products during cultivation or post-harvest. Bio-fungicides are primarily used as preventative treatments, as they are ineffective after infection. They should be used when climatic conditions are favorable for bio-fungicide colonization, before periods of high vine vulnerability, such as flowering and leaf plucking/trimming, and after veraison (Whipps and Lumsden, 2001; Elad et al., 2016). Certain climatic conditions are required for bio-fungicides colonization, and variations in these parameters limit efficacy (Elad and Stewart, 2004). As a result, biological control is less consistent than that achieved by synthetic fungicides (Elmer and Michailides, 2004; Elmer and Reglinski, 2006). Furthermore, unlike systemic fungicides, most bio-fungicides require direct contact with the pathogen, hence disease control efficiency is frequently reduced after bunch closure when their penetration becomes ineffective. The biological control agent is unlikely to remain inside the bunch after this time (Holz and Volkmann, 2002) when grapes are most susceptible to infection (Nicholas et al., 1994). The lack of field reliability of bio fungicides has been a major obstacle in the adoption of this technology, but these products can be used effectively in combination with other techniques, including synthetic fungicides (Fillinger and Elad, 2016).

Although there are few instances of stable performance (Calvo-Garrido et al., 2013; Ilhan and Karabulut, 2013), biological control is typically thought to be variable in field circumstances, either across various sites or from one growing season to the next (Nicot et al., 2011). The survival, establishment, and activity of BCAs in commercial production systems can be influenced by changing environmental conditions (Morandi et al., 2008). The characteristics of a BCA that affect its persistence on the plant determine how effective it is at protecting the plant. Suitable shelf life has been ensured by formulations that have been created to guarantee both the survival of the BC3A and its protective characteristics, as demonstrated for *Pseudomonas* (Janisiewicz and Jeffers, 1997), *A. pullulans* (Mounir et al., 2007) or various *Trichoderma* spp. (Ruocco et al., 2011). As demonstrated for *Rhodotorula* (Calvente et al., 2001) and *Pichia carribbica* (Zhao et al., 2012, 2013), production processes also have a significant effect (Jackson et al., 1991), and significant advances in protection efficacy can be gained by adjusting the nutritional substrate composition.

4.3. Cultural methods *Botrytis* spp. management

In addition to the application of chemical control and biocontrol treatments, different cultural methods aiming to decrease humidity around the plant and limit plant surface wetness can be combined for improved *Botrytis* disease control. The effect of leaf removal on gray mold control has been proven in previous research (Gubler et al., 1991). The effectiveness of this practice is influenced mainly by seasonal weather conditions. In comparatively dry seasons, leaf removal is highly effective, but in extremely rainy seasons, it is insufficient to achieve a high level of gray mold control. Leaf removal, on the other hand, improves the efficacy of fungicide application during extremely wet seasons (English et al., 1993). *B. cinerea* and another epiphytic fungus often found on grapefruit are reduced when leaves are removed (Duncan et al., 1995). It can also boost phytoalexin production, as well as epicuticular wax and cuticle formation, in exposed berries, preventing gray mold diseases (Percival et al., 1993).

The evaporative potential inside the vine canopy has been suggested as a simple method of determining canopy openness and drying conditions as a result of leaf removal (English et al., 1993). The evaporative capacity of a grape canopy is inversely proportional to canopy density, with 1 mL h^{-1} indicated as the minimal evaporative potential for reducing gray mold (English et al., 1993). Shoot reduction comprises the removal of an excessive number of shoots per vine to adjust microclimate conditions under the grape canopy, which minimizes the conditions permissive to gray mold (Savage and Sall, 1982; Bettiga et al., 1986). These viticultural approaches improve airflow and sunshine penetration within the grape canopy, which is particularly significant for table grapes trained as Pergola, which have a dense foliage canopy (Zoecklein et al., 1992). Cluster removal and thinning are required in most table grape cultivars to obtain quality fruit to meet market demand. These viticultural measures are also critical in the prevention of severe gray mold. Cluster removal during harvest reduces overcropping and bunch crowding, preventing cluster maturation delays and ensuring high-quality berries. Cluster thinning reduces cluster compactness, improves airflow within clusters, and reduces berry-to-berry contact, limiting cuticle formation at contact points and preventing berry split in the center of the clusters. Furthermore, as cluster compactness rises, fungicide spray coverage degrades (Zoecklein et al., 1992; Tardaguila et al., 2008; Hed et al., 2009). Additionally, cluster thinning has been shown to boost the total resveratrol level (Prajitna et al., 2007). As we observed above cultural management is another viable method of decreasing fruit rot damage. When extra branches and leaves are eliminated, sclerotia and/or conidia formation and spread

are minimized (Gubler, 1987). Cultural control, on the other hand, is frequently unrealistic in large-scale commercial farming. Gray mold thrives in conditions of high humidity, little light, and moderate temperature. As a result, creating an open canopy that allows for enough airflow and good light interception is useful in crop management, allowing rain or irrigation water droplets to dry as rapidly as possible. High relative humidity enhances conidial production by allowing germination and penetration of the host. Gray mold has a diverse set of cultural activities, many of which are tailored to certain species and cropping systems. Pruning perennial woody plants like grapevines is beneficial because it inhibits excessive vegetative growth (Gubler et al., 1987). Nitrogen fertilization in excess encourages rapid vegetative growth while also raising the risk of gray mold and other ailments. Rain shelters and tunnels made it possible to overcome some of the problems with soft fruit production induced by rain during the blossoming season, allowing for a huge increase in strawberry and raspberry crop area. Ninety percent of disease reductions in strawberries cultivated under plastic have been recorded when compared to field-grown plants (Xiao et al., 2001). However, increasing airflow inside these structures to reduce high relative humidity and prevent leaf soaking is still necessary. Infection of the leaves and stems remains when the plastic covers are removed in late summer, resulting in mycelium and sclerotia overwintering. In several crops, near-UV filters embedded in plastic coverings are efficient in minimizing conidiation and infection (Reuveni et al., 1989; Reuveni and Raviv, 1992; West et al., 2000). Fresh product post-harvest management relies significantly on 'cold-chain marketing' of slightly underripe and minimally injured fruits. Several plant defense systems are still active in the host tissues at this time; however, gray mold damage can be considerably reduced if the temperature during transportation is strictly controlled. In practice, the amount of inoculum accumulated throughout the growing season has a big impact on the spread of gray mold after harvest.

Outbreaks of *Botrytis*-induced gray mold are prevalent in open fields, orchards, and greenhouses. High humidity and the presence of a layer of water on vulnerable plant organs encourage infection, although these circumstances can be controlled to prevent infection. Heating greenhouses was once a popular method of managing humidity in those structures, and it is still practiced in some temperate locations. However, the high cost of active heating has forced farmers in some areas to abandon this disease-management strategy, resulting in an increase in the incidence and severity of gray mold as susceptible organs of crop plants remain wet for longer periods, leading to an increase in the incidence and severity of gray mold. Reduced planting density, crop canopy management to allow for aeration of the crop or

susceptible organs via passive and active ventilation (Trolinger and Strider, 1984; Elad and Shtienberg, 1995; Legard et al., 2000; Xiao et al., 2001), fertigation with increased potassium and calcium (Wojcik and Lewandowski, 2003; Yermiyahu et al., 2006), and reduced nitrogen (Yermiyahu et al., 2006), the use of soil mulch and passive solar heating of unheated greenhouses, timing fungicide applications for the best protection, and avoiding harvesting on rainy days are all cultural methods for controlling *Botrytis*-incited disease. Mineral nutrients are vital for plant growth, development, and reproduction. Nutrients can also affect plants' exposure to pathogens (Engelhard, 1989). The development of fruit and vines depends on calcium, which also influences many cell functions and boosts disease resistance (Conway, 1982; Ferguson, 1984; Conway et al., 1991; Volpin and Elad, 1991). It has been demonstrated that calcium in plant tissues reduces the severity of gray mold (Volpin and Elad, 1991; Chardonnet and Doneche, 1995; Bar-Tal et al., 2001; Yermiyahu et al., 2006). Likewise, increased calcium concentration in strawberries decreased the frequency of *Botrytis* infection (Cheour et al., 1990; Karp and Starast, 2002; Wojcik and Lewandowski, 2003). Similarly, gray mold incidence on chrysanthemum flowers increased quadratically when nitrogen was provided at concentrations of 1.5, 3.8, and 6.0 g/m^2 (Hobbs and Waters, 1964). In contrast, Verhoeff, 1968 discovered that the susceptibility of soil-grown tomatoes to gray mold increased with diminishing levels of nitrogen in the soil.

A harvest of cucumbers from a crop with two stems per plant had more cucumber gray mold on the fruits than a crop with one stem per plant (Elad and Shtienberg, 1995). *Exacum affine* flower parts grown in compacted conditions produced higher amounts of gray mold than those grown in less crowded conditions (Trolinger and Strider, 1984); similarly, densely planted strawberry plants showed comparable results (Legard et al., 2000). Gray mold-prone crops can be cultivated in polyethylene tunnels. *B. cinerea* sporulation is affected differently by several types of polyethylene (Reuveni et al., 1989). The use of UV-blocking film (up to 405 nm) instead of the conventional film decreased the incidence of infection during two seasons in primula and strawberry crops cultivated beneath polyethylene tunnels (West et al., 2000). After several weeks conidia production was minimal in tomato plants inoculated with *B. cinerea* due to the presence of polyethylene films that absorbed ultraviolet radiation (Nicot et al., 1996). According to studies on gray mold, it is best to utilize heating, aeration, and ventilation of the greenhouse to lower humidity levels and the amount of dew on susceptible plant tissues (Morgan, 1984; Jarvis, 1992; Elad and Shtienberg, 1995; Eden et al., 1996; Dik and Wubben, 2004). These findings highlight the value of ventilation in reducing humidity levels and

avoiding the occurrence of infection-promoting environments. Gray mold can be influenced by row and field positioning and direction, as these factors affect local air movement and temporal temperature fluctuations. A combination of treatments can suppress gray mold more effectively than individual treatments, and proper integration of management techniques can offer adequate disease control with little chemical fungicide use. Infections induced by pathogenic *Botrytis* species are more common in high-humidity environments, and cultural practices that reduce humidity can help to suppress those infections. As different findings show in some circumstances, cultural approaches improve the resistance of the crop to the disease. For better disease control, different cultural techniques can be integrated. Cultural approaches can also be used in conjunction with chemical botryticides or biocontrol treatments. A decision-support system, such as BOTMAN (*Botrytis* Manager) (Shtienberg and Elad, 1997), may be used in integrated crop management, including disease control.

4.4. Improving resistance of plants to diseases of *Botrytis* spp.

Enormous attempts to develop resistant varieties have encouraged researchers to look for new breeding technologies based on the knowledge of genes and genomes. To develop novel cultivars that address difficulties with disease resistance, many nations have created new breeding initiatives. Breeding-resistant plants are currently the most popular and sustainable strategy for fungal disease management. The development of disease-resistant varieties is possible via conventional breeding methods or genetic engineering by incorporating resistance mechanisms obtained from other plant species or pathogens (Hammond-Kosack and Parker, 2003). The genetic engineering method includes several methods that can purposefully alter the genome of plants to obtain resistant cultivars with the desired properties for growers and consumers (Holme et al., 2013; Grohmann et al., 2019; Villano and Aversano, 2020). As an example of breeding strategy in *Vitis* spp., some strong heritable host resistance sources against *B. cinerea* have been discovered. However, these resistant cultivars have undesirable economic traits such as thicker skins and greater epidermal waxes (Naegele, 2018). Similarly, Wan et al., 2015, examined the genotypes of cultivated *V. vinifera* and wild grape species from China for *B. cinerea* resistance. Consequently, the wild species from China were distinguished by low infection rates and strong fungus resistance. *B. cinerea* resistance, like resistance to other necrotrophic infections, is largely multigenic. In addition, fungicide resistance genes are inversely related to fungal pathogen resistance genes, making efficient breeding challenging (Naegele, 2018). Although traditional breeding for gray mold resistance in tomatoes has achieved significant progress

(Naegele, 2018), breeding for *B. cinerea* resistance has been difficult and unrewarding in most crops. As a model, the approaches employed to examine tomatoes could be applied to other plants. Wild *Solanum* species closely related to the cultivated tomato *S. lycopersicum* have partially resistant leaves and/or stems (Guimarães et al., 2004; ten Have et al., 2007). Thus, gray mold resistance was introduced into *S. lycopersicum* using the genotype LYC4 of *S. habrochaites*. Three quantitative trait loci (QTLs) for resistance were found in a segregating F2 population (Finkers et al., 2007a, 2007b). Seven novel QTLs were found in a population of 30 introgression lines containing separate well-defined sections of *S.habrochaites* LYC4 chromosomes in the genetic background of *S. lycopersicum* (Finkers et al., 2007b). One of the genotypes created in these studies included multiple QTLs and demonstrated an 85 percent reduction in gray mold disease parameters when compared to the susceptible parent (Finkers et al., 2007b). Because of the high disease load in these tests, partial resistance levels may confer absolute resistance in ordinary greenhouse cultures with lower disease pressure. The QTLs for gray mold resistance in tomatoes have the potential to improve disease control in tomatoes. The mechanisms underlying the enhanced resistance are still being studied, and an introgression line population is an excellent tool for examining resistance mechanisms governed by specific QTLs. It will be possible to use gene transfer techniques to strengthen the host response to infection without loosing other essential plant traits that agribusiness and consumers seek with a better understanding of the underlying mechanisms of genetic resistance. In rose flowers, RcERF099, a gene encoding a member of the AP2/ERF transcription factor family, was recently found to be involved in the regulation of resistance against *B. cinerea*. This finding can serve as a springboard for additional research aiming to increase gray mold disease resistance in roses (Li et al., 2020). Some plants naturally show resistance to some *Botrytis* species, for example, lettuce cultivars to *B. cinerea* (Ogilvie and Croxall, 1942), and onions to *B. squamosa* (Bergquist and Lorbeer, 1971), peanut cultivars (Alexander and Boush, 1964), as well as raspberry cultivars (Barritt, 1971; Mel'nikova, 1972), and strawberry cultivars (Barritt et al., 1971; Kolbe, 1971; Priedite and Ozolina, 1971; Barritt, 1972 and Naumova, 1972). Such resistance, however, is never absolute and is most likely of the polygenic type. Some plants have a habit that decreases the probability of infection. According to Darrow (1966), firm-fruited strawberry cultivars, as well as those with less dense foliage, are less susceptible to *B. cinerea* than soft-fruited cultivars, however, their exposed early flowers were more susceptible to frost damage. Tompkins, 1950 demonstrated that *Begonia* cultivars with red flowers and hairy stems were found to be more resistant to *B. cinerea* than cultivars with light-colored flowers and smooth branches. A similar remark was

made by Jennings, 1962 and Knight, 1962 in the case of raspberry canes; the more resistant cultivars have comparatively hairy, spineless, waxy, and non-pigmented canes and Jennings recognized escape, at least in part, to a greater runoff of surface water. Esmarch, 1926 indicated that strawberry cultivars with long stiff inflorescences that held flowers and fruit above the foliage canopy would be less susceptible to *B. cinerea*, and similarly, Koch, 1963 bred such a cultivar.

4.5. RNA interference (RNAi)

RNA interference (RNAi) or RNA-induced gene silencing, hailed as a breakthrough in molecular biology is a powerful genetic tool used to assess gene function by interfering with endogenous gene expression at the transcriptional or posttranscriptional level. It is considered a promising approach for plant protection against various biotic stresses, including fungal pathogens (Zotti et al., 2018; Schaefer et al., 2020), via host-induced gene silencing (HIGS) or spray-induced gene silencing (SIGS) (Nowara et al., 2010; Koch et al., 2013; Wang et al., 2016; Islam and Sherif, 2020; Gebremichael et al., 2021). In contrast to chemical fungicide-based control approaches, RNAi-based pathogen control methods offer eco-friendly strategies to combat fungal diseases (Wang et al., 2016; Gebremichael et al., 2021). The high specificity of the RNAi mechanism, the fact that it occurs in nearly all eukaryotes, and the regular consumption of RNA by humans in the form of fresh fruits and vegetables indicate that RNAi-based disease management is expected to be non-toxic (Fletcher et al., 2020). Neither HIGS nor SIGS entails the expression of foreign proteins, further reducing the toxic side effects following intake by animals/humans (Nunes and Dean, 2012; Koch et al., 2013; Koch et al., 2016; Davalos et al., 2019; Xiong et al., 2019).

Host-induced gene silencing (HIGS), an RNAi-based process in which RNA molecules (dsRNA or a hairpin-structured dsRNA) are produced by the host plant to target invader transcripts, has emerged as an effective strategy for enhancing plant resistance against phytopathogens (Nowara et al., 2010; Ghag, 2017; Sang and Kim, 2020). HIGS has been widely used to enhance resistance against *B. cinerea*, by expressing dsRNAs that target essential fungal genes in host plant species leading to disease resistance (Wang et al., 2016). In a study to control *B. cinerea*, Arabidopsis plants expressing hairpin RNA (hpRNA) targeting Dicer-like genes of *B. cinerea* (Wang et al., 2016) exhibited enhanced resistance to the pathogen. Similarly, transgenic potato and tomato plants expressing the dsRNA of the TOR

gene of *B. cinerea* strongly reduced the occurrence of gray mold in these host plants (Xiong et al., 2019). The results of HIGS mentioned above corroborate the hypothesis that RNA molecules can move from plants into fungal cells and effectively silence their target genes.

In addition to the production of RNA molecules in planta as noted in HIGS, SIGS can also be used by spray-applied biopesticides to control *B. cinerea*. Spray-induced gene silencing is a novel RNAi-based strategy for silencing target genes against phytopathogens using exogenous applications (i.e., dsRNA, siRNA, and hpRNA). In this approach, the exogenously administered dsRNA can either be directly taken up by the fungal cells (Koch et al., 2016; Wang et al., 2016; McLoughlin et al., 2018; Qiao et al., 2021) or transit through plant cells (cross-kingdom RNAi) (Wang et al., 2016; Cai et al., 2018). In SIGS, the dsRNA/siRNA that enter the fungal cells are processed by the fungal RNAi machinery for targeted gene silencing, causing fungal growth arrest (Dang et al., 2011). Several studies have demonstrated the ability of exogenously administered RNAi molecules to protect plants against various fungal diseases (Koch et al., 2016; Song et al., 2018). The exogenous administration of dsRNA and siRNA has been reported to decrease *B. cinerea* infection, thereby introducing a new era of RNAi-based fungicide strategies for controlling gray mold diseases. Exogenous applications of the dsRNAs that target *B. cinerea* genes, such as thioredoxin reductase, mitochondrial import inner membrane translocase subunit TIM44, peroxidase, pre-40S ribosomal particle, and necrosis- and ethylene-inducing peptide 2, reduced the severity of canola plants infection by *B. cinerea* (Mcloughlin et al., 2018). In another study, exogenous administration of dsRNAs and siRNAs targeting Dicer-like 1 and 2 (*DCL1* and *DCL2*) genes of *B. cinerea*, significantly reduced the gray mold diseases in fruits (e.g., tomato, strawberry, and grape) and vegetables (e.g., lettuce and onion) (Wang et al., 2016). The virulence genes of *B. cinerea* are a potential target for RNAi-based fungicides (Choquer et al., 2007). For example, mutations in the *B. cinerea* chitin synthase genes (*Bcchs3a*) (Soulie et al., 2006) or the genes engaged in signal transduction such as G subunits of G-proteins (Bcg1, Bcg2, and Bcg3) (Gronover et al., 2001; Doehlemann et al., 2006) decrease the virulence of *B. cinerea* in diverse plant species.

RNAi-based biopesticides have a lot of potential as an alternative to chemical-based control approaches since they target pathogens with specificity and accuracy. SIGS involves no stable genetic transformation (Machado et al., 2018), making it a more acceptable alternative to genetically modified organisms that need regulatory agency approval. Therefore, the exogenous method has the potential to be more readily accepted by the public and biosafety

regulators. In addition, SIGS does not need the development of efficient transformation techniques for each crop species, nor does it limit the technology to a particular gene or application (Koch et al., 2016; Wang and Jin, 2017; McLoughlin et al., 2018; Taning et al., 2020; Gebremichael et al., 2021). However, RNAi has its own limitations to overcome including target genes for RNAi-based fungicides are still limited, thorough transcriptome investigations are necessary to identify target genes for biotrophic and necrotrophic phases of *B. cinerea* infections. Moreover, the exogenous dsRNA uptake mechanisms by plants or fungi cells are still inexplicable. Our comprehension of the fundamental small RNA uptake mechanisms may be enhanced by a clearer understanding of the functions of membrane-bound proteins and receptors found in plant and fungal cells. The stability of naked dsRNA molecules under field conditions is another significant issue that could limit the application of SIGS-based disease management strategies (Landry and Mitter, 2019). These limitations might be solved by using nanoparticles and other stabilizers to improve the stability and sustained release of the RNAi-bio fungicides (Mujtaba et al., 2019; Avila-Quezada et al., 2022). According to Dubrovina and Kiselev, 2019, the effectiveness of naked RNA molecules typically lasts only a few days. However, plants sprayed with dsRNA loaded onto nanoparticles (such as BioClay) have demonstrated a better level of protection against pathogenic infections for 30 days after application, both on treated and newly emerged leaves (Mitter et al., 2017). Additionally, it has been noted that nanoparticles enhance the target genes' ability to be silenced and the uptake of dsRNA by root tips (Worrall et al., 2018). The development of affordable and scalable methods for the synthesis of RNAi-bio fungicides is likely the major challenge facing the practical agricultural utilization of this technology. However, in the future, these problems might be resolved by technologies like bacterially expressed small RNA (dsRNA, and hpRNA), and minicells. Since the sequence identity of the siRNAs and mRNA targets determines the RNAi specificity, there is a possibility of off-target effects that could result in the silencing of other transcripts with sufficient sequence identity (Casacuberta et al., 2015). Even in the host plant, this off-target is still possible (Papadopoulou et al., 2020). This can be reduced by careful design of dsRNAs on the target genes by identifying and avoiding the contagious matches to guarantee the reduction in homology to off-target transcripts (Naito et al., 2005; Gebremichael et al., 2021). In conclusion, SIGS RNAi-based bio fungicides have enormous potential for controlling deadly gray mold diseases. However, future research should concentrate on formulation, synthesis, stability, and application techniques for RNAi-based fungicides to enable the implementation of SIGS in open fields and to increase their effectiveness, applicability, and cost-effectiveness. On the other hand, despite the great potential of the HIGS

strategy to control Phytopathogens, there are a variety of drawbacks that must be considered. The costs associated with the development, registration, and maintenance of genetically modified crops, as well as the challenges related to the public acceptance of these plants and the lack of a stable genetic transformations system for several economically important crops (Wang et al., 2016), are the main problems that could be mentioned at the forefront. Due to these problems utilization of HIGS as a disease management strategy against *B. cinerea* is not currently attainable. A comprehensive review of SIGS techniques is presented in the third chapter.

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Chapter 2. Grape downy mildew and control methods

Abstract

The grapevine (*Vitis vinifera*) is one of the most important crops in the world based on cultivated area and economic value. Grapes can be used as fresh fruit, for the production of juices or dried grapes, as well as for the production of goods with a high added value such as wine and other alcoholic beverages. Like many other cultivated plant species, grapevines are targets for both biotic and abiotic stresses due to their adamant nature. These stresses have triggered major concern worldwide with substantial losses in crop yields and quality. Grapes are impacted by an onslaught of diseases that hinder their cultivation. Diseases of grapes, including *Plasmopara viticola*, *Erysiphe necator,* and *Botrytis cinerea* cause significant annual losses. Downy mildew represents one of the most serious diseases of the vine, particularly in mild and humid climates. The disease affects leaves, shoots, inflorescences, and infructescences. All the vine cultivars in Europe used for the production of grapes are susceptible to downy mildew, and infections of this disease cause enormous economic damage. An understanding of the *P. viticola* life cycle is essential for the management of the disease. *P. viticola* has both sexual and asexual reproductive forms, causing primary and secondary downy mildew infections, respectively. Its management primarily relies on intensive use of fungicides throughout the growing season, which leads to environmental pollution threatening both biodiversity and human health. Therefore, new disease management strategies must be developed. Those alternative approaches may include the cultivation of disease-resistant varieties, the use of biocontrol products, and the application of RNA interference technique.

Keywords: Grapevine, *P. viticola,* downy mildew, control method, RNAi

1. Introduction

Like many other cultivated plant species, the vine is susceptible to numerous biotic and abiotic stresses which often compromise both the productivity of the vineyards and the quality of the grapes produced, causing enormous economic damage not only to the growers but also to the entire stakeholders. Disease control is commonly based on the intense application of chemicals, often harmful to humans and the environment. In recent years, however, there has been strong social and legislative pressure that requires a gradual reduction in the use of chemical products in agriculture (European regulation 1107/2009). *Vitis vinifera* is susceptible to numerous pathogens, in particular fungi and oomycetes represent an important class of pathogens that compromise vine quality and yield (Delaunois et al., 2014). Among them, the biotrophic oomycete *Plasmopara viticola (P. viticola)* (causal agent of grape downy mildew), the biotrophic ascomycete *Erysiphe necator* (causal agent of grape powdery mildew), and the necrotrophic deuteromycete *Botrytis cinerea* (causal agent of gray rot) are the three main pathogens (Delaunois et al., 2014). Furthermore, fungi associated with grapevine wood diseases (e.g., *Phomopsis viticola*, *Botryosphaeria dothidea,* and *Eutypa lata*) are responsible for other important grapevine diseases (Ferreira et al., 2004). Due to its historical, social, and economic importance, the vine is one of the most studied agricultural plants for the prevention and protection from natural adversities and is therefore a "model" plant for the study of the mechanisms of plant-pathogen interaction (Delaunois et al., 2014). The following sections discuss grape downy mildew's general characteristics and methods of control.

2. General characteristics of grape downy mildew

2.1. *Plasmopara viticola*: causal agent of grape downy mildew

Downy mildew caused by *P. viticola* (Berkeley and Curtis) Barlesse and De Toni represents one of the most serious diseases of the vine, particularly in mild and humid climates (Gessler et al., 2011; Wilcox et al., 2015). All the vine cultivars used for the production of table or wine grapes are susceptible to downy mildew, and the infections of this disease cause enormous economic damage due to the reduction of production both from a quantitative and qualitative point of view. *P. viticola* affects leaves, shoots, inflorescences, and infructescences and is controlled mainly by the intense application of chemicals (Gessler et al., 2011). Estimating the total costs sustained in the EU or by its member states for fungicide treatments against *Plasmopara viticola* is difficult (Salinari et al., 2006). Even though few studies are addressing the economic aspects of this disease, a study conducted in the Piemonte region of Italy

estimated the annual cost of controlling downy mildew between 8 and 16 million euros (Salinari et al., 2006).

2.2. Taxonomic classification of *Plasmopara viticola*

P. viticola belongs to order *Peronosporales* and family *Peronosporaceae*. *P. viticola* is an obligate biotroph and therefore depends completely on a grape as a host to complete its life cycle. It was first identified and described in the northeastern United States. Lewis David de Schweinitz, an American botanist and mycologist, initially discovered *P. viticola* in 1834, and he erroneously included it among the microorganisms belonging to the species *Botrytis cana* (cited in Gregory, 1915). Subsequently, it was reclassified in 1848 by Henry William Ravenel and Miles Joseph Berkeley, as the new species *Botrytis viticola* (Hendrickx, 1948). The German microbiologist Anton De Bary described the asexual and sexual stages of the grape pathogen and classified it in a new genus *Peronospora* as *Peronospora viticola* (De Bary, 1863). Later, Schröder (Schröter, 1886) found differences between several *Peronospora* members and introduced a new genus of *Plasmopara* (Schröter, 1886). Using the Schröder classification scheme, Berlese and de Toni (Berlese and de Toni, 1888) gave its current name of *Plasmopara viticola*. Finally, oomycetes' strong association with photosynthetic organisms like brown algae and diatoms was recognized and the oomycetes moved from the Fungal Kingdom to the heterokonts (Van der Auwera et al., 1995). For instance, the cell walls of oomycetes are made of cellulose rather than chitin and septations are only present in older, coenocytic mycelia. Oomycetes have diploid nuclei when they are in the vegetative state, whereas analogous fungi have haploid (*Ascomycetes*) or di-karontic nuclei (*Basidiomycetes*). Many Oomycetes produce two-flagellated, self-motile zoospores (Situ et al., 2022).

2.3. Worldwide distribution of *Plasmopara viticola*

P. viticola was first observed in Europe in 1878. It most likely arrived in Europe together with the American grape cuttings that were used to replace the French vines that had been decimated by phylloxera (Müller and Sleumer, 1934). After *P. viticola* was found to be the causal agent of the disease by Thiemann, Plan-chon, and Farlow, it spread quickly throughout France, northern Italy, including South Tyrol, and surrounding Austrian territories (1879) (Gessler et al., 2011). Following its discovery in the Alsatian and Mosel region of Germany a year later, it spread to Eastern Europe, Turkey, and Greece in 1881 before heading west into Spain, Portugal, and Africa. In Europe, it was believed that the disease would only affect the foliage

of grapevines (Müller and Sleumer, 1934) but later it was confirmed it also affects the berry. Müller and Sleumer, 1934 proposed the theory that the timing of the infections in the vineyards was related to the movement of *Peronospora* from leaves to berries. The disease was unquestionably a major issue for European viticulture from the start of the 20th century (Gäumann, 1927). In Europe, epidemics occurred sporadically and irregularly when there were favorable weather conditions and insufficient control mechanisms were either not yet available or not used (1900, 1905. 1906, 1910, 1912, 1913, 1916, 1917, 1930, and 1932). Serious damage was caused to viticulture in France, Germany, and Switzerland. For instance, *P. viticola* destroyed 70% of the French crop harvest in 1915 (Cadoret, 1923, 1931). France lost 20 million hl of wine in 1930. Downy mildew caused a 33% decrease in the total area used for growing vines in the German province of Baden between 1907 and 1916 (Müller, 1938). In Italy, significant damage was documented in the years 1889, 1890, 1903, 1910, 1928, 1933, and 1934 (Baldacci, 1954). This disease caused considerable damage during the Second World War. However, the lack of copper due to war contributed to this situation more than adverse weather conditions (Hadorn, 1942; Stellwaag, 1943).

2.4. *Plasmopara viticola* infection cycle

An understanding of the *P. viticola* life cycle is essential to the epidemiology, pathology, and for the development of better management of the disease. *P. viticola* has both sexual and asexual reproductive forms (Figure 1), causing primary and secondary downy mildew infections, respectively. The primary infections are caused by oospores and the secondary infections are caused by sporangia. Oospores represent the sexual stage of *P. viticola* (Burruano, 2000; Rossi et al., 2013) and are produced from the fertilization of oogonia by antheridia (Conigliaro et al., 1996). Oospore development is not temperature-dependent but appears to be favored by dry circumstances (which prevent asexual sporulation) or leaf senescence (Gessler et al., 2011). Oospores overwinter in the leaf litter (Kennelly et al., 2007; Rossi et al., 2013) and acquire morphological maturity throughout the winter; i.e. the oospore wall thickens, the nuclei fuse, an ooplast forms, and large lipid globules divide into smaller ones (Vercesi et al., 1999). Dormancy (Galet, 1977; Rossi and Caffi, 2007), a process governed by the environment, nutritional permeability, and endogenous inhibitors, prevents the germination of morphologically mature oospores. When the dormancy is broken, oospores are regarded as physiologically mature, and they are capable of germinating in favorable environmental conditions (Rossi and Caffi, 2007). Oospores are the only source of inoculum

for primary infections of *P. viticola* and were long deemed to play a role only in triggering the epidemic in the early grapevine season. The formation of a macrosporangium containing zoospores marks the completion of oospore germination (Galbiati and Longhin, 1984). Oospore germination needs a minimum temperature of 12–13 °C (The ideal temperature range is 20–24 °C) (Laviola et al., 1986; Gessler et al., 2011). Asexual multiplication and secondary infections were then responsible for the subsequent explosive increase of the disease (Blaeser and Weltzien, 1979; Lafon and Clerjeau, 1988; Lalancette et al., 1988). The primary inoculum not only initiates epidemics but also aids in their progression, as evidenced using DNA microsatellites, which allows the identification of genotypes causing single Downy mildew leaf lesions. It was discovered that the new *P. viticola* genotypes enter the epidemic for most of the grape-growing season (Kump et al., 1998; Gobbin et al., 2003; Rumbou and Gessler, 2004; Gobbin et al., 2005; Gobbin et al., 2006). Oospores make up a sizable and varied inoculum pool, which results in a pathogen population with a high level of genotypic variation. Due to this diversity, the pathogen may adapt to a wide range of host species and microclimates (Rossi et al., 2013).

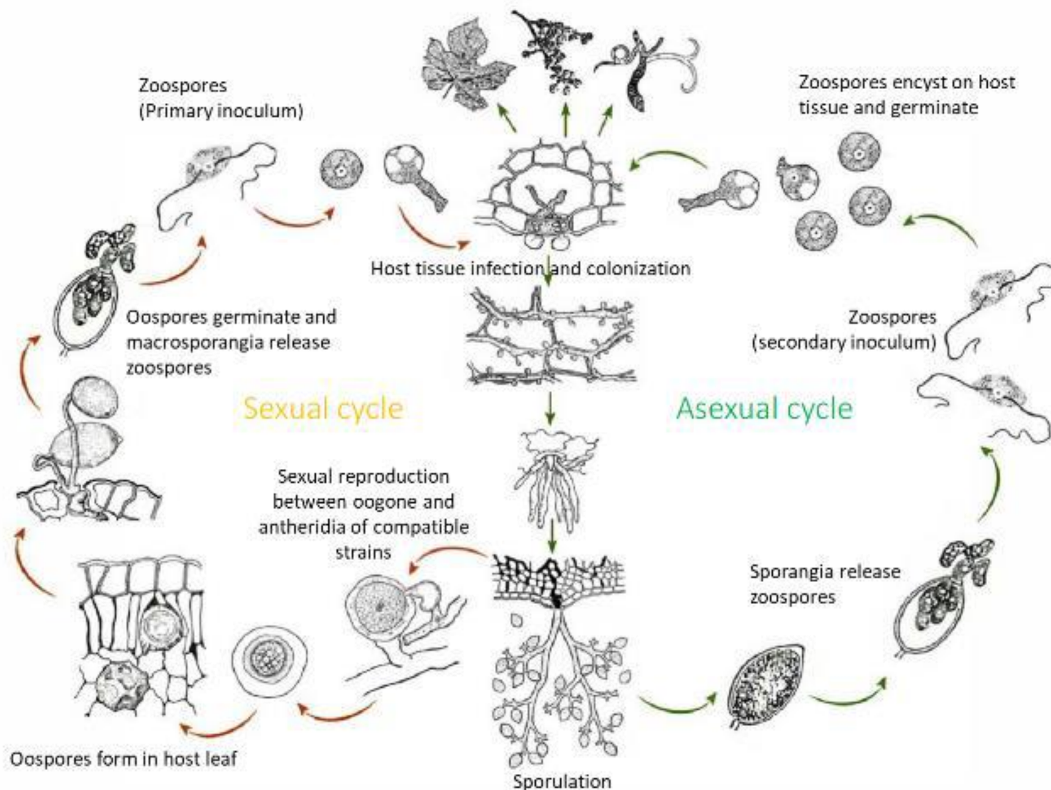

Figure 1. The life cycle of *Plasmopara viticola* (Poeydebat et al., 2022).

2.5. Symptoms of *Plasmopara viticola* infection

P. viticola infections have a wide range of negative effects on different organs of the plant. This obligate, biotrophic pathogen can infect all the green parts of grapevine plants, resulting in significant yield losses due to qualitative and quantitative damage (Yu et al., 2012) (Figure 2). Attacks on the bunches can lead to a substantial loss of production, while damage to the leaves causes a reduction of the photosynthetic activity and, therefore, of the accumulation of sugars and aromas (Pertot et al., 2005). Furthermore, the decrease in photosynthetic activity also affects the reserves in woody organs and roots (Pertot et al., 2005). The most severe infections can jeopardize the entire metabolic process of the plant and threaten vegetative growth the next year. "Oil spots," or transparent patches, are the defining sign of *P. viticola* infection on vine leaves (Figure 2a.). They occur after an incubation period of 4–18 days, depending on the surrounding temperature. Brownish margins start to appear at the edges of the oil stains, after which the fabrics turn brown and dry out, and the leaves finally fall off. Depending on the temperature, the incubation period of the disease can range from a minimum of 4-5 days to a maximum of 15-18 days. Young leaves are immune to infection because they have undifferentiated stomatal holes and a diameter of less than 2 cm (Pertot et al., 2005). Leaves older than 50-60 days become less vulnerable to attacks by the pathogen, primarily due to the structural resistance of the fabrics and a higher content of active polyphenols antimicrobial (Pertot et al., 2005). Downy mildew attacks on grapes are extremely dangerous due to their effect on production. The inflorescences are very sensitive to the pathogen starting from their differentiation up to flowering. Like what happens to leaves, symptoms on the inflorescences appear as a mold that covers the flowers. Early in the attack, the entire inflorescence may necrotize or distort, taking on the distinctive "shape of an S," and the affected areas may darken (Pertot et al., 2005). When the grapes reach a diameter of 2 mm, the stomata atrophy, and the grapes become less sensitive and can no longer be directly affected. However, the pathogen can penetrate through the stomata of the pedicels and also spread inside the acini. In this case, in the absence of stomata, downy mildew cannot manifest itself by producing the typical white mold, the affected berries turn blue-brown, harden, and tend to fall off (brown rot or downy mildew) (Pertot et al., 2005).

Figure 2. *P. viticola* infection a) Old oilspot with necrosis on the adaxial leaf Surface b) Sporulation of *P. viticola* on abaxial leaf surface c) Sporulation on a grape cluster before Bloom d) Symptoms caused by *P. viticola* on a developed cluster of grape e) *Plasmopara viticola* on newly emerged shoot f) *Plasmopara viticola* on clusters (Pertot et al., 2005; Carisse, 2016).

3. Control methods

As we mentioned above downy mildew triggered by the oomycete *P. viticola* is one of the most damaging diseases of grapevine (*Vitis vinifera*) worldwide. Its management mostly relies on the heavy application of fungicides throughout the growing season, endangering biodiversity, and human health in the process. Therefore, new disease management strategies must be developed. Those alternative approaches may include the cultivation of disease-resistant varieties (breeding), the use of biocontrol products, and RNA interferences.

3.1. Chemical control

Because of the polycyclic nature of the disease and the importance of oospores as initial inoculum, the management of grape downy mildew generally relies on fungicide applications early in the season to control primary infections and to avoid infections of inflorescences, flowers, and young berries. Several fungicide treatments, particularly those based on copper (Cu), are frequently used in vineyards to manage the disease (Wong et al., 2001; Gessler et al., 2011; Fontaine et al., 2019). However, there are rising concerns about the possible damage that

synthetic chemical fungicides and copper may cause to humans and the environment (Wightwick et al., 2008; Brunetto et.al., 2016). Cu, in particular, can start building up on the surface horizon of the soil and be detrimental to both plants and the soil biota (Kandeler et al., 1996; Merrington et al., 2002; Brunetto et al., 2016; Ambrosini et al., 2018; De Conti et al., 2018; Keiblinger et al., 2018; Marastoni et al., 2019a; Marastoni et al.,2019b; Schwalbert et al., 2019; Hammerschmitt et al., 2020). Due to excessive usage of synthetic agrochemicals *P. viticola* became recently resistant to many fungicides (Gisi and Sierotzki, 2008). Therefore, the development of more eco-friendly approaches to control *P. viticola* infections in *viticulture* is strongly encouraged to reduce the effects of synthetic chemicals and Cu-based fungicides.

3.2. Breeding and deploying disease-resistant cultivars

The main goals of plant breeding projects are to improve quality traits to make crops more productive and nutritious, as well as to increase plant resilience against biotic or abiotic stress. Breeding of grapevine for disease resistance is a worldwide trend that aims to decrease the application of fungicides in crop cultivation. Even though there was variation in susceptibility to *P. viticola* in the range of available *V. vinifera* cultivars breeders focused on crossing *V. vinifera* with American and Asian species (Pee-Laby, 1926). American species with a high level of disease resistance, *Muscadinia*, *rotundifolia*, *V. rupestris*, *V. riparia*, and *V. berlandieri* (Merdinoglu et al., 2003; Marguerit et al., 2009), as well as Asian species with a high level of disease resistance, primarily *V. amurensis* (Blasi et al., 2011; Moreira et al., 2011; Schwander et al., 2012; Venuti et al., 2013), have been used in the breeding programs to create resistant cultivars. Identification of the genomic regions linked to disease resistance has been made possible by genetic mapping. To apply marker-assisted selection, these studies have produced molecular markers flanking genomic regions that provide resistance to diseases (Julius Kühn-Institut, 2022). The genomic regions known as "resistance to *P. viticola* (*Rpv*)" that provide resistance to downy mildew have also been mapped (Julius Kühn-Institut, 2022). Chromosomes 12 and 18 include the *Rpv1* and *Rpv3* resistance loci, respectively (Merdinoglu et al., 2003; Fischer et al., 2004; Welter et al., 2007; Moroldo et al., 2008; Bellin et al., 2009; Zyprian et al., 2016), and *Rpv1* was found to be responsible for the resistance derived from *M. rotundifolia* (Merdinoglu et al., 2003; Peressotti et al., 2010), as a qualitative trait loci (QTL) identified in the same region in *V. riparia* (Marguerit et al., 2009). Whereas *Rpv3* was found to be responsible for the onset of a hypersensitive response (Bellin et al., 2009). In marker-assisted selection (MAS), these regions' related molecular markers were used to pyramid these

resistance loci (Eibach et al., 2007). Many breeding programs that aim to increase the persistence of genetic resistance, such as those for *Rpv1* and *Rpv3.1* (Eibach et al., 2007), *Rpv3.1* and *Rpv10* (Schwander et al., 2012), and *Rpv12* and *Rpv3.1*, have started to pyramid the resistance loci for grapevine downy mildew (Venuti et al., 2013). Rpv3.n characterized loci are more than 1. See Vezzulli et al. (2022) Genomic Designing for Biotic Stress Resistant grapevine as complete overview.

QTLs with major downy mildew resistance effects have been identified in linkage groups (LGs) 9 and 12 in *V. riparia* (Marguerit et al., 2009), LG 14 in *V. amurensis* (Blasi et al., 2011) and in LGs 4 and 18 in the resistant grapevine 'regent' (Fischer et al., 2004; Welter et al., 2007). A major QTL was also identified on LG 7, together with additional QTLs on LGs 8, 12, and 17 in a segregating population of *V. vinifera* × *V. viparia* (Moreira et al., 2011). Other QTLs were identified on LGs 1, 6 and 7 in a cross between two interspecific hybrids inheriting *V. rotundifoglia* and *V. amurensis* traits (Moreira et al., 2011). To date, hybrids or non-*vinifera* species have been used to cross *V. vinifera* cultivars to create downy mildew-resistant grapevine varieties. Efforts to introgress resistant traits into cultivated *V. vinifera* genotypes using conventional breeding techniques (Eibach et al., 2007) have yielded some resistant interspecific hybrids, but further work is needed to couple strong resistance with high-quality wine production traits (Burger et al., 2009). However, the breeding technique is quite time-consuming since it requires numerous cycles of backcrossing with susceptible cultivars to minimize the background of non-*vinifera* species. S-genes mediate susceptibility to a pathogen, and their knocking down is associated to resistance. S-gene study in grapevines is still in its infancy. Two *MLO* genes for *V. vinifera* susceptibility to powdery mildew were found by Pessina et al., 2016, and some more *MLO* genes for downy mildew were also suggested (Toffolatti et al., 2020; Pirrello et al., 2021). Recently, it was discovered that the *V. vinifera* cultivar Mgaloblishvili from Georgia (Southern Caucasus) exhibits resistance to *P. viticola* (Toffolatti et al., 2016).

3.3. Biological control and natural products

Theoretically, utilizing microorganisms to manage plant diseases through biocontrol is a powerful alternative to using chemical pesticides. According to Arnone et al., 2008, numerous microorganisms have been chosen over the years for their antagonistic activities toward *P. viticola*, but none of them have been effective as bio fungicides. Here, a few illustrations are

provided. *Trichothecium plasmoparae*, is a hyperparasite that affects sporulating *P. viticola* and results in pink mildew patches. Despite being aggressive, it was never further researched for use as a biocontrol agent (Arpai et al., 1957). Similarly, although it was discovered that *Erwinia herbicola* significantly inhibited the germination of *P. viticola* sporangia in laboratory conditions and that using it reduced the number of zoospores surrounding stomata in liquid cultures, no additional research on this microorganism has been recorded (Tilcher et al., 1994). In another study, the endophytic fungus *Acremonium byssoides* was discovered in 34 grapevine types over two years after being initially isolated from the leaves of the grapevine Regina Bianca cultivar (Burruano et al., 2008). This fungus actively parasitizes *P. viticola* and is thought to naturally colonize grapevines. *A. byssoides* crude extract and culture filtrates suppressed the pathogen (Burruano et al., 2008).

From grapevine leaves that exhibited abnormal downy mildew symptoms, 125 additional microorganisms and a strain of *Alternaria alternata* that inhibits *P. viticola* were isolated (collected in Tuscany, Italy). Cytological investigations highlighted the fact that *P. viticola* mycelia exposed to this fungus had severe ultrastructural alterations, including the existence of expanded vacuoles or vacuoles harboring electron-dense precipitates, even without direct contact with *A.alternata*. Their haustoria appeared necrotic and irregularly shaped or coated with callose-like substances. In light of these findings, researchers hypothesized that *A. alternata* might be toxic to *P. viticola* (Musetti et al., 2006). Their haustoria had irregular shapes, a necrotic appearance, or were covered in callose-like materials. Therefore, because of these observations, researchers speculated that *A. alternata* would be toxic to *P. viticola* (Musetti et al., 2006). In another study, an aqueous extract of the dry mycelium of *Penicillium chrysogenum* provided grapevine downy mildew control equivalent to that offered by copper (Thuerig et al., 2006). The extract was thought to protect plants by triggering their defense mechanisms even though it exhibited no significant direct fungicidal effects. *Trichoderma harzianum* T39 was initially developed as a commercial fungicide with the trade name Trichodex (Makhteshim Agan, Chemi-cal Works LTD, Be'er Sheva, Israel). This strain protects susceptible grapevine cultivars without directly inhibiting the germination of sporangia, through a plant-mediated resistance mechanism. The systemic resistance is homogeneously activated, independent of leaf position (Perazzolli et al., 2008). The commercial fungicide Trichodex, originally developed by Makhteshim Agan of Chemical Works LTD in Be'er Sheva, Israel, is from *Trichoderma harzianum* species. Through a plant-mediated resistance mechanism, this strain defends sensitive grapevine cultivars without

directly preventing sporangia from germinating. The systemic resistance is homogeneously activated, independent of leaf position (Perazzolli et al., 2008). In conclusion, despite the good activity of microorganisms against *P. viticola* demonstrated under controlled conditions, microbial biocontrol agents have never shown good and consistent activity against *P. viticola* in the field. Once the pathogen infects plants, it can no longer be controlled by antagonists (Pertot and Gessler, 2007). All of the microorganisms that have been examined so far have been discovered to be active for a short period after application.

Extensive tests on the impact of natural products have lately been conducted (Dagostin et al., 2011). Several examples of testing have been conducted on natural products. In one study 58 plant extracts were examined and only a few of these extracts—those from *Chloris virgata, Dalbergia hupeana, Pinus massoniana, Paeonia suffruticosa, and Robinia pseudo-acacia*—inhibited germination of sporangia and successfully managed the plant disease (Chen et al., 2002). In a different investigation, it was discovered that sage (Salvia officinalis) extract can prevent grapevine downy mildew in outdoor conditions (Dagostin et al., 2010). Additionally, it has been demonstrated that an oily paste extract of *Inula viscosa* leaves can successfully prevent downy mildew in outdoor situations (Cohen et al., 2006). But the use of this extract as a fungicide is constrained by its severe phytotoxicity towards the grapevine. The use of plant extracts is generally constrained by their expensive costs, limited availability in large amounts, low persistence, and low level of rain fastness (Dagostin et al., 2010). The impacts of an extract of the brown alga *Ascophyllum nodosum* on grapevine interactions with *P. viticola* were observed. Lizzi et al., 1998 demonstrated that downy mildew outbreaks were significantly decreased when they spray the brown alga *Ascophyllum nodosum* extracts on grape leaves. In another study, it has been explained that the brown alga *Laminaria digitata* can significantly minimize *P. viticola* damage by inducing defense responses in grapevine cells and plants (Aziz et al., 2003). Similarly, it has been demonstrated that sulfated laminarin stimulates plant defense mechanisms and guards the grapevine against downy mildew (Trouvelot et al., 2008). A deacetylated derivative of chitin known as chitosan has also been demonstrated to enhance plant defense responses and considerably reduce the degree of infection on grapevine leaves (Aziz et al., 2006). According to reports, the non-protein amino acid BABA (DL-3-ami-no-*n*-butanoic acid, beta-aminobutyric acid), causes downy mildew resistance both locally and systemically in grapevine leaves. BABA was able to prevent fungal colonization even when administered to leaf discs after infection. The resistance of BABA endured for more than 14 days. BABA has been demonstrated to provide systemic protection (Cohen et al., 1999;

Slaughter et al., 2008). Although it is still unknown if inducers of grapevine resistance to *P. viticola* can serve as a substitute for copper in the management of downy mildew, inducers are expected to help reduce plant susceptibility to infection, hence enhancing the outcomes of other weak natural control agents.

3.4. RNA interference (RNAi) applied to control oomycetes

In eukaryotes, RNA silencing sometimes referred to as RNA interference or RNAi, is a common method of controlling the expression of genes. Small non-coding RNAs (sRNAs) are key players in RNA silencing. They direct sequence-specific repression of target genes through transcriptional gene silencing by mediating DNA, histone, and chromatin modifications and post-transcriptional gene silencing by mediating transcript cleavage and translational repression (Ghildiyal and Zamore, 2009; Castel and Martienssen, 2013). In plants, sRNAs have been thoroughly investigated for their regulatory roles in abiotic stress response (Sunkar et al., 2007), development (Chen, 2012), and anti-viral defense (Ding, 2010). However, there is a growing amount of evidence pointing to the possibility that sRNAs also play an important role during interactions with filamentous eukaryotic pathogens including fungi and oomycetes. Specifically, plant mutants in the main RNAi pathway show altered resistance (Ellendorff et al., 2009; Qiao et al., 2015; Zhang et al., 2015); and specific plant sRNAs influence host-pathogen interactions (Liu et al., 2014; Ouyang et al., 2014; Wong et al., 2014). Recently, oomycete infections in the genus *Phytophthora* produced effector proteins with RNAi suppression activity (Qiao et al., 2013). The discovery of *Phytophthora* suppressors of RNA silencing suggests that manipulating the host RNA silencing process is a common virulence strategy employed by pathogens across the kingdoms to facilitate infection, along with the well-characterized viral suppressors and the previously reported bacterial suppressors (Navarro et al., 2008; Pumplin and Voinnet, 2013; Zhao et al., 2016). These results also suggest that sRNAs are essential parts of the plant defense system and play a significant role in how plants interact with *Phytophthora* or possibly other filamentous eukaryotic pathogens including other oomycetes and fungi (Baulcombe, 2015). A comprehensive review of the SIGS approach is presented in the third chapter.

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Chapter 3: RNA Interference Strategies for Future Management of Plant Pathogenic Fungi: Prospects and Challenges

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Abstract

Plant pathogenic fungi are the largest group of disease-causing agents on crop plants and represent a persistent and significant threat to agriculture worldwide. Conventional approaches based on the use of pesticides raise social concern for the impact on the environment and human health and alternative control methods are urgently needed. The rapid improvement and extensive implementation of RNA interference (RNAi) technology for various model and non-model organisms has provided the initial framework to adapt this post-transcriptional gene silencing technology for the management of fungal pathogens. Recent studies showed that the exogenous application of double-stranded RNA (dsRNA) molecules on plants targeting fungal growth and virulence-related genes provided disease attenuation of pathogens like *Botrytis cinerea, Sclerotinia sclerotiorum* and *Fusarium graminearum* in different hosts. Such results highlight that the exogenous RNAi holds great potential for RNAi-mediated plant pathogenic fungal disease control. Production of dsRNA can be possible by using either in-vitro or in-vivo synthesis. In this review, we describe exogenous RNAi involved in plant pathogenic fungi and discuss dsRNA production, formulation, and RNAi delivery methods. Potential challenges that are faced while developing a RNAi strategy for fungal pathogens, such as off-target and epigenetic effects, with their possible solutions are also discussed.

Keywords: RNA interference; dsRNA delivery; small RNA production; dsRNA formulation

1. Introduction

Pathogens have decreased the productivity of crops since the advent of agriculture, and farmers have been exploring ways of safeguarding their crops from these organisms. The use of synthetic pesticides is currently an indispensable means of intensive agricultural systems to guarantee food supply worldwide, protecting crops from pathogens, which otherwise would cause more than 30% yield losses (Alexander et al., 2017; Keulemans et al., 2019). There is a long tradition of using synthetic pesticides which have been developed and applied to control pathogens. However, the evolution of pathogens resistance to pesticides, together with the concern for the environment and human health, has stimulated demand for more selective, environmentally friendly, and cost-effective alternative control methods for pathogens and pests (Nicolopoulou-Stamati et al., 2016). Scientists have allocated a great deal of intellectual energy into seeking alternative strategies to reduce crop losses, such as the development of tolerant/resistant plants to pathogens and pests and with increased quality products by using conventional breeding and plant biotechnological tools (Sabbadini et al., 2021). More recently, gene silencing through RNA interference (RNAi) is offering a new opportunity for precision breeding and for the development of new products for protecting plants from pathogens and pests. RNAi is a conserved eukaryotic mechanism triggered by double-stranded RNA (dsRNA) molecules. It is associated with diverse eukaryotic regulatory processes, including protection against viral infection, control of transposon movement, regulation of genome stability, gene expression, and heterochromatin formation (Ketting, 2011; Castel and Martienssen, 2013).

RNAi was first reported by Napoli and colleagues (Napoli et al., 1990) to produce violet petunias, the *chalcone synthase* gene (*CHS*), encoding for a key enzyme in flavonoid biosynthesis, was overexpressed by introducing a transgene that resulted in an unintended white petunia phenotype. Further analysis revealed declined expression of both the endogenous and exogenously introduced *CHS* gene, which led to the conclusion that the transgene co-suppressed the endogenous *CHS* gene. A similar phenomenon was reported in the filamentous fungus *Neurospora crassa* (Romano and Macino, 1992), where the introduction of the transgene '*albino-1*' resulted in the quelling of the endogenous gene. Similarly, in *Caenorhabditis elegans*, the injection of dsRNAs led to the silencing of *unc-22* gene, highly homologous in sequence to the delivered dsRNA molecules (Fire et al., 1998). Over the last two decades, the understanding of RNAi has evolved from initial observation of unexpected patterns of expression to a deeper understanding of a multi-faceted network of mechanisms

that regulate gene expression in many organisms (Koch and Kogel, 2014; Wang et al., 2016; Brilli et al., 2018). Consequently, RNAi is getting research attention also as an environmentally friendly alternative to agricultural pest and pathogen control. In fact, because of its sequence-dependent mode of action, RNAi technology has an enormous range of potential as plant protection application, including control against insects (Zotti et al., 2013), mite pests (Niu et al., 2018; Bensoussan et al., 2020), plant pathogens (Jahan et al., 2015; Koch et al., 2016; Wang et al., 2016; Nerva et al., 2020), nematodes, and weeds (Hollomon, 2012; Koch and Kogel, 2014; Shaner and Beckie, 2014; Iqbal et al., 2020).

The concept is based on the administration of small RNA (dsRNA/siRNA) molecules that induce the silencing of key genes in pathogenic organisms, thereby limiting/stopping their growth. Delivering dsRNAs to a target organism is a crucial aspect that determines the success of the RNAi technology in crop protection. Delivery can be achieved through host-induced gene silencing (HIGS) RNAi approach, corresponding to in-planta expression of siRNA targeting key genes of the pest/pathogen. Besides HIGS, exogenous delivery of dsRNA can be considered as an alternative approach. In this review selected research findings on RNAi approaches through exogenous delivery of small RNA molecules targeting plant pathogenic fungi will be discussed. Small RNA production techniques, potential limitations, and solutions for the application of RNAi for fungal disease control are also discussed.

2. RNAi for Resistance against Plant Pathogenic Fungi

In the past, RNAi in plants has been mainly used to improve resistance to diseases by silencing susceptibility genes, those genes that negatively regulate plant defense responses (Brodersen and Voinnet, 2006). During the last decade, however, RNAi has been more exploited to provide plants with so-called "pathogen-derived resistance", where resistance is achieved through small interfering RNAs (siRNAs) able to silence genes that are important for infection or the life cycle of the pathogen (Govindarajulu et al., 2015; Kettles et al., 2019; Su et al., 2020). The silencing process starts with the cleavage of dsRNAs into 21–25-nucleotide-long double-stranded siRNAs in cytoplasm by Dicer or Dicer-like homologs and sRNA-specific RNase III family enzyme. Dicer protein contains an N-terminal helicase domain, a Piwi/Argonaute/Zwille (PAZ) motif, a dsRNA binding domain, and two RNase III motifs at the C-terminus. Dicer-generated siRNAs are then incorporated into a multi-component protein complex, the RNA-induced silencing complex (RISC), which becomes activated on ATP-

dependent unwinding of the siRNA duplex (Vaucheret et al., 2004). RISC contains an Argonaute protein that has a sRNA-binding domain and an endo-nucleolytic activity for cleavage of target RNAs (Vaucheret et al., 2004). Once the siRNA is incorporated into RISC, it will be unzipped into the guide and passenger strands, the latter will be degraded, and the guide strand will bind to the target mRNA sequence and stimulate its endo-nucleolytic cleavage or will inhibit translation (Limera et al., 2017). Although greatly diminished, residual mRNA levels can be detected. Therefore, the RNAi-mediated silencing of a particular gene is commonly referred to as a 'knockdown' rather than a 'knockout' (Preall and Sontheimer, 2005; Wilson and Doudna, 2013). Within the fungal kingdom, the mechanistic facets of RNAi were studied in *N. crassa* (Romano and Macino, 1992; Catalanotto et al., 2000). Since then, RNAi machinery has been recognized in a wide range of fungal species. The use of RNAi as a tool for reverse genetics, targeted at modification of fungal gene expression, is continually growing with a large number of fungal species already proved to be responsive (Dang et al., 2011). Furthermore, the functionality of absorbed exogenous RNAi molecules offers excellent adaptability and flexibility in securing the required effects on gene expression of fungi, even without the need to genetically modify the targeted pathogen (Nakayashiki and Nguyen, 2008; Wang et al., 2016). This homology-based gene silencing stimulated by transgenes (co-suppression), antisense, or dsRNAs has been demonstrated in several plant pathogenic fungi/oomycetes, including different mold fungi, such as *Botrytis cinerea*, *Neurospora crassa, and Sclerotinia sclerotiorum* (Goldoni et al., 2004; Wang et al., 2016 ; McLoughlin et al., 2018; Nerva et al., 2020); blast, blight, and rust fungi, such as *Fusarium asiaticum*, *Fusarium graminearum*, *Magnaporthe oryzae,* and *Puccinia striiformis f. sp. tritici* (Nakayashiki, 2005; Koch et al., 2016; Zhu et al., 2017; Gu et al., 2019; Werner et al., 2020); mildew, and others, such as *Blumeria graminis*, *Cochliobolus sativus,* and *Venturia inaequalis* (Leng et al., 2010; Nowara et al., 2010; Fitzgerald et al., 2019). Over the past few years, a variety of target genes have been used to test whether RNAi is functional in plant-fungal pathogens (Tables 1). To date, the number of successful candidate genes studied that led to reduced fungal growth development is limited, and includes effectors, cell wall elongation, chitinase, and hexose transporter genes. Much work remains to be done to identify suitable fungal candidate genes. Fortunately, opportunities exist to establish high-throughput screening pipelines to find strong candidates.

Table 1. Representative potential target genes tested for controlling pathogenic fungi and oomycetes

Species	Target Gene(s)	Host Plant	References
Alternaria alternata	*Putative hydrolase (ACTT2), a host-selective ACT-toxin*	Tangerine	Miyamoto et al., 2008
	Enoyl-reductase (ACTTS2), a host-selective ACT-toxin	Tangerine	Ajiro et al., 2010
A. flavus and A. parasiticus	Transcription factor (aflR)	Corn and wheat	McDonald et al., 2005
A. flavus	*aflS, aflR, aflC, pes1, aflep*	Peanut	Arias et al., 2015
	aflR	Maize	Masanga et al., 2015
Blumeria graminis f. sp. tritici	MLO	Wheat	Riechen et al., 2007
Bipolaris oryzae	Polyketide synthase gene (PKS1)	-	Moriwaki et al., 2007
Blumeria graminis	*Avira10*	Barley and wheat	Nowara et al., 2010
	BEC1011, BEC1054, BEC1038, BEC1016, BEC1005, BEC1019, BEC1040, and BEC1018	Barley	Pliego et al., 2013
Botrytis cinerea	*Superoxide dismutase (BCSOD1)*	French bean	Patel et al., 2008
	Dicer-like 1 and Dicer-like 2	Arabidopsis, tomato, strawberry, grapes, lettuce, onion, and rose	Wang et al., 2016
Bremia lactucae	Cellulose synthase 1, Highly abundant message #34 (HAM34)	lettuce	Govindarajulu et al., 2015
Cladosporium fulvum	Hydrophobin gene (HCf-1)	-	Hamada and Spanu, 1998
	First exons of six hydrophobin coding genes	-	Lacroix and Spanu, 2008
Cochliobolus sativus	*GFP, a host-selective toxin (ToxA) and a polyketide synthase (CsPKS1)*	Wheat	Leng et al., 2010
Colletotrichum gloeosporioides	*Transcription factor (PAC1)*	-	Shafran et al., 2008
Fusarium culmorum	*FcGls1*	Wheat	Chen et al., 2016

Table 1 cont .

Species	**Target Gene(s)**	**Host Plant**	**References**
Fusarium graminearum	*Transcription factor (Tri6)*	Corn and wheat	McDonald et al., 2005
	Cytochrome P450 lanosterol C-14α-demethylase genes CYP51A, CYP51B and CYP51C	Arabidopsis and barley	Koch et al., 2016
	Chs3b	Wheat	Cheng et al., 2015
Fusarium oxysporum f. sp. cubense (fusarium wilt)	*Velvet, Fusarium transcription factor 1*	Banana	Ghag et al., 2014
F. oxysporum f. sp.	*FRP1, FOW2, OPR*	Arabidopsis	Hu et al., 2015
Fusarium solani f.sp. pisi	*ß (1,3)-D-glucan synthase (FsFKS1)*	-	Ha et al., 2006
Fusarium solani	*Chitosanase (CSN1)*	Pea	Liu et al., 2010
F. verticillioides	*GUS (ß glucuronidase)*	Tobacco	Tinoco et al., 2010
Glomus species	*Monosaccharide transporter 2*	Potato	Helber et al., 2011
Magnaporthe oryzae	*MPG1 and PKS-like gene*		Nakayashiki et al., 2005
	37 genes involved in calcium signalling	Barley and wheat	Nguyen et al., 2008
Melampsora lini	*Effector protein (AvrL567)*	Flax	Lawrence et al., 2009
Moniliophthora perniciosa	*GFP, hydrophobin (MpHYD3) and 1-cys peroxiredoxin (MpPRX1)*	-	Caribé dos Santos et al., 2009
Mucor circinelloides	*Carotenogenic gene (carB)*	-	Nicolas et al., 2003
Mycosphaerella fijiensis, Fusarium oxysporum	*Nuclear condensin, coatomer alpha, DNA-directed RNA polymerase, actin cortical patch 2/3, coatomer zeta, CAP Methyltransferase, GTP ASE binding protein, proteasome PRE4, Ribosomal RNA, DNA Polymerase alpha/delta subunit, Adenylase cyclase, Protein kinase C, FRQ-interacting RNA helicase*	-	Mumbanza et al., 2013

Table 1. Cont.

Species	Target Gene(s)	Host Plant	References
Ophiostoma novo-ulmi	*Endopolygalacturonase (Epg1)*	-	Carneiro et al., 2010
Puccinia triticina	*MAPK, cyclophilin (CYC1), and a calcineurin (CNB) regulatory subunit gene*	Wheat	Panwar et al., 2013
Puccinia striiformis f. sp. tritici	*PsCPK1, PsFuz7*	Wheat	Zhu et al., 2017
Phytophthora infestans	*G-protein b-subunit encoding gene (Pigpb1)*	Potato	Latijnhouwers and Govers, 2003
	Cdc 14 coding gene (PiCdc14)	-	Fong and Judelson, 2003
	G-protein a-subunit gene (Pigpa1)	Potato	Latijnhouwers et al., 2004
	cdc14	-	Whisson et al., 2005
Phytophthora infestans	*bZIP transcription factor (Pibzp1)*	Tomato	Blanco and Judelson, 2005
	Nuclear LIM interactor-interacting factors (NIFC1 andNIFC2)	Tomato	Judelson and Tani, 2007
	Inf1		Ah-Fong et al., 2008
	Putative glycosylated protein (Pihmp1)	Potato	Avrova et al., 2008
	Putative ATP-dependent DEAD-box RNA-helicase gene (Pi-RNH1)	Potato	Walker et al., 2008
	Four members of the CesA encoding for cellulose synthase genes	Potato	Grenville-Briggs et al., 2008
	Effector protein (PiAVR3a)	Tobacco and potato	Bos et al., 2010
	SYR1	Potato	Eschen-Lippold et al., 2012
	Cutinase	Potato	Niblett and Bailey, 2012
	Dicer-like (Pidcl1), Argonaute (Piago1/2), Histone deacetylase (Pihda1)	Potato	Vetukuri et al., 2011
	G protein β*-subunit* (*GPB1*), *Cellulose synthase A2, Pectinesterase, Glyceraldehyde 3-phosphate*	Potato	Jahan et al., 2015
	DCL1, HMP1-,PGB1-, and *DCTN1+SAC1*	Potato	Qiao et al., 2021

Table 1. Cont.

Species	Target Gene(s)	Host Plant	References
P. parasitica var.nicotianae	*A coding gene considered to be involved in cellulose-binding (CB), elicitor (E) of defence in plants and lectin (L)-like activities (CBEL)*	Tobacco	Gaulin et al., 2002
	GST	Tobacco	Hernández et al., 2009
Phytophthora nicotianae, Peronospora tabacina	*Cutinase*	Tobacco	Niblett and Bailey, 2012
Phytophthora sojae	*Heterotrimeric G-protein a subunit (PsGPA1)*	Soybean	Hua et al., 2008
	C2H2 zinc finger transcription factor (PsCZF1)	Soybean	Wang et al., 2009
	MAP kinase encoding gene	Soybean	Li et al., 2010
	(PsSAK1)	Soybean	Wang et al., 2009
	Putative seven-transmembrane G-protein-coupled receptor (GPR11)	Soybean	Zhao et al., 2011
	PsYKT6, a conserved member gene of the soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs)	Tobacco and soybean	Liu et al., 2011
	Crinkling- and necrosis-inducing proteins (CRN) (PsCRN63 and PsCRN115)	Glycine max	Erental et al., 2007
Puccinia striiformis f. sp.tritici	PsCPK1, PsFuz7	Wheat	Zhu et al., 2017
Puccinia striiformis f. sp.tritici	PsCNA1 and PsCNB1	Barley and wheat	Wang et al., 2016
Puccinia triticina	*MAP kinase (PtMAPK1), cyclophilin (PtCYC1), calcineurin B (PtCNB*	Wheat	Panwar et al., 2013
Sclerotinia sclerotiorum	*B regulatory subunit (rgb1) of 2A*	Tomato	Erental et al., 2007
	phosphoprotein phosphatase (PP2A)	Tobacco	Andrade et al., 2016
Ustilago hordei	*GUS and mating-type gene (bW)*		Laurie et al., 2008
Verticillium dahliae	*Ave1, SIX gene expression 1 (Sge1) and necrosis and ethylene-inducing-like protein (NLP1)*	Tomato and Arabidopsis	Song and Thomma, 2016
	V. dahliae hygrophobins1	Cotton	Zhang et al., 2016
Verticillium longisporum	*Chorismate synthase (Vlaro2)*	Arabidopsis and rapeseed	Singh et al., 2010
Venturia inaequalis	*Trihydroxynaphthalene reductase (THN)*	Apple	Fitzgerald et al., 2004

3. Small RNA Production Technologies

At present, exogenous application of dsRNA seems a new promising strategy to deploy RNAi for pathogen control in agriculture. To carry out exogenous approaches, silencing experiments have been successfully performed using sequence-specific small RNA molecules produced by different methods (Table 2). Production of dsRNAs can be possible by employing either in-vitro (Sohail et al., 2003; Koch et al., 2016; Wang et al., 2016; Nwokeoji et al., 2019) or in-vivo synthesis (Yin et al., 2009; Huang et al., 2013). Studies have shown that the application of in-vitro synthesized dsRNAs targeting essential fungal genes onto the plant leaf surface attenuated fungal infection by inhibiting fungal growth, altering fungal morphology, and reducing pathogenicity, leading to the development of weaker plant disease symptoms (Goldoni et al., 2004; Koch et al., 2016; Wang et al., 2016; Gu et al., 2019). In-vitro methods consist of either enzymatic transcription or chemical synthesis with advantages and disadvantages for both. The enzymatic transcription approach is cost-effective for producing both short and long dsRNA molecules. This method is a source of pure dsRNA based on the annealing of two single-stranded (sense and antisense) RNAs (ssRNAs). Based on the principle of in-vitro transcription, on linearized DNA templates, or PCR-generated templates, the use of commercially available kits to produce dsRNA is widely used. Using in-vitro methods for dsRNA production, fungal resistance has been achieved in a plethora of cases as listed in Table 3. However, these kits are expensive when the production of large amounts of dsRNA is needed (Koch et al., 2016; Ahn et al., 2019). For RNAi studies on large-scale application, the enzymatic transcription method is therefore not a practical means of dsRNA production. Chemical synthesis, on the other hand, can produce a large yield of high purity dsRNA, but it is more expensive with the cost of synthesis increasing considerably as the length of the dsRNA increases (Beaucage and Reese, 2009). Chemical synthesis of siRNA enables control over the quantity and purity of siRNA and it also allows chemical modifications to enhance stability, an important feature needed for delivery. Chemically synthesized siRNAs can be labeled for evaluating siRNA uptake or localization by fluorescence microscopy (Ahmadzada et al., 2018).

In-vivo production of dsRNA using genetically engineered bacteria (for ex. *Escherichia coli* and *Pseudomonas syringae*) and yeast (*Yarrowia lipolytica*) (Voloudakis et al., 2015; Álvarez-Sánchez et al., 2018) emerged as an alternative approach to produce large quantities of dsRNAs at low cost. Concerning the costs, for example, it is possible to buy a fungus-derived dsRNA sequence produced in bacteria (*E. coli*) with about $1 USD per 1 g from low-cost companies

(Taning et al., 2020). These systems are able to produce large amounts of dsRNA molecules needed for field trial applications. Tenllado et al. 2003 demonstrated that crude extracts of bacterially expressed dsRNAs are effective in protecting plants from virus infections when sprayed onto plant surfaces by a simple procedure. The use of recombinant bacteria to produce dsRNA is an efficient technique due to their ease of handling, ability to maintain plasmid, and the fast growth rate of bacteria (Terpe et al., 2006). Among the available *E. coli* strains, *HT115* (DE3) is widely used to produce large amounts of dsRNA for exogenous application studies. The *E. coli HT115* (DE3) harbors the pro-phage λDE3 encoding the Isopropyl β-D-1-thiogalactopyranoside (IPTG) inducible T7 polymerase gene for dsRNA transcription (Tenllado et al., 2003; Ongvarraspone et al., 2007; Yin et al., 2009; Thammasorn et al., 2015). Even though, the bacterial production systems may contain bacterial homologous DNA molecules; that may affect the RNA quality and applicability, crude extracts of dsRNA can be applied on plants to test its efficiency against plant pathogens and pests (Zhu et al., 2016; Nerva et al., 2020). Researchers demonstrated that bacterially expressed dsRNAs can be used to induce RNAi in fungus (Nerva et al., 2020), virus (Pliego et al., 2013), worms (Newmark et al., 2003), and in insect pests (Tian et al.,2009; Ghag et al., 2014). Researchers are also using in-vivo dsRNA amplification employing *P. syringae* harboring the bacteriophage phi6 RNA-dependent RNA polymerase complex (Sun et al., 2004; Aalto et al., 2007; Niehl et al., 2018). Niehl and colleagues (2018) demonstrated that the in-vivo dsRNA production by *P. syringae* has great potential to allow therapeutic dsRNAs to be designed and produced for large-scale crop protection against different fungal and viral pathogens, and insect pests. However, the use of *E. coli* is still controversial because even if used as lysate containing the dsRNA, its residuals may have an impact on animal and human health (Somchai et al., 2016). Therefore, alternatives for expressing dsRNA in organisms are being explored, especially those that are generally considered safe for human consumption, which do not produce endotoxins or pose risks to health or the environment. One organism that possesses this characteristic is yeast (*Y. lipolytica*), which can provide unique advantages for the production of dsRNA. Alvarez-Sanchez et al. 2018 observed that *Y. lipolytica* is a convenient host for producing and delivering dsRNA-ORF89 that can protect shrimp from white spot syndrome virus attack. Besides other factors, the role of RNAi-based products for controlling fungal pathogens depends on the cost of production. Taking the cost trend into account, it is expected that small RNA production costs will decrease substantially in the future, with commercial companies investing in dsRNA production capacity. Over the past few years, a declining trend in the dsRNA production cost has been recorded. For example, the cost for producing 1 g of dsRNA using in-vitro nucleoside

triphosphate (NTP) synthesis fell from $12,500 USD in 2008 to $60 USD in 2018 (Jahan et al., 2015; Ghosh et al., 2018). For field-scale pest and pathogen management, metric tons of dsRNA will be required. It is conceivable that such a huge demand cannot be satisfied only by an in-vitro dsRNA transcription system. For this reason, some industrial companies have achieved low-cost (almost $2 USD per 1 g of dsRNA) and large-scale production of dsRNA using bacteria (Hamada and Spanu, 1998; Dalakouras et al., 2020).

Table 2. Advantages and disadvantages of different methods of double-stranded RNAs (dsRNAs)/small interfering RNAs (siRNAs) production.

Methods	Advantage	Disadvantages	Fungal Pathogen Tested with the Technology and References
In vitro			
Enzymatic synthesis	Less expensive No need to test individual siRNA separately	Purity and specificity are variable	Wang et al., 2016; McLoughlin et al., 2018: Werner et al., 2020
Chemical synthesis	Fast/Rapid High purity	Expensive	
In vivo			
Escherichia coli/ Pseudomonas syringae	Produce large quantities of dsRNAs at low cost	Labor intensive	Nerva et al., 2020
Yarrowia lipolytica	Produce large quantities of dsRNAs at low cost	Labor intensive	

Table 3. **Summary of exogenously applied RNA molecules to plant pathogenic fungi/ascomycetes**

Host Plant	Species	Target Gene(s)	Role(s) of Target(s) Gene(s)	Method of Production	References
Cereals					
Barley	*Fusarium graminearum*	*CYP51A, CYP51B,* and *CYP51C*	Ergosterol biosynthesis	*In vitro* (MEGA script® RNAi Kit	Koch et al., 2016
Barley	*Fusarium asiaticum*	*ß2 tubulin*	Fungal growth	*In vitro* (MEGA script® RNAi Kit)	Gu et al., 2019
Barley	*Fusarium graminearum*	*ARGONAUTE* and *DICER*	Fungal vegetative and generative growth, mycotoxin production, antiviral response	*In vitro* (MEGA script® RNAi Kit)	Werner et al., 2020
Rice	*Rhizoctonia solani*	*DCTN1, SAC1, polygalacturonase (PG)*	Vesicle trafficking pathway genes and virulence factor	*In vitro (MEGA script® RNAi Kit)*	Qiao et al., 2021
Wheat	*Fusarium asiaticum*	*Myosin 5 gene*	Cytokinesis and actin filaments organization	*In vitro* (MEGA script® RNAi Kit)	Song et al., 2018
Wheat	*Fusarium asiaticum*	*Myosin 5 gene*	Cytokinesis and actin filaments organization	*In vitro* (MEGA script® RNAi Kit)	Song et al., 2018
Wheat	*Fusarium asiaticum*	*ß2 tubulin*	Fungal growth	*In vitro* (MEGA script® RNAi Kit)	Gu et al., 2019
Wheat	*Fusarium graminearum*	*RdRP1, AGO1, QDE3, QIP, AGO2, DCL1, RdRP2, RdRP3, RdRP4,* and *DCL2*	Sexual reproduction AGO generative development DCL1		Gaffar et al., 2019

Table 3. Cont.

Host Plant	Species	Target Gene(s)	Role(s) of Target(s) Gene(s)	Method of Production	References
Vegetable					
Cucumber	*Fusarium asiaticum*	*ß2 tubulin*	Fungal growth	*In vitro (MEGA script® RNAi Kit)*	Gu et al., 2019
Tomato	*Aspergillus niger*	*VPS51, DCTN1, SAC1, pgxB*	Vesicle trafficking pathway genes and virulence factor	*In vitro (MEGA script® RNAi Kit)*	Qiao et al., 2021
	Botrytis cinerea	*DCL1 and DCL2*	Effectors	*In vitro* (MEGA script® RNAi Kit)	Wang et al., 2016
		VPS51, DCTN1, SAC1	Vesicle trafficking pathway genes	*In vitro (MEGA script® RNAi Kit)*	Qiao et al., 2021
	Colletotrichum gloeosporioides	*DCL1-2, VPS51, DCTN1, SAC1*	Effectors and vesicle trafficking pathway genes	*In vitro (MEGA script® RNAi Kit)*	Qiao et al., 2021
Lettuce	*Botrytis cinerea*	*DCL1 and DCL2*	Effectors	*In vitro* (MEGA script® RNAi Kit)	Wang et al., 2016
		VPS51, DCTN1, SAC1	Vesicle trafficking pathway genes	*In vitro (MEGA script® RNAi Kit)*	Qiao et al., 2021
	Sclerotinia sclerotiorum	*VPS, DCTN1, SAC1*	Vesicle trafficking pathway genes	*In vitro (MEGA script® RNAi Kit)*	Qiao et al., 2021
Collard green	*Sclerotinia sclerotiorum*	*VPS, DCTN1, SAC1*	Vesicle trafficking pathway genes	*In vitro (MEGA script® RNAi Kit)*	Qiao et al., 2021

Table 3. Cont.

Host Plant	**Species**	**Target Gene(s)**	**Role(s) of Target(s) Gene(s)**	**Method of Production**	**References**
Oil Crops					
Onion	*Botrytis cinerea*	*DCL1* and *DCL2*	Effectors	*In vitro* (MEGA script® RNAi Kit)	Wang et al., 2016
Soya	*Fusarium asiaticum*	*β2 tubulin*	Fungal growth	*In vitro* (MEGA script® RNAi Kit)	Gu et al., 2019
Canola	*Sclerotinia sclerotiorum*	59 target genes	Cell wall modification, mitochondria, ROS response, protein modification, pathogenicity factors, transcription, splicing, and translation	*In vitro* (MEGA script® RNAi Kit)	McLoughlin et al., 2018
Fruit Crops					
Apple	*Aspergillus niger*	*VPS51, DCTN1, SAC1, pgxB,*	Vesicle trafficking pathway genes and virulence factor	*In vitro (MEGA script*® *RNAi Kit)*	Qiao et al., 2021
	Colletotrichum gloeosporioides	*DCL 1-2, VPS51, DCTN1, SAC1*	Effectors and vesicle trafficking pathway genes	*In vitro (MEGA script*® *RNAi Kit)*	Qiao et al., 2021

Table 3. Cont.

Host Plant	Species	Target Gene(s)	Role(s) of Target(s) Gene(s)	Method of Production	References
Fruit Crops					
Banana	*Mycosphaerella fijiensis, Fusarium oxysporum*	*Nuclear condensing, Coatomer alpha, DNA directed RNA polymerase, ARP 2/3, Coatomer zeta, Cap methyltransferase, GTPase-binding protein, Proteasome Pre4, Ribosomal RNA, DNA polymerase alpha subunit, DNA polymerase delta Subunit, Adenylase cyclase, Protein kinase C, FRQ-interacting RNA helicase*	Spore germination	*In vitro* (MEGA script® RNAi Kit)	Mumbanza et al., 2013
Cherry	*Colletotrichum gloeosporioides*	*DCL 1-2, VPS51, DCTN1, SAC1*	Effectors and vesicle trafficking pathway genes	*In vitro (MEGA script® RNAi Kit)*	Qiao et al., 2021
Grape	*Botrytis cinerea*	*BcCYP51, Bcchs1,* and *BcEF2*	Elongation factor, ergosterol and chitinase biosynthesis	*In vivo* (*HT115* (DE3) *E. Coli)*	Nerva et al., 2020
		DCL1 and *DCL2*	Effectors	*In vitro* (MEGA script® RNAi Kit)	Wang et al., 2016
		VPS51, DCTN1, SAC1	Vesicle trafficking pathway genes	*In vitro (MEGA script® RNAi Kit)*	Qiao et al., 2021
	Aspergillus niger	*VPS51, DCTN1, SAC1, pgxB,*	Vesicle trafficking pathway genes and virulence factor	*In vitro (MEGA script® RNAi Kit)*	Qiao et al., 2021
Strawberry	*Botrytis cinerea*	*DCL1* and *DCL2*	Effectors	*In vitro* (MEGA script® RNAi Kit)	Wang et al., 2016
Flowers					
Rose	*Botrytis cinerea*	*DCL1* and *DCL2* *DCL1* and *DCL2*	Effectors	*In vitro* (MEGA script® RNAi Kit)	Wang et al., 2016
		VPS51, DCTN1, SAC1	Vesicle trafficking pathway genes	*In vitro (MEGA script® RNAi Kit)*	Qiao et al., 2021

Table 3. Cont.

Host Plant	Species	Target Gene(s)	Role(s) of Target(s) Gene(s)	Method of Production	References
Model Plant					
Arabidopsis	*Botrytis cinerea*	*DCL1* and *DCL2*	Effectors	*In vitro* (MEGA script® RNAi Kit)	Wang et al., 2016
Arabidopsis	*Sclerotinia sclerotiorum*	59 target genes	Differentially upregulated genes	*In vitro* (MEGA script® RNAi Kit)	McLoughlin etal., 2018
Arabidopsis	*Fusarium graminearum*	*CYP51*	Ergosterol biosynthesis	*In vitro* (MEGA script® RNAi Kit)	Höfle et al., 2020
Arabidopsis	*Verticillium dahliae*	*DCL 1-2, DCTN1, SAC1*	Effectors and vesicle trafficking pathway genes	*In vitro (MEGA script® RNAi Kit)*	Qiao et al.,2021
Arabidopsis	*Macrophomina phaseolina*	*Chitin synthase (MpCHS) gene*	Catalyze the *β-1,4* polymerization of *N-acetylglucosamine*		Forster and Shuai, 2020

4. Exogenous Delivery of Small RNA for Controlling Fungal Pathogens of Plants

The exogenous delivery method is certainly the most promising approach for the application of RNAi technology in the field (Yin et al., 2009; Dalakouras et al., 2016). This method avoids any modification of crop genomes and can be exploited against virtually any microbial pathogen that is responsive to RNAi approaches (Wang et al., 2016; Wang and Jin, 2017). Hence, the exogenous method can be an alternative method to HIGS, more easily accepted by public and biosafety authority, and faster to optimize than the obtainment of a HIGS plant. The first observation, explaining exogenous delivery of dsRNA molecules on plants, inducing RNAi of a plant gene, was reported in *Nicotiana benthamiana* plants pre-treated with the surfactant Silwet L-77 (Sammons et al., 2011). In this study, in-vitro-transcribed 685 bp dsRNAs and/or chemically synthesized 21-nt sRNAs targeting the endogenous phytoene desaturase mRNA was sprayed on plant surfaces resulting in extensive phytoene desaturase downregulation (Sammons et al., 2011). In an exogenous RNAi mechanism, to induce RNAi and achieve successful protection against pathogens, two prerequisites are fundamental: i) the sensitivity of the target organism to the silencing process stimulated by dsRNA, and ii) the capability to uptake external RNA molecules from the environment by fungal pathogens (Koch et al., 2016; Wang et al., 2016; Wang and Jin, 2017), viruses (Mitter et al., 2017; Niehl et al., 2018; Vadlamudi et al., 2020), and insects (Whangbo and Hunter, 2008; Li et al., 2015; Ghosh et al., 2018). Plants and fungi are capable of taking up externally applied dsRNAs and siRNAs. Reports showed that fungi can uptake 21nt sRNA duplexes as well as long dsRNAs of at least up to 800 nt (Koch et al., 2016; Wang et al., 2016). The presence of Dicer, Argonaute, and RdRP proteins in several fungal species suggests that they should be capable to display active RNAi mechanisms (Dang et al., 2011; Gaffar et al., 2019). However, exogenous delivery of small RNA to fungi can be tricky and for some fungal species has not been achieved yet. The reason underneath reluctance of RNA uptake by some fungal species can be difficult to explore and can be associated with different biological aspects, including the cell wall or membrane biochemical components (Wang et al., 2016). For example, *Zymoseptoria tritici* encodes the core components of the RNAi machinery but still is dsRNA insensitive (Kettles et al., 2019). The authors have demonstrated through live-cell imaging that the conidiospores of *Z. tritici* were unable to absorb dsRNAs, suggesting that there may not be an encoded dsRNA receptor or a defect in the uptake pathway. Wang and co-workers reported rapid dsRNA uptake from the environment by *Botrytis cinerea* and that these RNAs were able to suppress fungal genes in a sequence-specific manner (Wang et al., 2016). In *Sclerotinia sclerotiorum,* a scientific

study demonstrated that the uptake of dsRNA occurs through clathrin-mediated endocytosis (Wytinck et al., 2020). One of the few recent studies reported that various beneficial or pathogenic fungal and oomycetes organisms have diverse capacity to adsorb fluorescein-labeled dsRNA from the environment, and this competence seems to have an influence on the efficacy of the RNAi when virulence-related gene were targeted through a spray-induced gene silencing (SIGS) approach for the defense of the hosts. The authors showed that *Colletotrichum gloeosporioides* cannot uptake dsRNA, whereas in *Trichoderma virens* and *Phytophthora infestans* RNA uptake was limited. The situation is different in *Botrytis cinerea*, *Sclerotinia sclerotiorum*, *Rhizoctonia solani*, *Aspergillus niger*, and *Verticillium dahliae* in which fluorescent dsRNAs are already inside the fungal cells within 6 hours after administration of specific long dsRNA (Qiao et al., 2021). Overall, information on dsRNA uptake in fungi is scarce, which is due to the limited number of studies conducted on the efficacy of exogenous RNAi against phytopathogenic fungi so far.

4.1. Formulation of Small RNA

The overall success of using exogenous RNAi is dependent on the mode of delivery of RNA molecules, application methods, length and/or concentration of dsRNAs, plant-organ specific activities, and stability under unsuitable environmental conditions (Dalakouras et al., 2016; Mitter et al., 2017; Dubrovina et al., 2019). The main constraint of exogenous applications of naked-dsRNAs is their short-term stability. Complexation of dsRNA with carrier molecules is a solution widely used to overcome this limitation (Numata et al., 2014; Lichtenberg et al., 2019; Christiaens et al., 2020). Although most studies of dsRNA carriers for plant protection have concentrated on insects (Zotti and Smagghe, 2015), the improved stability and penetrability of some formulations may also be applied to phytopathogenic fungi. It is tricky to predict when a fungal outbreak will occur and, thus, the longer the protective antifungal treatment on the surface of the plant will remain intact, the more likely it will be successful when the infection occurs. Furthermore, a variety of necrotrophic pathogens, such as *S. sclerotiorum*, can become systemic in a matter of days within the plant (Girard et al., 2017). This underlines the importance of getting the optimized load of dsRNA into the fungus as quickly as possible, and this can be done by carriers that enhance penetrability. In order to increase stability and uptake efficiency, dsRNA can be incorporated into nanoparticles. Nanoparticles are the most common choice made in order to deliver the unstable naked dsRNA/siRNA to the targeted sites since they protect the dsRNA/siRNA from degradation.

Besides, they can be used by adding target-specific ligands to their surface for targeted delivery (Young et al., 2016). Chitosan (poly β-1,4-Dglucosamine) is one of the most widely used polymers to generate nanoparticles to protect and deliver dsRNA/siRNA to target cells (Lichtenberg et al., 2020). Chitosan has been the topic of many studies, due to its inexpensive production from marine waste, low toxicity, and a wide variety of molecular weights and modifications available (Kean and Thanou, 2010; Vázquez et al., 2013). It has been shown that chitosan-based formulations boost endonuclease stability and uptake in a variety of species of insects (Zhang et al., 2010; Wang et al., 2020). Another means to obtain an increased RNAi efficiency is through the use of layered double hydroxide clay nanosheets. Positively charged nanosheet stacks bind the dsRNA negative charges electrostatically and provide enhanced protection against environmental factors and nucleases. Mitter et al., 2017 reported that loading RNAi inducing dsRNAs into layered double hydroxide clay nanosheets and applying to plant surface enabled sustained release of the dsRNA for up to 30 days. The formulated dsRNAs (Bioclay) offered protection against virus for up to 20 days post spraying, compared to naked dsRNA which offered 5 days protection window. Owing to this increased period of bioactivity, this technology also holds the potential to be useful in insect and fungal defense. Interestingly, this formulation also seems to facilitate uptake and systemic dissemination within the sprayed host plant (Mitter et al., 2017). The use of a class of very small nanoparticles, called carbon dots, for the delivery of siRNA to the *Nicotiana benthamiana* and tomato plants, has also been reported (Schwartz et al., 2020). In addition, a liposome-based delivery method has been applied in insects, fungi, and nematodes (Lin et al., 2016; Nami et al., 2017; Adams et al., 2019) with success in altering gene expression and/or mortality. It should be stated here that, although carrier compounds considerably facilitate RNA delivery, they are also quite expensive and/or difficult to synthesize. Different administration strategies have been reported in mammalian cells, such as conjugation of dsRNAs to cholesterol, cationic lipids, and cell-penetrating peptides (Rettig and Behlke, 2013; Kim and Rossi, 2007). Future studies are required to determine whether they also improve dsRNA uptake and efficiency in fungal pathogens.

4.2. Delivery Methods

Different application/delivery strategies have been studied in various agricultural pest species and the main dsRNA application methods tested so far include high-pressure spray, injection into trunks, soil application, petiole absorption, brush-mediated application, infiltration, injection, root soaking, soil/root drench, and postharvest spraying of bunches (Whangbo and

Hunter, 2008; Jiang et al., 2014; Numata et al., 2014; Li et al., 2015; Dalakouras et al., 2016; Koch et al., 2016; Wang et al., 2016; Dalakouras et al., 2018; Ghosh et al., 2018; Dubrovina et al., 2019; Nerva et al., 2020). When high-pressure spraying was used for the exogenous application of siRNAs, it was successful in inducing local and systemic silencing of the green fluorescent protein (GFP) transgene in *N. benthamiana* (Dalakouras et al., 2016). Here, high-pressure spraying was more effective compared to wiping, infiltration, and gene gun techniques. Direct exogenous application of dsRNA, by spreading with sterile individual soft brushes without using any additional techniques, was also observed successful in inducing efficient suppression of enhanced *green fluorescent protein* (*eGFP*) and *neomycin phosphotransferase–II* (*NPTII*) transgenes in *Arabidopsis* (Dubrovina et al., 2019). The authors analyzed the effects of different dsRNA concentrations (0.1, 0.35, and 1.0 μg/μl) and the concentration at 0.35 μg/μl had a higher significant influence on transgene-silencing efficiency (Dubrovina et al., 2019). The effects of different lengths of dsRNAs (315, 596, and 977-bp) targeting different virus genes were also investigated in *N. tabacum* leaves, and results indicated that shorter dsRNAs showed reduced antiviral activity, indicating that dsRNA length could influence its efficacy (Tenllado et al., 2001). Overall, fungal uptake of environmental RNAs appears less dependent on RNA size, as both short sRNA duplexes and long dsRNAs are taken up and stimulate strong gene silencing in the fungal cells.

The efficient delivery of dsRNA is crucial in moving RNAi-based fungal control from laboratory to field. dsRNAs not only move within a fungus but they can also transfer from the environment to the fungus (environmental uptake), and between interaction of plants and fungus (cross-kingdom dsRNA trafficking), thereby subsequently inducing gene silencing in the fungal organism (Wytinck et al., 2020). Exogenous RNAs derived from plant fungal pathogens gene sequences can either be directly internalized into fungal cells or indirectly via passage through plant tissue before transport into targeted fungal cells (Weiberg et al., 2013; Koch et al., 2016; Wang et al., 2016; Song et al., 2018). The vascular system of plants translocates RNAs (Melnyk et al., 2011); indeed, RNAi in plants is linked with the production of a mobile signal that can move from cell-to-cell and over long distances. This fact can therefore be useful in the establishment of targeted strategies for the control of pathogens (Hunter et al., 2012; De Andrade and Hunter, 2016). With respect to HIGS-in planta stable resistance, exogenous dsRNA applications offer shorter-term protection from fungal infections, but they could be particularly beneficial to shield agricultural food products during

post-harvest storage and protecting plant species for which not defined nor efficient transformation protocols are available (Wang and Jin, 2017).

Studies conducted on exogenous RNAi concerning fungal pathogens, summarized in Table 3, showed that exogenous application is effective in suppressing fungal growth. For example, a recent study by Werner and colleagues (Werner et al., 2020) showed that using spray-induced gene silencing (SIGS), targeting *Argonaute* and *Dicer* genes of *F. graminearum*, afforded protection of barley leaves from infection by *F. graminearum*. Similarly, *F. asiaticum* virulence decreased when in-vitro-transcribed dsRNA targeting its *myosin 5* gene was sprayed on wounded wheat coleoptiles (Song et al., 2018). In another study, foliar applications of in-vitro transcribed dsRNAs on canola (*Brassica napus*), targeting 59 genes of necrotrophic fungi reduced *S. sclerotiorum* and *B. cinerea* leaves infection (McLoughlin et al., 2018). Spraying of detached barley leaves with dsRNA, 791nt long, targeting three ergosterol biosynthesis genes *CYP51A, CYP51B,* and *CYP51C* of *F. graminearum*, effectively inhibited the fungal growth both in local areas, where the dsRNA was sprayed and in non-sprayed distal leaf parts (Koch et al., 2016). These results demonstrate that dsRNA can translocate within the plant. Topical application of dsRNA and sRNAs targeting *Dicer-like* (*DCL*) genes of *B. cinerea* (*BcDCL1* and *BcDCL2*) on the surface of tomato, strawberry, fox grape (*Vitis labrusca*), iceberg lettuce, onion, rose, and *Arabidopsis* leaves, effectively suppressed gray mold disease (Wang et al., 2016). On the other hand, the capacity of exogenously applied dsRNAs to prevent and counteract infection of *B. cinerea* was tested on grapevine (*Vitis vinifera*). Three separate approaches for dsRNA delivery into plants were applied, namely, high-pressure spraying of leaves, petiole adsorption of dsRNAs, and postharvest spraying of bunches. The results demonstrated that, independently from the method of application, the exogenous method can decrease the virulence of *Botrytis cinerea* (Nerva et al., 2020). These successful experiments of exogenous application indicated that exogenously supplied dsRNA could form the basis for the development of a new tool aimed at protecting crops against fungal diseases. The exogenous application of dsRNA can be very interesting also on horticultural produces at the postharvest stage (Wang et al., 2016) and against fungal pathogens, which are capable of producing mycotoxins very harmful to animal and human health (McDonald et al., 2005; Power et al., 2020). Their control at the disposition stage is strictly limited to a few active ingredients due to residue concerns. With regard to postharvest pathogens, the halted growth of *B. cinerea* on the surface of fruits, vegetables, and flowers due to dsRNAs and sRNAs of *BcDCL1/2* (Wang et al., 2016) shows the potential of externally applied small RNA as a new generation

of sustainable and environmentally friendly products for controlling postharvest pathogens. In addition, it should be recalled that post-harvest products are not exposed to open field environmental conditions such as UV light that promote degradation of dsRNAs and this makes them more suitable for protection during post-harvest.

5. Challenges of dsRNA-based Products for Disease Management Strategy in Plants

Exogenous application of dsRNA molecules has been largely successful to induce RNAi (Table 3), and the studies outlined above highlight several critical aspects that need to be addressed before the development of RNAi-based products against fungal pathogens. Some considerations are required concerning the future application of exogenous RNA molecules against fungi and addressing the major issues that presently limit the viability of RNAi for fungal pathogen control.

5.1. Epigenetic Effect

As mentioned above, exogenous RNAi is an efficient transgene-free approach in modern crop protection platforms. In SIGS approaches, RNA molecules are externally applied on plants in order to selectively trigger the degradation of target mRNAs. However, once present in the plant cell, the applied dsRNAs may be processed by *DCL4* into 21-nt siRNAs, which slice complementary mRNAs in a process termed post-transcriptional gene silencing (Hamilton and Baulcombe, 1999), and by *DCL2* into 22-nt siRNAs, which either recruit RNA-directed RNA polymerase 6 (RDR6) on the complementary mRNA for the generation of secondary siRNAs or repress mRNA's translation (Chen et al., 2010; Wu et al., 2020). Finally, *DCL3* processes the dsRNA into 24-nt siRNAs, that are involved in RNA-directed DNA methylation (RdDM) of cognate DNA sequences (Chan et al., 2004). Thus, in exogenous RNAi methods, the applied dsRNAs can trigger unexpected epigenetic alterations and lead to epigenetically modified plants. DNA methylation refers to the addition of a methyl group to the fifth carbon of the six-ring cytosine residue. DNA methylation was expected to be caused by DNA: DNA interactions for a long time, until a groundbreaking study showed that RNA: DNA interactions cause DNA methylation in viroid-infected tobacco plants, which was thus called RdDM (Wassenegger et al., 1994). Dubrovina and colleagues (Schwartz et al., 2020) applied in-vitro transcribed dsRNA targeting *GFP* and *NPTII* genes in transgenic *Arabidopsis thaliana* carrying a *GFP/NPTII* cassette. They observed that not only were *GFP* and *NPTII* mRNAs downregulated, but also DNA methylation occurred in the corresponding coding region 7

days after administration (Dubrovina et al., 2019). Therefore, the information from Dubrovina and colleagues 2019, seem to reflect a more general mechanism and support a more careful consideration of possible epigenetic changes in the application of exogenous RNAi, because plants treated with exogenous dsRNAs may still contain no transgenes, but they are still epigenetically modified. In general, the occurrence of epigenetic changes in the genome after the application of exogenous RNAi should be resolved and clarified. This will help better interpret the exogenous RNAi data obtained.

5.2. Biosafety Considerations

Because of its sequence-dependent mode of action, there is increasing interest to use RNAi, both in academia and the commercial sector, in the management strategies for a large number of agricultural pests and pathogens as either in planta stable expression or in topical application (Taning et al., 2020). RNAi-based plants have been already approved at the commercial level (corn and potato) and others are ready for submission (plum). The main issues for developing the risk assessment on these plants have been already defined (Arpaia et al., 2020). The same biosafety approaches can be used to assess and approve new RNAi-based products for topical application. Below, we try to synthesize the most important aspects that need to be addressed in the risk assessment of plants during exogenous RNAi application. Although the binding of dsRNA/siRNA is believed to be highly specific (Dillin, 2003), the siRNAs can bind to off-target genes that have sufficient sequence homology to the target gene (Lundgren and Duan, 2013). The binding of siRNA somewhere else within the target genome may not be a problem, but concerns increase if off-target binding happens in non-target organisms.

However, to reduce possible effects on non-target species, it is possible to use the sequence-dependent nature of RNAi as an advantage to tailor the design of dsRNA sequences (Schwartz et al., 2020). In fact, at the beginning of the development phase of the exogenous-RNAi mechanism, a thoughtful design of dsRNA will restrict the possibility of non-target effects due to sequence similarity. Designing a unique siRNA/dsRNA, which does not share high DNA identity with other genetic loci greatly limits the probability of off-target effects (Jackson et al., 2003; Davidson and Mccray, 2011). Current siRNA and dsRNA design guidelines for RNAi experiments suggest BLAST similarity searches (http://www.ncbi.nlm.nih.gov/BLAST) (Altschul et al., 1990) against sequence databases to pinpoint potential off-target genes to increase the probability that only the intended gene is targeted (Elbashir et al., 2002). However,

the BLAST algorithm was not specifically designed to assess RNAi off-target effects. Therefore, dedicated bioinformatics programs, like the open-access siRNA finder (si-FI) software (https://github.com/snowformatics/siFi21;Lücketal., 2019), ERNAi (https://www.dkfz.de/signaling/e-rnai3/) and dsCheck (http://dscheck.rnai.jp/http://dscheck.rnai.jp/), can also be used to screen the candidate dsRNA/siRNA sequences for complementarity with other genes.

6. Future Prospects and Concluding Remarks

Food security is threatened by production constraints including diseases. Crop protection against pathogens relies mostly on the widespread use of chemical pesticides that are applied to the environment in large amounts yearly. Some of these chemicals have been in use for almost half a century. Therefore, there is a need for novel tools that are more sustainable and less detrimental to the environment. RNAi is a novel and promising method that is gaining pace as a technique to cope with pathogens in many economically important crop plants. Despite few limitations, the applicability of RNAi to improve crop resistance, especially against pathogens, is expected to be the most reliable and significant approach in the future, as shown by a plethora of studies. Generally, RNAi has emerged as one of the most promising potential control mechanisms for plant pathogens and insects. Although still a lot remains to be explored and understood about the molecular process of RNAi in plants and their pathogens, the current knowledge available and the studies reviewed in this paper have proved that exogenous RNAi technology is an essential tool for identifying gene functions and targeting critical genes to control plant pathogenic fungal development. In-planta stable expression offers a possible long-term stable resistance to diseases. In-planta stable expression offers the benefits of a long-term stable resistance to diseases, but it is clearly classified as a GMO and needs to follow rules applied for this type of modified plants (Arpaia et al., 2020). Topical application, on the other hand, offers a more flexible solution for developing new dsRNA-based products to be used to protect crops in agricultural systems. Although information on external RNA uptake in fungi is limited, interesting progress has been achieved in *B. cinerea, F. asiaticum, F. graminearum, F. Oxysporum, M. phaseolina, M. fijiensis,* and *S. Sclerotiorum*. RNAi technology using the topical application of RNA molecules has emerged as a potential tool for improving various agronomically important plants. RNA-based biocontrol compounds are already under development and there is the perspective that new RNAi based formulates soon will reach the market, with a good cost-benefit balance for their application in different agriculture sectors.

This objective now seems quite achievable considering the availability of first documents, the most important one from OECD (http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/jm/mono (2020) 26&doclanguage=en), which indicate risk assessment and regulatory approaches for these new RNAi-based products in line with those applied for the authorization of new biological pesticides (Taning et al., 2020).

To develop dsRNA-based products, besides the identification of effective dsRNA sequences, we need to develop appropriate formulates and delivering systems depending on the type of fungi and plants. Technological advancement in the field of biotechnology has offered new understandings to detect distinctive target genes. In fungi, the formulation, uptake, and processing of dsRNAs remains relatively undescribed. Analyzing the stability and delivery methods of dsRNAs, and more specifically the uptake of these dsRNAs into the target organism, remains ready for investigation. The delivery of dsRNA via nanoparticle complexes has novel potential for crop protection against pests, especially those refractories to RNAi. The topical use of dsRNA/nanoparticle complexes is expected to be the future of RNAi-mediated control of pests/pathogens without genetic modification of crops. Although carrier compounds considerably facilitate RNA delivery, they are also quite expensive and/or difficult to synthesize. Biosafety approaches already adopted to approve RNAi-based plants can be used for developing the risk assessment for new dsRNA-based products. Existing legislation should be implemented to consider the approval of new dsRNA-based products. Taking into account these aspects, we can think of a very important role in the development of this technology to improve the systems of protection of plants from diseases in a more compatible way with the environment, as foreseen by the new lines expected from the green deal indicated by Europe and of interest in the world (Taning et al., 2020).

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Chapter 4: Exogenous dsRNAs against chitin synthase and glucan synthase genes suppress the growth of the pathogenic fungus *Botrytis cinerea*

Abstract

Botrytis cinerea, the causative agent of the gray mold disease, causes crop failures of many economically important crops worldwide and its control is usually difficult. This study aims to evaluate the effectiveness of RNA interference (RNAi)-based control strategy to suppress the growth of *B. cinerea*, through exogenous application of double-strand RNA (dsRNA) targeting three chitin synthase (*BcCHSI, BcCHSIIIa, BcCHSVI, BcCHSI/IIIa/VI*) genes' expression and two glucan synthase (*alpha-1,3-glucan synthase (BcAlgl), β-1,3-glucan synthase (BcBegl), BcAlgl/Begl*) genes' expression. Since chitin and glucan synthases are the main components in the fungal cell wall, we hypothesized that suppressing the expression of the above mentioned genes through RNAi would negatively affect the growth of the fungus, and ultimately its virulence. Our results show that the virulence of the fungs was indeed reduced due to dsRNAs treatments and that the reduction in virulence was found correlating with the downregulation of *BcCHSI, BcCHSIIIa, BcCHSVI,* and *BcCHSI/IIIa/VI, BcAlgl, BcBegl,* and *BcAlgl/Begl* genes. Overall, our data indicate that exogenous application of dsRNAs can compromise the virulence of *B. cinereal* and that RNAi-based strategy targeting genes important for cell wall synthesis may provide effective means to halt growth of *B. cinerea* and consequently affecting compatible interaction with its hosts to cause infection.

Keywords: *RNAi-based plant protection, Botrytis cinerea, fungal cell wall genes, fungal pathogen control.*

1. Introduction

Fresh fruits and vegetables are an essential source of nutrients in the human diet. Postharvest losses of fresh vegetables are expected to be 20%-40% globally, decreasing the availability of fruits and vegetables (Mari et al., 2016; Henz, 2017). Several pre- and post-harvest fungal pathogens such as *Botrytis, Colletotrichum, Fusarium, and Penicillium spp.* affect the quality of fresh fruits and vegetables during production, transport, and processing (Li et al., 2015). Diseases caused by the genus *Botrytis*, are among the most common diseases of berry and fruit crops, vegetables, ornamentals, and some field crops (Droby and Lichter, 2004). *B. cinerea* is a generalist necrotrophic fungal pathogen, which causes a gray mold on more than 1400 different plant species including many economically important crops such as grapes and tomatoes (Williamson et al., 2007; Dean et al., 2012; Elad et al., 2016). Each year gray mold disease caused by *B. cinerea* has been estimated to cause annual losses of $10 billion to $100 billion globally (Boddy, 2016).

About its management, chemical control, germplasm improvement, biological control, and crop management practices have so far been the main management strategies in grapes and tomatoes to combat gray mold contamination (Nicot et al., 2011; Fillinger and Walker, 2016), though the problem persists. The use of synthetic fungicides, with a global investment of over $1 billion, remains the principal method to control gray mold caused by *B. cinerea* (Hua et al., 2018). However, none of the methods produced results that were very encouraging because cultural practices can be ineffective due to the formation of overwintering structures, which can remain in the soil for several years, and the method requires time and resources (Derbyshire and Denton-Giles, 2016), the use of conventional fungicides has risks on human health and the environment (Pearson et al., 2016). Furthermore, the excessive and repeated use of conventional fungicides has resulted in the development of resistant strains to various fungicides with different modes of action (Chapeland et al., 1999; Rupp et al., 2017; Shao et al., 2021). Biological methods have not produced consistent or effective results against the fungus (Nicot et al., 2011). Plant breeding, which entails fixing the target gene in the desired plant over generations, is a viable and sustainable method for producing variations, but it is also very reliant on the gene pool of a small number of resistant cultivars (Bradshaw, 2017). Therefore, a new, effective, environmentally friendly, and species-specific alternative method is urgently required to help reduce crop losses due to gray mold. Recently, advances have been made in the RNA-based gene regulation approach i.e., RNA interference (RNAi), a sequence-

specific post-transcriptional gene silencing mechanism found in eukaryotes, in which the expression of a gene is specifically inhibited by its cognate double-stranded (dsRNA) (Fire et al., 1998; Montgomery and Fire, 1998; Pathak and Gogoi, 2016). It is a conserved biological response to double-stranded RNA, which provides resistance to both endogenous parasitic and exogenous pathogenic nucleic acids, as well as controls the expression of protein-coding genes (Hannon, 2002). Since its first report in petunias (Napoli et al., 1990), RNA interference (RNAi) has been studied as a potential nucleic acid-based treatment for many plant diseases. In RNAi-based pathogen management, a target gene is typically selected based on its importance in growth and/or pathogenicity. For instance, the knockdown of genes involved in the production of toxins (Johnson et al., 2018), pathogenicity factors (Tiwari et al., 2017), and growth (Alhawatema et al., 2017) has proved successful in reducing infection or limiting host symptoms caused by fungal infections. Delivering dsRNAs to a target organism is a crucial aspect that determines the success of RNAi technology in crop protection. Delivery can be achieved through either host-induced gene silencing (HIGS) RNAi approach or exogenous delivery of dsRNA (Spray-Induced Gene Silencing (SIGS)) (Koch et al., 2013; Wang et al., 2016).

Host-induced gene silencing (HIGS), an RNAi-based process in which RNA molecules are produced by the host plant to target invader transcripts, has emerged as an effective strategy for enhancing plant resistance against fungal pathogens (Klimes and Dobinson, 2006; Hernandez et al., 2009; Nowara et al., 2010; Tinoco et al., 2010; Koch et al., 2013; Panwar et al., 2013; Jahan et al., 2015; Ghag et al., 2014; Masanga et al., 2015; Song and Thomma, 2016, Zhang et al., 2016), oomycetes (Govindarajulu et al., 2015; Jahan et al., 2015), viruses (Waterhouse et al., 1998), bacteria (Escobar et al., 2001), insects (Baum et al., 2007; Mao et al., 2007) and nematodes (Huang et al., 2006; Chen et al., 2015; Shivakumara et al., 2017). In addition to the production of RNA molecules in planta as noted in HIGS, the exogenous delivery method has been used in several strategies against plant pathogen infections, with varying success. This method avoids any modification of crop genomes and can be exploited against virtually any microbial pathogen that is responsive to RNAi approaches (Wang et al., 2016; Limera et al., 2017). Hence, the exogenous method can be an alternative method to HIGS, more easily accepted by the public and biosafety authorities, and faster to optimize than the obtainment of a HIGS plant. Uptake of RNAs from the environment, a process known as environmental RNAi, was observed in fungi (Wang et al., 2016), *C. elegans* (Feinberg and

Hunter, 2003; Winston et al., 2007; Whangbo et al., 2008; McEwan et al., 2012), and insects (Ivashuta et al., 2015; San Miguel and Scott, 2015).

In this approach, the exogenously administered dsRNA can either be directly taken up by the fungal cells (Koch et al., 2016; Wang et al., 2016; McLoughlin et al., 2018; Qiao et al., 2021) or transit through plant cells (cross-kingdom RNAi) (Wang et al., 2016; Cai et al., 2018). There are reports of the successful implementation of exogenously administered RNAi molecules to protect plants against plant pathogenic fungi (Koch et al., 2016; Song et al., 2018). The growth of *Fusarium graminearum* was inhibited when dsRNA targeting fungal cytochrome P450 was topically administered to barley (*Hordeum vulgare* L.) leaves (Koch et al., 2016). Options to reduce gray mold production using RNAi in crops have had varying levels of success. Exogenous applications of the dsRNAs that target *B. cinerea* genes, such as thioredoxin reductase, mitochondrial import inner membrane translocase subunit TIM44, peroxidase, pre-40S ribosomal particle, and necrosis-and ethylene-inducing peptide 2, reduced the severity of *Brassica napus* infection by *B. cinerea* (Mcloughlin et al., 2018). Similarly topical application of dsRNA targeting virulence-related genes in *Botrytis cinerea*, *Sclerotinia sclerotiorum*, *Rhizoctonia solani*, *Aspergillus niger*, and *Verticillium dahlia* significantly inhibited plant disease symptoms (Qiao et al., 2021). In another study, exogenous administration of dsRNAs and siRNAs targeting Dicer-like 1 and 2 (*DCL1* and *DCL2*) genes of *B. cinerea*, significantly reduced the gray mold diseases in fruits (e.g., tomato, strawberry, and grape) and vegetables (e.g., lettuce and onion) (Wang et al., 2016). Nerva et al. 2020 also demonstrated the suppression of *B. cinerea CYP51, Bcchs1*, transcripts by application of spray-induced gene silencing methods using three independent dsRNA delivery approaches namely, high-pressure spraying of leaves and petiole adsorption of dsRNAs on the grapevine. These studies established the feasibility of exogenous applications of dsRNA to control pathogenic fungi. Although spray-induced gene silencing strategy has shown promising results in fungal species, the number of successful candidate genes that led to reduced *B. cinerea* growth development is limited, which is due to the limited number of studies conducted on the efficacy of exogenous RNAi against *B. cinerea* especially on fruit surfaces using exogenous applications of dsRNA molecules. Therefore, much work remains to be done to identify suitable target genes for RNAi-based fungicides against gray mold disease. In this study, RNAi activity in *B. cinerea* was investigated by testing the effectiveness of exogenously-applied dsRNAs to suppress fungal growth through targeted knockdown of chitin synthase genes (named *BcCHSI*, *BcCHSIIIa*, *BcCHSVI*, *BcCHSI/IIIa/VI* hereafter), and glucan synthase genes ((alpha-1,3-

glucan synthase (named *BcAlgl* hereafter)), and 1,3-β-D-glucan synthase ((named *BcBegl* hereafter), *BcAlgl/Begl*)), which are core components of the fungal cell wall (Rappleye et al., 2004; Ha et al., 2006). It is hypothesized that reduced expression of the chitin synthase and glucan synthase genes through RNAi will suppress the fungus' ability to grow, which may affect the capability of *B. cinerea* to infect host plants. We found that growth of dsRNA-treated fungi was suppressed, as indicated by smaller growth area. Real-time quantitative PCR (RT-qPCR) analysis revealed the growth phenotype was well correlated with significant decreases in *BcCHSI*, *BcCHSIIIa*, *BcCHSVI*, *BcCHSI/IIIa/Vi*, *BcAlgl, BcBegl, and BcAlgl/ BcBegl* transcript abundances. This study strongly supports an effort to apply an RNAi-based strategy to target *B. cinerea* in grapes and tomatoes and shows that RNAi is a viable technique to apply toward the control of gray mold disease in the future.

2. Materials and methods

2.1. Fungal isolate, bacterial strains, and plant material

Botrytis cinerea strain B05.10 was used in this study. The fungal strain was routinely cultured on Potato dextrose agar (PDA) medium (24g of potato dextrose broth and 15g of agar per liter) at room temperature for two weeks. The bacterial strain used for the in vivo production of dsRNA molecules was the *E. coli* strain *HT115* (DE3), which has the RNase III gene disrupted by a Tn10 transposon carrying a tetracycline-resistance marker, as well as an IPTG-inducible T7 RNA polymerase gene contained within a stable insertion of a modified lambda prophage DE3 (Timmons et al., 2001). For all other molecular biology manipulations, the *E. coli* strain DH5α was used. The bioassays were performed on grape and tomato fruits. Grape and tomato fruits purchased from local supermarkets were used as experimental host plant materials. Only fruit similar in size and free from any mechanical defects and diseases were selected for the experiment. Fruits were divided into two groups. One group was kept as a control (water and negative control (*αCOP*(*Cop*)), *Drosophila suzukii* gene), whereas the other ones were inoculated with the *B. cinerea* constructs. Before inoculation, the samples were surface sterilized by immersing them in 1% sodium hypochlorite solution for 30s (Wang et al., 2012). To remove any sodium hypochlorite residue, the samples were then washed three times in sterile distilled water and air-dried. For *B. cinerea* infection, the fungal spores were diluted in 0.01 g/ml PDB to a final concentration of 1×10^6 spores/ml, for drop inoculation of grape and tomato fruits.

2.2. Target Gene Identification and Selection

Nucleotide sequences of *B. cinerea* B05.10 strain *BcCHSI*, *BcCHSIIIa*, *BcCHSVI*, *BcAlgl* (alpha-1,3-glucan synthase), and *BcBegl* (1,3-beta-D-glucan synthase) genes were downloaded from Ensemble (https://fungi.ensembl.org/index.html). A selection of target genes (Table 1) was chosen based on fungicide sites of action found in the literature (Liu et al., 2018; Fishel and Dewdney, 2021), and based on previous reports on their effectiveness in the killing of other fungal species (Rappleye et al., 2004). SiRNAs targeting the genes were designed by using siFi software ((https://github.com/snowformatics/siFi21; Lück et al., 2019). Each resulting dsRNA and its complement were subjected to BLAST searches against the GenBank database NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to check for identical matches in species other than *B. cinerea*, which could lead to off-target binding. Final selections were made and used for the synthesis of the construct.

Table 1: Target genes in *B. cinerea* and their biological functions

Gene ID	Target gene name	Function	References
Bcin09g01210	BcCHSI	Fungal cell wall integrity	Soulie et al., 2003; Liu et al., 2018; Fishel and Dewdney, 2021
Bcin04g03120	BcCHSIIIa	Fungal cell wall integrity	Soulie et al., 2006; Liu et al., 2018; Fishel and Dewdney, 2021
Bcin12g05360	BcCHSVI	Fungal cell wall integrity	Cui et al., 2013; Liu et al., 2018; Fishel and Dewdney, 2021
Bcin08g02140	Bc-alpha-1,3-glucan synthase	Fungal cell wall integrity	Rappleye et al., 2004
Bcin02g06930	1,3-beta -D-glucan synthase, 1,3-beta -glucan synthase	Fungal cell wall integrity	Liu et al., 2018; Chen et al., 2016

2.3. Total RNA Extraction, cDNA Synthesis, PCR Amplification

The obtained sequences (mentioned in section 2.2) were introduced into the Primer 3 selection tool (http://primer3.ut.ee) for primer design to amplify the targeted portions of the aforementioned genes. All primers sequences were submitted to the manufacturer for synthesis (Eurofins Genomics). Total RNA was extracted from *B. cinerea* B05.10 mycelia using

Spectrum™ Planta Total RNA kit (Sigma Aldrich, USA) according to the manufacturer's instructions; complementary DNA (cDNA) molecules were synthesized by employing ImProm-II™ reverse transcriptase (Promega, USA). To Produce dsRNA templates using the cDNA, target sequences *BcCHSI* (390bp), *BcCHSIIIa* (381bp), *BcCHSVI* (395bp), *Bc*-alpha-1,3-glucan synthase (202bp), *Bc*-1,3-beta-D-glucan synthase,1,3-beta-glucan synthase (399bp), chimeric *BcCHSI/IIIa/VI* (643bp) and chimeric *Bc*-alpha-1,3-glucan synthase/1,3-beta -D-glucan synthase, 1,3-beta -glucan synthase (426bp) were amplified using gene-specific primers with restriction enzymes (Table 2). Then we constructed *BcCHSI/IIIa/VI* chimeric RNAi fragment obtained by integrating *BcCHSI* (202bp), *BcCHSIIIa* (222bp), and *BcCHSVI* (219bp), by overlapping PCR. In addition, we also constructed *Bc*-alpha-1,3-glucan synthase/1,3-beta-D-glucan synthase, 1,3-beta-glucan synthase chimeric RNAi fragment obtained by integrating *Bc*-alpha-1,3-glucan synthase (202bp) and *Bc*-1,3-beta-D-glucan synthase,1,3-beta-glucan synthase (224bp) *via* overlapping PCR. Restriction enzyme sites for BamH1 (GGATCC) and EcoR1 (GAATTC) were added to the 5' end of the forward and the reverse primers, respectively. Primers were designed to generate PCR amplicons of 202–643bp in length for the tool-designed construct, corresponding to exons of selected target genes. PCR conditions were maintained and each PCR reaction was carried out in a 50µl reaction volume containing, double-distilled water, dNTPs (10 mM), 10x Buffer, 10 ng of each forward and reverse primers, 0.5 units of Taq DNA polymerase, and 10 ng of DNA. The temperature profile used for PCR amplification comprised a denaturation step at 95 °C for 1 minute, followed by primer annealing temperature at 61-69 °C for 1 minute, and elongation at 72°C for 1 minute. After 40 cycles, the reaction was terminated with 10 min at 72 °C for the final extension. The PCR reaction was carried out under the same conditions for all the primers except for the annealing temperatures.

2.4. Construction of Recombinant Litmus 28i Vector and Double-stranded RNA synthesis in bacteria

The PCR product was cleaned using the nucleospin® gel and PCR clean-up kit (Macherey-Nagel, Germany) following manufacturer instructions, and the concentration was quantified using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). Double restriction enzyme digestion was then performed with EcoRI and BamHI (New England bio lab, GMbH, England) to clone the PCR amplicon into the multiple cloning site (MCS) of the plasmid Litmus28i vector, which contains a double and convergent T7 promoter to produce dsRNA.

The Litmus28i plasmid is a small (2800 bp), high-copy number *E.coli* plasmid vector designed for efficient transcription of dsRNA. The concentration was quantified using a Nanodrop2000 spectrophotometer (Thermo Fisher Scientific). Litmus28i was previously linearized with EcoRI and BamHI and dephosphorylated with alkaline phosphatase (New England bio lab, GMbH, England) to prevent self-ligation. After ligation DNA purification was done and then ligation products were transformed into chemically competent DH5α *E. coli* cells. To confirm the correctness of plasmid sequences, positive colonies were grown overnight in 4mL of LB plus antibiotic (100μg/mL ampicillin), subjected to plasmid extraction using the NucleoSpin Plasmid, Mini kit for plasmid DNA Kit (Macherey-Nagel, Germany). The recombinant vectors were validated by restriction digestion (BamHI and EcoRI) and sequencing before transformation into the RNase III-deficient *HT115* (DE3) *E. coli* strain (Kindly donated by Dr. Nji Tizi Clauvis, Department of Crop Protection, Faculty of Bioscience Engineering, Ghent Uni- versity, Ghent, Belgium). The plasmid vector containing the PCR product was transformed into chemically competent *HT115* (DE3) cells, an RNase III-deficient *E. coli* strain with IPTG-inducible T7 polymerase activity. The bacterial cells were plated on LB media supplemented with 12.5 μg/mL of tetracycline and 100 μg/mL of ampicillin as selection markers. Positive colonies were then screened by PCR and stored at −20 °C for the subsequent operation. Single colonies of *E. coli* containing Litmus 28i vector plus insert, cultured on Luria–Bertani (LB) agar plates, were inoculated into 4 ml of LB medium containing 4 ul of ampicillin (100 μg/ml) and 4 ul of tetracycline (12.5 μg/ml), and cultured overnight at 37 ^{0}C while shaking at 200 rpm. The bacterial solution was then diluted 100 times by transferring 250 μl of the overnight culture into 25 ml of fresh LB medium containing ampicillin (100 μg/ml) and tetracycline (12.5 μg/ml), and allowed to grow to an $OD_{600} = 0.4$**.** Then, expression of T7 RNA polymerase was induced by adding a final concentration of 12 μl of 1M of isopropyl-ß- D-1-thiogalactopyranoside (IPTG) and then incubated for an additional 3 hrs at the same conditions. The culture medium was centrifuged at 6000g for 10 min to harvest the bacteria cells, which were then suspended in 0.8 % NaCl solution and stored at -80 ^{0}C until the purification of the dsRNA from the bacteria cells. To purify and analyze the dsRNA synthesized in the bacteria, dsRNA was extracted from the bacterial cells using TRI reagent (Sigma-Aldrich) with some modifications. After the cell lysis step from the TRI reagent protocol, an extra step where single-stranded RNA was removed by incubating the lysate with 5 μl RNAse A (1000U/ll) and 25 μl of 10X RNase A buffer (4M NaCl, 0.1 M Tris–HCl) at 37 ^{0}C for 25 min was added. After purification, the dsRNA pellets were re-suspended in 25 μl nuclease-free water, and the concentration of the dsRNA was quantified using a Nanodrop

2000 Spectrophotometer (Thermo Scientific, Wilmington, Delaware, USA). The synthesized dsRNA was also evaluated by loading the suspension onto a 1.5% agarose gel, stained with gel red, and photographed to determine the integrity and estimate the quantity relative to the standard marker. In parallel, as a negative control of *B. cinerea* inoculation assay, Litmus28i vector containing a 398bp long coatomer subunit alpha gene (*αCOP/COP*) of *Drosophila suzukii* was used to synthesize dsRNA of *αCOP/COP*.

2.5. External application of RNAs on the surface of plant materials

The bioassays were performed on grape and tomato fruits to evaluate the protective effect of the dsRNA produced in vivo against *B. cinerea* infection. All dsRNAs were adjusted to a concentration of 120 ng μl^{-1} with RNase-free water before use. The grape and tomato fruits were treated with the dsRNA molecules produced in this study and then *B. cinera* inoculum (1x 10^6 ml $^{-1}$). Application of the dsRNA and the *B. cinerea* inoculum on grape and tomato fruits was performed by drop inoculation on the fruit surface with one drop of 20 µl of 120 ng/µl of dsRNA or water, and then after one hour one drop of 20 µl of 1 x10^6 ml^{-1} of sporangia solution on top of the droplet. Disease progress was evaluated until 7days post inoculation (dpi) for grape and 11 days post inoculation (dpi) for tomato in five and three biological replicates, respectively. A single fruit was considered a biological replicate. The controls used included: (a) sterile distilled water plus *B. cinerea* inoculum, and (b) in vivo produced coatomer subunit alpha gene *αCOP/COP-dsRNA* of *Drosophila suzukii* plus *B. cinerea* inoculum. In the bioassay, fruits treated only with sterile distilled water plus *B. cinerea* inoculum were used as a control to assess the infectivity of the fungal inoculum used. In the bioassay, fruits treated with *αCOP-dsRNA* plus *B. cinerea* inoculum were used as a negative control to assess the infectivity of the *B. cinerea* inoculum used.

2.6. Statistical analysis

All experiments were performed in triplicate. The statistical analyses were performed using one-way ANOVA to compare the dsRNA data in time, and $p < 005$ was considered statistically significant. The data were analysed using Sigma plot software (Systat Software, Inc., San Jose California USA). To assess the progress of the pathogen, fruit area covered by *B. cinerea* (in square millimetres) was measured from the digital images using the free software ImageJ program. Fruit area covered by the pathogen and disease progress rate data were analysed using analysis of variance. Means were separated by Tukey’s honestly significant difference test.

2.7. Total RNA extraction and Reverse transcription quantitative PCR (RT-qPCR)

B. cinerea strain B05.10 were grown on PDA plates at room temperature for 15 days. Grape and tomato fruits were bought from local markets. Each treatment contained three biological replicates. The surface of experimental grape and tomato fruits that were treated with dsRNA or water were excised at 3dpi and immediately frozen in liquid nitrogen, and kept at -80 ^{0}C until further purification and transcript analysis. For RNA extraction, ten grape and tomato fruits for each treatments were ground to fine powder with liquid nitrogen by using a precooled mortar and pestle. Total RNA was extracted using a rapid cetyltrimethylammonium bromide (CTAB) method (Gambino et al., 2008). The integrity of the extracted nucleic acids was determined by analysing 1uL of the extract using 1% TAE, agarose gel electrophoresis (Invitrogen), stained with 1X GelRed (Biotium Inc., Fremont, CA, USA) and visualized under UV light using a Gel Logic 212 Pro System (Carestream Molecular Imaging, New Haven, CT, USA), and the quantity and quality of total RNA was estimated using a Nanodrop 2000 Spectrophotometer (Thermo Scientific, Wilmington, Delaware, USA) with the OD260 nm/OD280 nm ratio expected to be between 1.8 and 2 and OD260nm/230nm >2 (indicating good RNA quality). For all the samples, one µg of total RNA, pre-treated with TURBO DNA-free KitTM (Invitrogen, CA, United States), was reverse transcribed by employing ImProm-IITM reverse transcriptase (Promega, USA) according to the manufacturer's protocol. The quantity of the final cDNA was assessed using a Nanodrop 2000 Spectrophotometer/2% agarose gel, and final cDNA products were aliquoted and stored at -20 ^{0}C until use. Purified cDNA samples were diluted properly with RNase-free water before used as templates in the qRT-PCR process. RNA extraction and cDNA synthesis from all samples were performed with three biological replicates.

2.8. Primers and real-time PCR

Quantitative-PCR (qRT-PCR) primers for the above five genes were designed using Primer 3 selection tool (http://primer3.ut.ee) (Table 3) with the melting temperature between 55 and 62 ^{0}C and a primer length of 20–21 bp. All primers sequences were submitted to the manufacturer for synthesis (Eurofins Genomics). The length of amplicons ranged from 130 to 144 bp (Table 3). We selected a reference gene (Bcin01g09620 (*BcRPL5*)) used previously in *B. cinerea* gene expression studies (Zhang and vanKan, 2013; Haile, 2017). Specific information for each primer is listed in Table 3. To assay the gene expression qPCR reactions were carried out in 96-well plates using SYBR Green-based PCR assay, MX3000 thermocycler (Stratagene CA,

United States CA, United States). The quantitative PCR reactions had a final volume of 12.5 µl and contained the following components: 2.5 µl of diluted cDNA as a template in 6.25 µl of SYBR Green /ROX qPCR 2x supermix (PE Applied Biosystems, Foster City, CA, USA), 3.25 µl of ultrapure water and 0.5 µl of 5 ng of specific forward and reverse primers (Table 3). Annealing temperatures were optimized according to individual genes and primers by testing several annealing temperatures ranging from 55 ^{0}C to 60 ^{0}C around the respective primer Tm, and the annealing temperature with the best efficiency was chosen. Reaction mixtures were incubated for 5 min at 95 ^{0}C, followed by 40 amplification cycles of 15s at 95 ^{0}C and 25s at 56-58 ^{0}C, and 30s at 72 ^{0}C. All samples were amplified in three biological replicates and two technical replicates. A negative control without cDNA template was also done at the same time. A standard curve for each gene was generated using tenfold serial dilutions of pooled cDNAs. A melting curve was established from 55 ^{0}C to 90 ^{0}C by changing 0.5 ^{0}C every 10s. All data were normalized against a reference gene (Bcin01g09620 (*BcRPL5*)). The efficiency of the five pairs of primers in qRT-PCR was calculated using Lin-RegPCR (Ramakers et al. 2003). The amplification efficiency value obtained was used to calculate the relative quantity (RQ) and normalized RQ (NRQ) according to Pfaffl (2001). Statistical analyses of the qPCR results were made after log(NRQ) transformation (Rieu and Powers, 2009). Statistical analyses were performed using SAS. The data are means ± standard errors of three biological replicates, and significant differences were determined by Student's t-test or Tukey–Kramer's honestly significant difference test.

Table 2. Primers used to amplify templates for dsRNA production

Gene name	Primer	Primer sequence (5'-3')	Annealing Temperature
BcCHSI	BcS_Chit1_Fw	TATA**GAATTC**CAATCCCAGTCAAAGCTACG	61 ºC
	BcS_Chit1_Rev	TATA**GGATCC**GGTGGTGCATGATTGACTTG	
BcCHSIIIa	BcS_Chit3_Fw	TATA**GAATTC**CAACAGCCCTTACGACTCTC	61 ºC
	BcS_Chit3_Rev	TATA**GGATCC**CCACCCTCCAAATCATTTCT	
BcCHSVI	BcS_Chit6_Fw	TATA**GAATTC**CCATCGCACCATCAAGAAAT	61 ºC
	BcS_Chit6_Rev	TATA**GGATCC**AGCGACTTCTTTTGCATTCC	
BcAlgl	BcS_alpha_Fw	TATA**GAATTC**AGACCATCATCGCCAACTC	63 ºC
	BcS_alpha_Rev2	TATA**GGATCC**TTCGGGCATCTTCATCATC	
BcBegl	BcS_beta_Fw	TATA**GAATTC**CGTATCACCAAGAGGGAGGT	66 ºC
	BcS_beta_Rev	TATA**GGATCC**GATATGGCTCCTTGGATCGT	
BcCHSI/IIIa/VI	BcChi_Chi1_FW	TATA**GAATTC**CAATCCCAGTCAAAGCTACG	69 ºC
	BcChi_Chi1_Rev_Ovlp	TCCTTGGGAGTGAGAGTCGTATCTGGCCCATAAGGTTCAT	
	BcChi_Chit3_FW_Ovlp	ATGAACCTTATGGGCCAGATACGACTCTCACTCCCAAGGA	
	BcChi_Chit3_Rev_Ovlp	ATTTCTTGATGGTGCGATGGGTTGTCTCCATGCCTCTGTG	
	BcChi_Chit6_FW_Ovlp	CACAGAGGCATGGAGACAACCCATCGCACCATCAAGAAAT	
	BcChi_Chit6_Rev	TATA**GGATCC**GGAATGAAGGAATCCACCAC	

Table 2 cont.

Gene name	Primer	Primer sequence (5’-3’)	Annealing Temperature
BcAlgl/Begl	BcgluA_C_FW	TATA**GAATTC**AGACCATCATCGCCAACTC	69 ºC
	BcgluA_C_Rev_Ovlp2	ACCTCCCTCTTGGTGATACGTTCGGGCATCTTCATCATC	
	BcgluB_C_Fw2	GATGATGAAGATGCCCGAACGTATCACCAAGAGGGAGGT	
	BcgluB_Rev	TATA**GGATCC**GCGGCCATAGTAGTCCATATC	
αCOP (Cop)	αCOP_Fw	TATA**GAATTC**AACTAAACTAAGGGGTCTCGC	60 ºC
	αCOP_Rv	TATA**GGATCC**GAATTACAAGACGGCCGCC	

Table 3. List of primers used for qRTPCR expression analysis in infected fruits

Gene ID	Gene name	Primers	
Bcin09g01210	*BcCHSI*	RPCR_Chit1_FW	CAAAGCTACGACTCGGAATA
		RPCR_Chit1_Rv	TCATCATTGTAAGCGTAGGG
Bcin04g03120	*BcCHSIIIa*	RPCR_Chit3_Fw	ACAACTCATCCCACACATAC
		RPCR_Chit3_Rv	CATAACGTTTGAGACCTCCA
Bcin12g05360	*BcCHSVI*	RPCR_Chit6_FW	TGAGAATGGAGAAATGGTGG
		RPCR_Chit6_Rv	ATGAGAGGAGGAATGAAGGA
Bcin08g02140	*BcAlgl* (Bc-alpha-1,3-glucan synthase)	RPCR_Alpha_Fw	TGCTAGTACCTCACAACTTG
		RPCR_Alpha_RV	CAGCTCTGTATCCCAAAGAA
Bcin02g06930	*BcBegl* (Bc-1,3-Beta Glucan)	RPCR_Beta_Fw	CTACAACGACCAGTACTACG
		RPCR_Beta_Rv	TCGAGCCATATCTGATCTCA
Housekeeping genes and primers used			
Bcin01g09620	*BcRPL5*	Bcrpl5-F	GATGAGACCGTCAAATGGTTC
		Bcrpl5-R	CAGAAGCCCACGTTACGACA

Table 4 . Sequences used for double-strand RNA synthesis

Gene name	Gene ID	Sequence
BcCHSI	Bcin09g01210	CAATCCCAGTCAAAGCTACGACTCGGAATATTCTTTAGATCCTAACGCGCATCACGATGCTTACTA CCAACCTCCATACCAACCCTCTCCTCATGAAGAACACCCTCTACAGAACTATGCCCCAGGGCAAGA CCCCTACGCTTACAATGATGATGATGACCATCAACCAATTCTACAATCGCATGAACCTTATGGGCC AGATCCGCACTCAGCTAGTGGCACTGATTACAAAGGTGGTTATGACGGGACGGTACAATCTCCATC AGCGACACCTGTACCTGCGTTAAGAAGATACAAGACGGTCAAGGAAGTCCAACTGTTCAATGGAA ATCTCGTACTCGATTGTCCGATTCCTCCCAAGCTTTTAAATCAAGTCAATCATGCACCACC
BcCHSIIIa	Bcin04g03120	CAACAGCCCTTACGACTCTCACTCCCAAGGAGGCCTTCGAGCGAATACTCCTCCAGTCAGACCTGT TTCTGCCTACAGTCTTACAGAAACGTATGCGAACGATCCACAACCATACAGCAGCGATTACAACTC ATCCCACACATACAATGAGCAGTTAGAAGATAACCCATACCCACAAACCGACACTCCTTTGTCAAG AGCCGGGACTACCTCCACAGAGGCATGGAGACAACGACAAGCCCCTCAGGCTGGAGGTCTCAAAC GTTATGCTACAAGAAAGGTTAAGCTTGTCCAGGGAAGCGTCTTGAGTGTCGATCATCCAGTACCCA GTGCTATCAAAAATGCAATTCAGCAAAAATACAGAAATGATTTGGAGGGTGG
BcCHSVI	Bcin12g05360	CCATCGCACCATCAAGAAATATCAGTGGTGGTGACTATGGACTCGGCTTCAAGGGAGATAATACGA GGGGCCAGGTTTATTACACTGATGAGAATGGAGAAATGGTGGATGATCCCGAAGGTGGCACCCCG AAAGCTGTCGTCAGCGAGGCAGCAACAAACCAGCGTCGTATCTGGGTCGCCATTACCTGGGCTTTC ACGTGGTGGATTCCTTCATTCCTCCTCTCATTTATTGGTCGGATGAAACGACCGGATGTACGCATGG CTTGGAGAGAAAAGCTAGTGTTGTGTTTCTTTATCCTCTTCATCAATGCCTTGGTTATTTCTGGATT ATTGAATTTGGTAAACTCCTCTGTCCAAATTCCGATAAAGCGTGGAATGCAAAAGAAGTCGCT
BcCHSI/IIIa/VI	-	AATCCCAGTCAAAGCTACGACTCGGAATATTCTTTAGATCCTAACGCGCATCACGATGCTTACTAC CAACCTCCATACCAACCCTCTCCTCATGAAGAACACCCTCTACAGAACTATGCCCCAGGGCAAGAC CCCTACGCTTACAATGATGATGATGACCATCAACCAATTCTACAATCGCATGAACCTTATGGGCCA GATACGACTCTCACTCCCAAGGAGGCCTTCGAGCGAATACTCCTCCAGTCAGACCTGTTTCTGCCTA CAGTCTTACAGAAACGTATGCGAACGATCCACAACCATACAGCAGCGATTACAACTCATCCCACAC ATACAATGAGCAGTTAGAAGATAACCCATACCCACAAACCGACACTCCTTTGTCAAGAGCCGGGA CTACCTCCACAGAGGCATGGAGACAACCCATCGCACCATCAAGAAATATCAGTGGTGGTGACTATG GACTCGGCTTCAAGGGAGATAATACGAGGGGCCAGGTTTATTACACTGATGAGAATGGAGAAATG GTGGATGATCCCGAAGGTGGCACCCCGAAAGCTGTCGTCAGCGAGGCAGCAACAAACCAGCGTCG TATCTGGGTCGCCATTACCTGGGCTTTCACGTGGTGGATTCCTTCATTCC

Table 5. Sequences used for double strand RNA synthesis

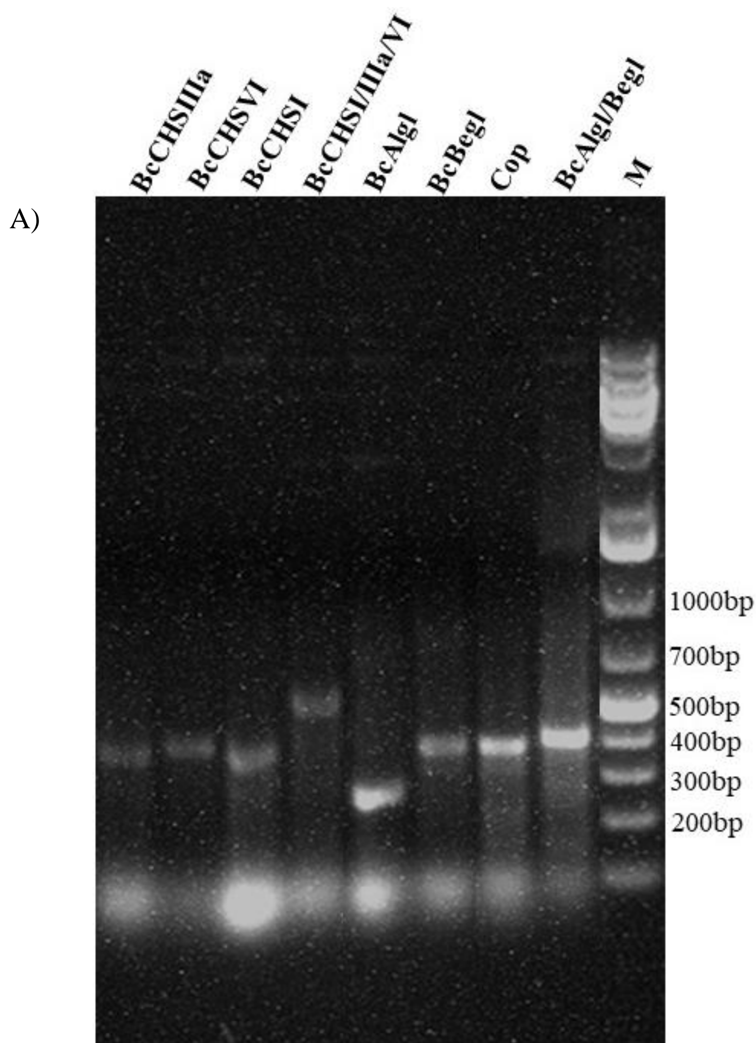
Gene name	Gene ID	Sequences
BcAlgl (Bc-alpha-1,3-glucan synthase)	Bcin08g02140	AGACCATCATCGCCAACTCGTGTTGAACAGCCACCGGCAGGAGGTGGACTGAGCCGAAAGC TTTCTTTGGGATACAGAGCTGGCCCGGGACATCGCGTAACGAAAAATAGATTCAATGGCAG TAATGCACCGGGTGCTGGAATCAAAAATGACGAAGGACTTACCGATGTGGATGAGGACAGC GATGATGAAGATGCCCGAA
BcBegl (1,3-beta -D-glucan synthase, 1,3-beta -glucan synthase)	Bcin02g06930	CGTATCACCAAGAGGGAGGTTATTACGAGGGTGGTGATCAAAACCAAGGCCAATATCAAGA TGAATACTACAACGACCAGTACTACGAGCAGGGTGGTGCAGCAGCTGGCGAAGCACCTCAA GCCAAGCGTCGGGGCGATTCAGAGGAGGATTCTGAGACTTTCAGCGACTTCACTATGAGAT CAGATATGGCTCGAGCCACCGATATGGACTACTATGGCCGCGGCGATGAGAGATACAACAG CTACAACGAGAGCCAAATGGGTGGTCGTGGCTACAGACCGCCATCTTCGCAGGTCTCTTAT GGTGGCAACAGATCATCCGGAGCATCAACGCCAAATTACGGAATGGACTACAACAATGTAC TTCCTGCCGGGCAACGATCCAAGGAGCCATATC
BcAlgl/Begl	-	AGACCATCATCGCCAACTCGTGTTGAACAGCCACCGGCAGGAGGTGGACTGAGCCGAAAGC TTTCTTTGGGATACAGAGCTGGCCCGGGACATCGCGTAACGAAAAATAGATTCAATGGCAG TAATGCACCGGGTGCTGGAATCAAAAATGACGAAGGACTTACCGATGTGGATGAGGACAGC GATGATGAAGATGCCCGAACGTATCACCAAGAGGGAGGTTATTACGAGGGTGGTGATCAAA ACCAAGGCCAATATCAAGATGAATACTACAACGACCAGTACTACGAGCAGGGTGGTGCAGC AGCTGGCGAAGCACCTCAAGCCAAGCGTCGGGGCGATTCAGAGGAGGATTCTGAGACTTTC AGCGACTTCACTATGAGATCAGATATGGCTCGAGCCACCGATATGGACTACTATGGCCGC
αCOP/COP	-	AACTAAACTAAGGGGTCTCGCTTGGCGTGGAGGAAACGTAAATATTGGAAAGCAAACAAG CCTGTCCGCCCTAGCGGAATTGCAGATTGGAGATCCGCAGTCCGATGCTATCCTTGCCGATC TGGCTCACCTCGCAAACGGTGCAGAGACTTCCCTTGAACTGCGGGTCGTAGGAGGAGGAGC AGAAGGGACAGGTCACCTCGGGTTTGCCCCTGTACAACGGCTTCCAGCTGATGCCGCAGAT TGTGAATGGATTAAACTCCTCGTACTGCAGCTGATGCTCATCCACGGGGTTCACCTCGCAAG CCTGCAGGATCTTCCGCACTTGCTGGGCGACATCTGGGCGAGGAGCCAACTCCAGAAGACG GCGGGCAAAAGAGGCGGCCGTCTTGTAATTC

3. Results

3.1. Identification of target genes and dsRNA production

B. cinerea chitin synthase and glucan synthase genes (Table 1) were identified and downloaded from the Ensemble database (https://fungi.ensembl.org/index.html), which were later named *BcCHSI (Bcin09g01210)*, *BcCHSIIIa (Bcin04g03120)*, *BcCHSVI (Bcin12g05360)*, *BcAlgl* ((*Bcin08g02140*) alpha-1,3-glucan synthase) and *BcBegl* ((*Bcin02g06930*)1,3-beta-D-glucan synthase). *BcCHSI*-dsRNA, *BcCHSIIIa*-dsRNA, and *BcCHSVI*-dsRNA were 390bp, 381bp, and 395bp long, respectively (Table 4). Whereas *BcAlgl*-dsRNA and *BcBegl*-dsRNA were 202bp and 399bp long, respectively (Table 5). The dsRNA templates used for cloning were produced by PCR using cDNA and gene-specific primers with restriction enzyme sites for BamH1 (GGATCC) and EcoR1 (GAATTC) added to the 5' end of the forward and the reverse primers, respectively. In addition, chimeric *BcCHSI/IIIa/VI* (643bp) gene, constructed by integrating *BcCHSI* (202bp), *BcCHSIIIa* (222bp), and *BcCHSVI* (219bp), and chimeric *BcAlgl/Begl* (426bp) gene obtained by integrating *BcAlgl* (202bp) and *BcBegl* (224bp), using overlapping PCR. Bacterial systems employed for the production of dsRNA molecules (Figure.1). The dsRNAs were produced by transforming an RNase III-deficient *E. coli* strain (*HT115)* having an IPTG-inducible T7 polymerase activity, with a litmus 28i plasmid (2814 bp) containing the gene-specific sequence to be transcribed.

A)



B)

dsRNA	Conc (ng/µl)	260/280	260/280
BcCHSI	1320.4	2.00	1.97
BcCHSIIIa	1521.2	2.00	2.00
BcCHSVI	1841.7	2.00	2.00
BcCHSI/IIIa/VI	2417	2.00	2.00
BcAlgl	1278.4	2.00	2.01
BcBegl	1921.5	2.0	1.99
BcAlgl/Begl	2013.2	2.0	2.03
Cop	1627.4	1.99	2.00

Figure 1. (A). Agarose gel electrophoretic analysis of *BcCHSI*, *BcCHSIIIa*, *BcCHSVI*, *BcCHSI/IIIa/VI*, *BcAlgl*, *BcBegl*, Cop, *BcAlgl/BcBegl*-dsRNA synthesized by *E.coli* (*HT115*). Samples (3 µl of 1/100 dilution + 2 µl of pure dsRNA extracted) were loaded as 5 µl. M: size molecular marker. (B). The quality of the dsRNA, as measured by 260/280 and 260/230 absorbance, was quantified by NanoDrop 1000 Spectrophotometer (Thermo scientific, Waltham, USA).

3.2. Preliminary inoculation assay to determine the minimum concentration of dsRNA

To ascertain the minimum effective concentration of the dsRNAs, *B. cinerea* was treated with 50 ng/µl and 90 ng/µl of chimeric *BcCHSI/IIIa/VI-dsRNA*, a fragment that was obtained by integrating *BcCHSI, BcCHSIIIa, and BcCHSVI,* and chimeric *BcAlgl/Begl-dsRNA* a fragment that was obtained by integrating *BcAlgl* and *BcBegl*. After the treatment with 50 ng/µl and 90ng/µl of *BcCHSI/IIIa/VI-dsRNA* and *BcAlgl/Begl-dsRNA*, and water, as control, tomato and grapes were challenged with 20 µl *B. cinerea* conidia (1 x10^6 ml^{-1}). Inoculated fruits were monitored for 7 days for grapefruits and 11 days for tomato fruits. Interestingly, the effects of dsRNAs on the *B. cinerea* varied with concentration (Fig 2, Fig 3, Fig 4, Fig 5). At 7dpi and 11 dpi for grape and tomato, respectively, the rate of disease progression was relatively slower in fruits that received 90 ng/µl of *BcCHSI/IIIa/VI-dsRNA* or *BcAlgl/Begl-dsRNA* (Fig. 2, Fig. 3, Fig.4, Fig. 5), indicating that pathogen control efficiency can increase with higher concentrations. Therefore, the ability of dsRNA to control *B. cinerea* growth in this treatment was assessed using a higher concentration (i.e., 120 ng/µl).

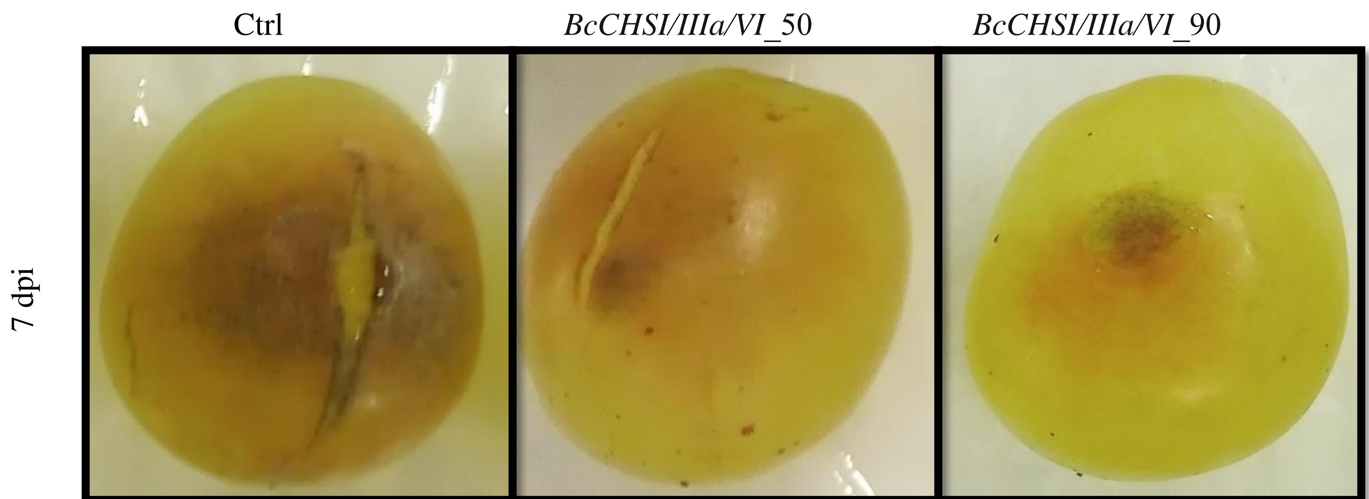


Figure 2. Externally applied *BcCHSI/IIIa/VI* dsRNA on grapefruits inhibited *Botrytis cinerea* infection. Fruits were treated with 20 µl of water (ctrl) or dsRNA before being inoculated with 20 µl of 1x10^6 mL^{-1} sporangia. *BcCHSI/IIIa/VI*_50: *BcCHSI/IIIa/VI dsRNA* at 50 ng μl^{-1} concentration; *BcCHSI/IIIa/VI*_90: *BcCHSI/IIIa/VI dsRNA* at 90 ng μl^{-1} concentration; dpi: days post-inoculation.

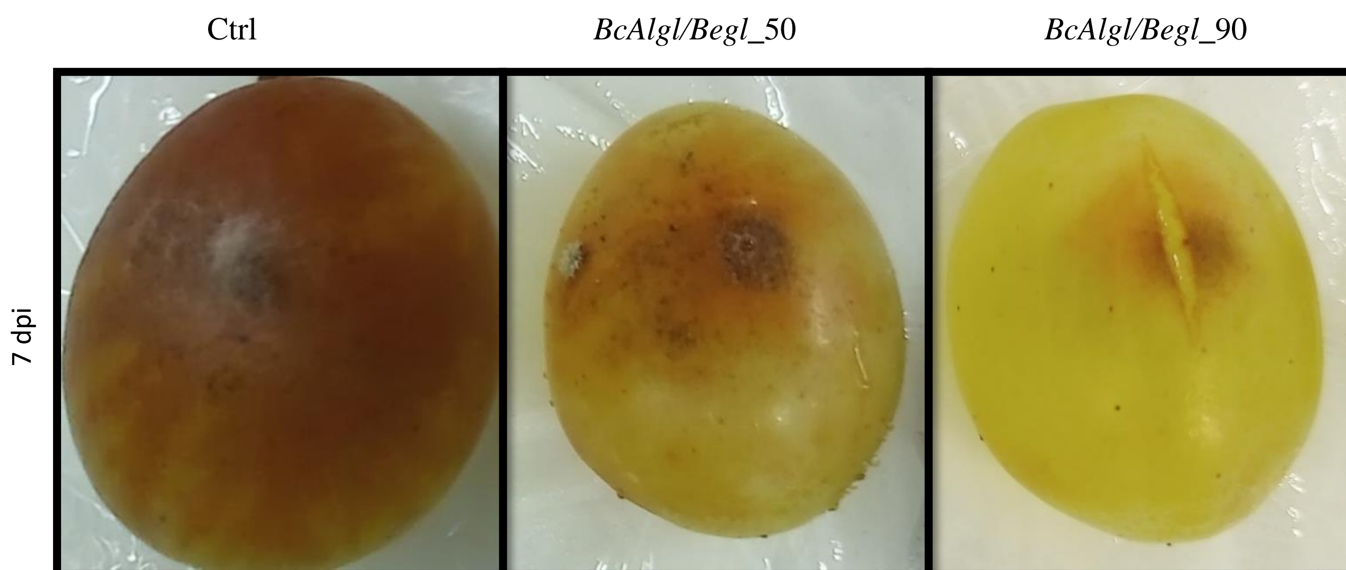


Figure 3 . Externally applied *BcAlgl/Begl* dsRNA on grape fruits inhibited *Botrytis cinerea* infection. Fruits were treated with 20 µl of water (ctrl) or dsRNA before inoculated with 20 µl of a $1x10^6$ mL^{-1} sporangia. *BcAlgl/Begl*_50 : *BcAlgl/Begl* at 50 ng $µl^{-1}$ concentration; *BcAlgl/Begl* _90: *BcAlgl/Begl* at ng $µl^{-1}$ concentration; dpi: days post inoculation.

Figure 4. Externally applied *BcCHSI/IIIa/VI* dsRNA on tomato fruits inhibited *Botrytis cinerea* infection. Fruits were treated with 20 µl of water (ctrl) or dsRNA before being inoculated with 20 µl of $1x10^6$ mL^{-1} sporangia. *BcCHSI/IIIa/VI*_50: *BcCHSI/IIIa/VI dsRNA* at 50 ng $µl^{-1}$ concentration; *BcCHSI/IIIa/VI*_90: *BcCHSI/IIIa/VI dsRNA* at 90 ng $µl^{-1}$ concentration; dpi: days post-inoculation.

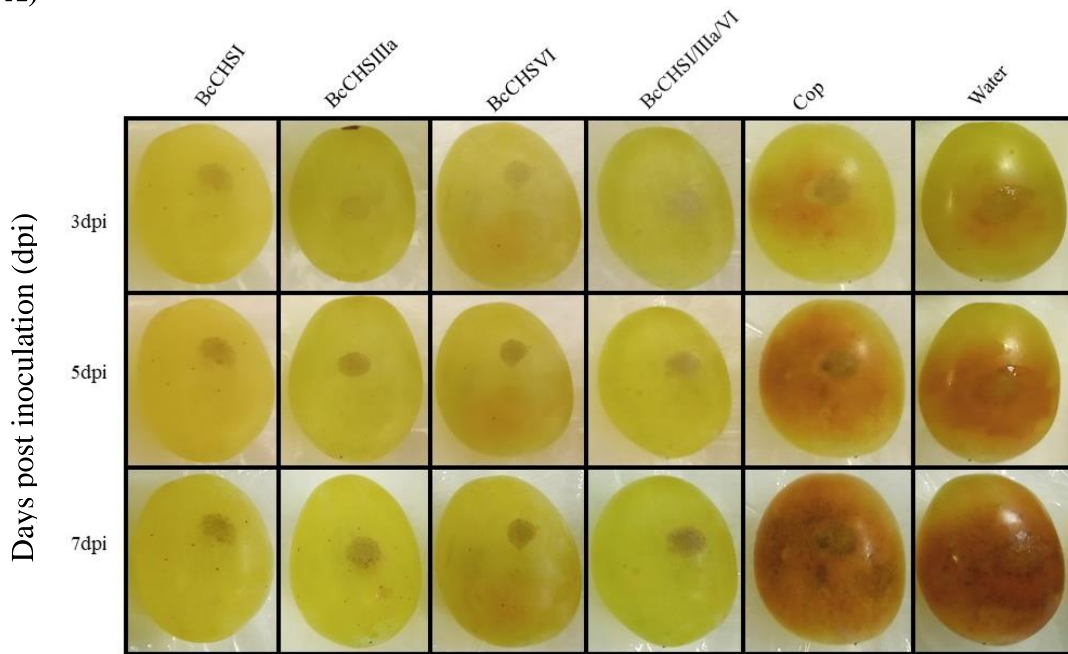
Figure 5. Externally applied *BcAlgl/Begl-dsRNA* on tomato fruits inhibited *Botrytis cinerea* infection. Fruits were treated with 20 µl of water (Ctrl) or *dsRNA* before inoculated with 20 µl of a 1 x10^6 mL^{-1} sporangia. *BcAlgl/Begl_50 dsRNA* at 50 ng $µl^{-1}$ concentration; *BcAlgl/Begl_90* at 90 ng $µl^{-1}$ concentration; dpi: days post inoculation.

3.3. Spray-Induced Gene Silencing of Chitin and Glucan Synthase Genes of *B. cinerea* and its Effect on Grape Infection

To explore the potential of spray-induced gene silencing, grapes were treated with *BcCHSI-dsRNA, BcCHSIIIa-dsRNA, BcCHSVI-dsRNA*, *BcCHSI/IIIa/VI-dsRNA, BcAlgl-dsRNA, BcBegl-dsRNA and BcAlgl/Begl-dsRNA*, which targets *B. cinerea* chitin synthase and glucan synthase genes. Effects of these dsRNAs on *B. cinerea* growth was assessed by comparing the fruit lesion area of the dsRNA-treated fruits to that treated with water or *Cop/αCOP-dsRNA*. *αCOP-dsRNA* was used as a negative control. Each dsRNA was tested (drop-inoculated) at the same concentration (120 ng/µl), followed by drop inoculation with 1 x10^6 ml^{-1} *B. cinerea* conidia (1hr later) directly onto the dsRNA treated area. The dynamics of fruit infection were estimated at 3, 5 and 7dpi . At 3dpi, 5dpi, and 7 dpi, *BcCHSI-dsRNA, BcCHSIIIa-dsRNA, BcCHSVI-dsRNA, BcCHSI/IIIa/VI-dsRNA, BcAlgl-dsRNA, BcBegl-dsRNA, and BcAlgl/Begl–dsRNAs*-treated fruits developed lesions that were substantially smaller than those on water or *Cop-dsRNA*-drop inoculated fruits that served as controls in this experiment (Fig 6A, Figure 7A). Both controls developed strong gray mold symptoms. As a consequence, the fruit area covered by *B. cinerea* infection at 3, 5, and 7 dpi were significantly and consistently lower in grapefruits treated with *BcCHSI-dsRNA*, *BcCHSIIIa-dsRNA*, *BcCHSVI-dsRNA*, *BcAlgl-dsRNA, BcBegl-dsRNA, BcCHSI/IIIa/VI-dsRNA,* and *BcAlgl/Begl–dsRNAs* than in those treated with *Cop-dsRNA* or water (Figure 6B, Figure 7B), confirming that topical application of *BcCHSI-dsRNA, BcCHSIIIa-dsRNA, BcCHSVI-dsRNA, BcAlgl-dsRNA, BcBegl-dsRNA,*

BcCHSI/IIIa/VI-dsRNA and BcAlgl/Begl–dsRNAs hampered *B. cinerea* growth. At 7dpi, *BcCHSI-dsRNA*, *BcCHSIIIa-dsRNA*, *BcCHSVI-dsRNA*, *BcAlgl-dsRNA, BcBegl-dsRNA, BcCHSI/IIIa/VI-dsRNA,* and *BcAlgl/Begl–dsRNAs* treated fungi (Fig 6A, Fig 7A) grew to cover 4.8%, 4.3%, 3.2%,4.5%, 5.4%, 5.1% and 6.8% of the area of the negative control (*αCOP/Cop*), respectively, (Fig.6B, Fig.7B), and similarly 4.7%, 4.3%, 3.2%, 4.5%, 5.4%, 5.1% and 6.8% of the area of the control (water), respectively. Although *BcCHSI* from chitin synthase genes group showed a slight advantage on *B. cinerea* growth suppression than *BcCHSVI, BcCHSIIIa and BcCHSI/IIIa/VI-dsRNA,* the difference among them was not statistically significant. Likewise, from glucan synthase genes *BcBegl* showed a slight advantage on *B. cinerea* growth suppression than *BcAlgl* and *BcAlgl/BcBegl*, but the difference among them was not statistically significant.

A)



B)

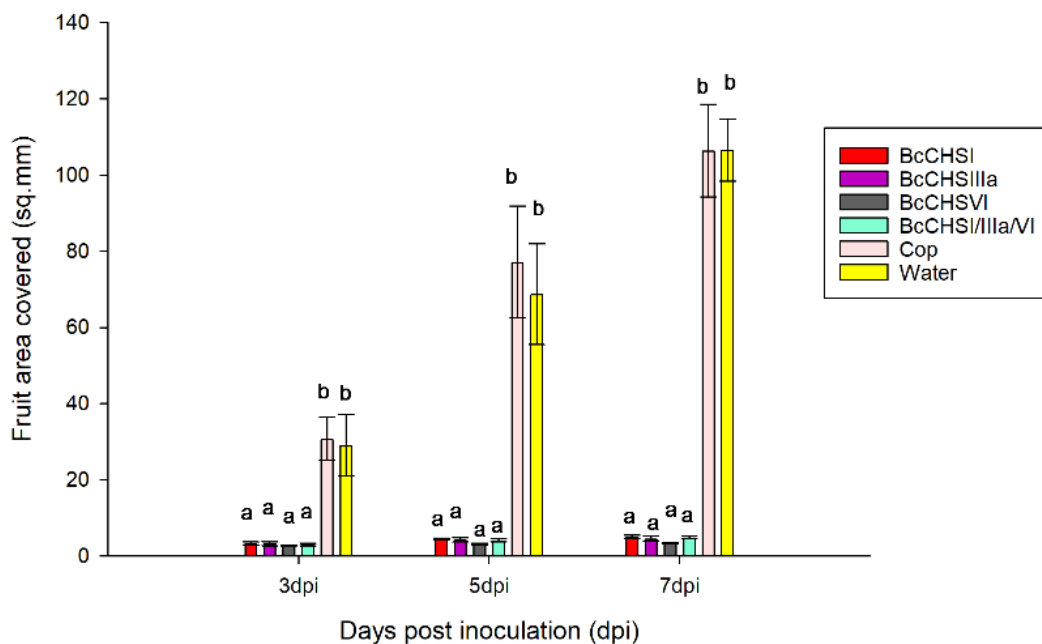
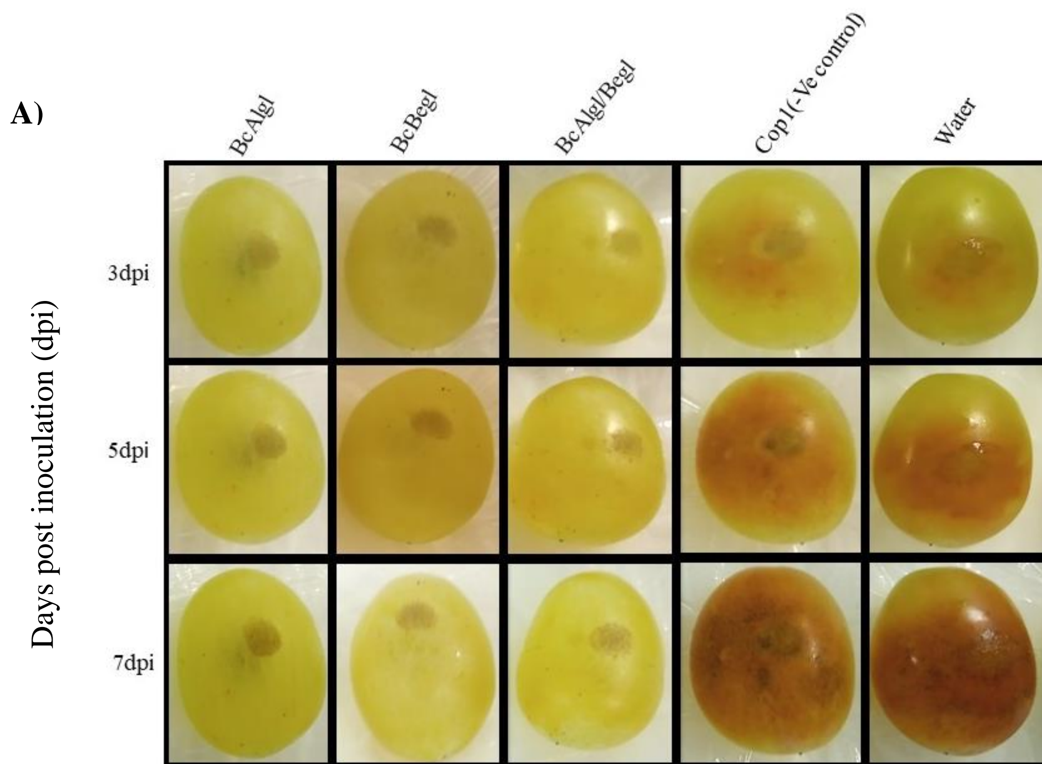


Figure 6. Externally applied *BcCHSI, BcCHSIIIa, BcCHSVI,* and *BcCHSI/IIIa/VI* double-stranded RNA (dsRNA) on grapefruits and *Botrytis cinerea* infection. (A) Progress of *B. cinerea* infection on grapefruits at 3, 5, and 7-days post inoculation (dpi). Fruits were treated with 20µl of water (ctrl) or dsRNA (120 ng/µl dsRNA of *BcCHSI, BcCHSIIIa, BcCHSVI, BcCHSI/IIIa/VI*, *αCOP/Cop* (negative control)) before being inoculated with 20 µl of a 1 x 10^6 ml^{-1} sporangia. (B) Disease progression of *B. cinerea* expressed as fruit area covered at 3,5 and 7 days of post-inoculation (dpi) relative to the control group water and cop. For panel (B) the error bars in the figure indicates standard error. Means at each dpi (at each days of post inoculation) followed by a common letter are significantly not different according to Tukey's honestly significant difference test ($P < 0.05$).

A)



B)

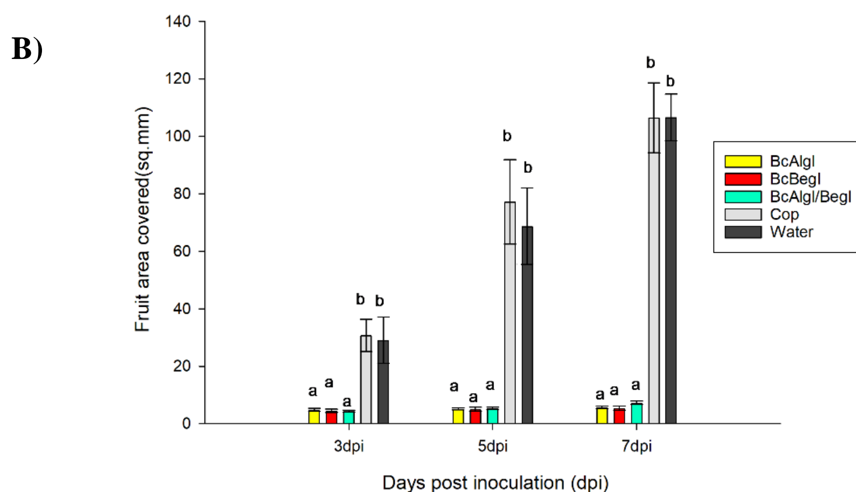


Figure 7. Externally applied *BcAlgl, BcBegl, and BcAlgl/Begl* double-stranded RNA (dsRNA) on grapefruits and *Botrytis cinerea* infection. (A) Progress of *B. cinerea* infection on grapefruits at 3-, 5-, and 7-days post inoculation (dpi). Fruits were treated with 20µl of water (ctrl) or dsRNA (120 ng/µl dsRNA of *BcAlgl, BcBegl, and BcAlgl/Begl*, *αCOP*/*Cop* (negative control)) before being inoculated with 20 µl of a 1 x 10^6 ml^{-1} sporangia. (B) Disease progression of *B. cinerea* expressed as fruit area covered at 3,5 and 7 days of post-inoculation (dpi) relative to the control group (Water and *Cop/αCOP*). For panel (B) the error bars in the figure indicates standard error. Means at each dpi followed by a common letter are significantly not different according to Tukey's honestly significant difference test (P< 0.05).

3.4. The Effect of SIGS on gray mold development on tomato fruits

To validate whether spray induced gene silencing of chitin synthase and glucan synthase genes could control gray mold disease, we tested gene construct of *BcCHSI-dsRNA* (390bp), *BcCHSIIIa-dsRNA* (381bp), *BcCHSVI-dsRNA* (395bp), and chimeric *BcCHSI/IIIa/VI-dsRNA* (643bp) designed by integrating *BcCHSI* (202bp), *BcCHSIIIa* (222bp), *and BcCHSVI* (219bp), via overlap PCR. In addition, we tested a single gene construct of *BcAlgl*-dsRNA (202bp), *BcBegl-dsRNA* (399bp), and chimeric *BcAlgl/Begl-dsRNA* (426bp) a fragment constructed by integrating *BcAlgl* (202bp) and *BcBegl* (224bp) via overlapping PCR. *BcCHSI-dsRNA, BcCHSIIIa-dsRNA, BcCHSVI-dsRNA* and *BcCHSI/IIIa/VI-dsRNA* dsRNA (20 µl of 120 ng μl^{-1} in RNase-free water) targeting *B. cinerea* chitin synthase transcripts*;* and *BcAlgl-dsRNA, BcBegl-dsRNA, and BcAlgl/Begl-dsRNA* (20 µl of 120 ng μl^{-1} in RNase-free water) targeting *B. cinerea* glucan synthase transcripts were separately applied to tomato fruits. After dsRNA treatment, tomato fruits were then inoculated with *B. cinerea* (20 µl of $1x10^{6}$ ml^{-1} conidia) at the dsRNA-treated area. Effects of these dsRNAs on *B. cinerea* growth virulence was assessed by comparing the fruit lesion area of the dsRNA-treated fruits to that treated with water or *αCOP*/*COP*. Gray mold progress was contained on tomato fruits received chitinase and glucanase dsRNA constructs, while progressive development of gray mold, with longer dpi, was observed on tomato fruits treated with water and *αCOP/COP-dsRNA* (Fig. 8a, Fig.8b, Fig.9a, Fig.9b). No difference was observed between water and *αCOP/Cop-dsRNA*-treated fruits, *αCOP/COP-dsRNA* was used as a negative control. At 11 dpi *BcCHSI, BcCHSIIIa, BcCHSVI, BcCHSI/IIIa/VI, BcAlgl, BcBegl, BcAlgl/Begl*, treated fungi (Fig 8a, Fig 9a) grew to cover 2.9%, 3.8%, 4.2%, 3.1%, 3.9%, 3.2%, and 3.7% of the area the negative control (*αCOP*/*COP*) , respectively, (Fig 8b, Fig 9b) , and similarly, 3.4%, 4.4%, 4.8%, 3.6% ,4.5%, 3.7% and 4.3% of the area of the control (water) , respectively. Thus, external application of *BcCHSI-dsRNA, BcCHSIIIa-dsRNA, BcCHSVI-dsRNA, BcCHSI/IIIa/VI-dsRNA, BcAlgl-dsRNA, BcBegl-dsRNA, and BcAlgl/Begl-dsRNA* inhibits infection of *B. cinerea* by targeting and silencing *B. cinerea* genes. Although *BcCHSI* from chitin synthase genes showed a slight advantage on *B. cinerea* growth suppression than *BcCHSVI, BcCHSIIIa and BcCHSI/IIIa/VI-dsRNA,* the difference among them was not statistically significant. Likewise, from glucan synthase genes *BcBegl* showed a slight advantage on *B. cinerea* growth suppression than *BcAlgl* and *BcAlgl/BcBegl*, but the difference among them was not statistically significant.

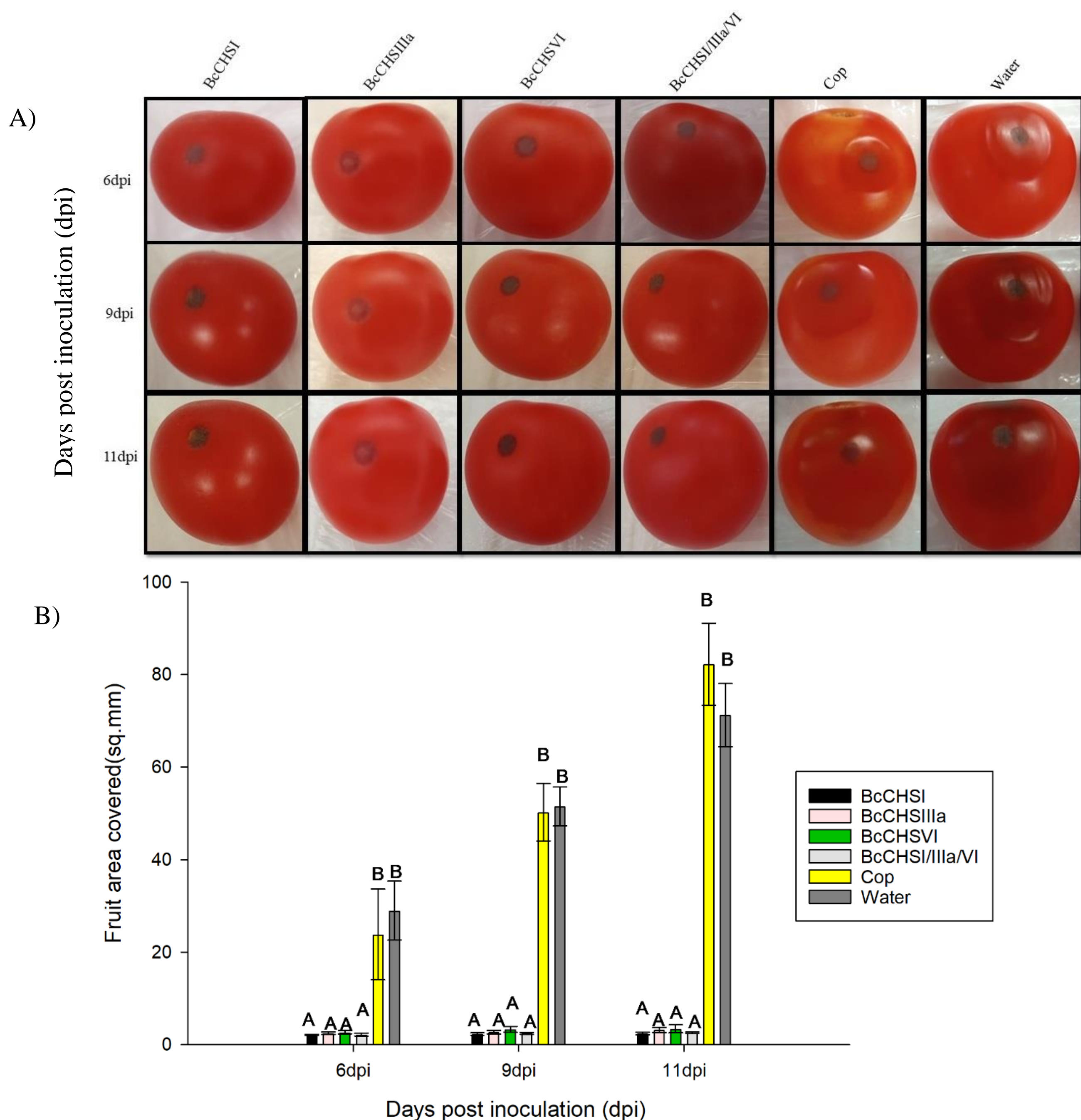
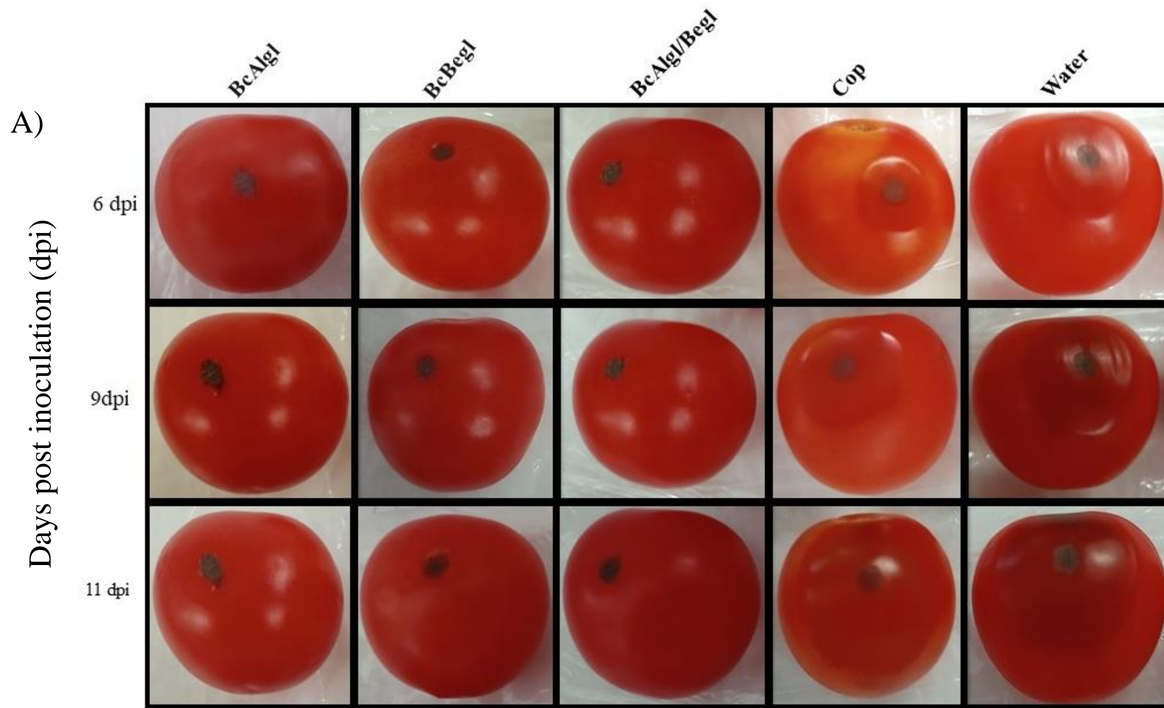


Figure 8. *B. cinerea* treated with externally applied *BcCHSI, BcCHSIIIa, BcCHSVI and BcCHSI/IIIa/VI* double-stranded RNA (dsRNA) on tomato fruits showed less growth than that treated with controls (water or *αCOP/COP-dsRNA*). (A) Images of *B. cinerea* infection on tomato fruits taken at 6, 9 and 11-days post inoculation (dpi). Fruits were treated with 20µl of water (ctrl) or dsRNA (120 ng/µl dsRNA of *BcCHSI, BcCHSIIIa, BcCHSVI, BcCHSI/IIIa/VI*, Cop/αCOP (negative control)) before being inoculated with 20 µl of a 1 x 10^6 ml^{-1} sporangia. (B) Disease progression of *B. cinerea* expressed as fruit growth area covered at 6 ,9 and 11 days of post inoculation (dpi) relative to the control group (Water and *Cop/αCOP-dsRNA*). For panel (B) the error bars in the figure indicates standard error between replicates. Means at each dpi (days of post inoculation) followed by a common letter are significantly not different according to Tukey's honestly significant difference test ($P < 0.05$).



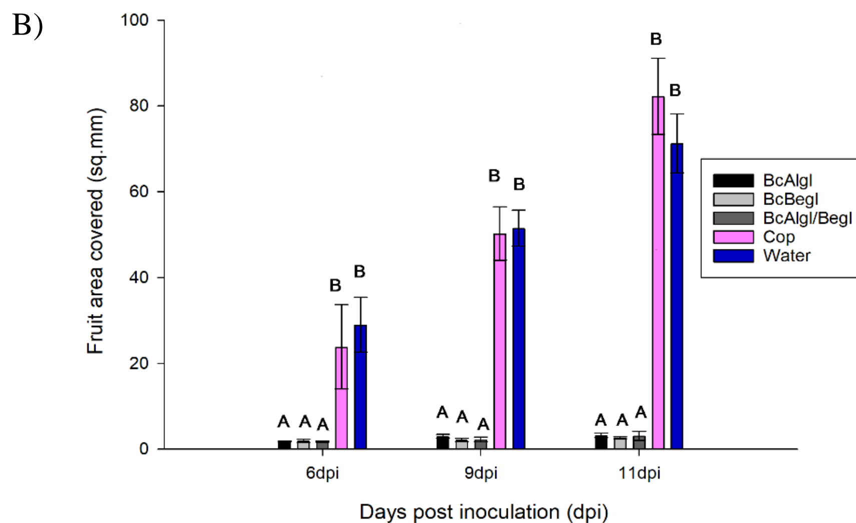


Figure 9. *B. cinerea* treated with externally applied *BcAlgl, BcBegl and BcAlgl/Begl* double-stranded RNA (dsRNA) on tomato fruits showed less growth than that treated with controls (water or cop-dsRNA) (A) Images of *B. cinerea* infection on tomato fruits taken at 6, 9 and 11-days post inoculation (dpi). Fruits were treated with 20µl of water (ctrl) or dsRNA (120 ng/µl dsRNA of *BcAlgl, BcBegl, and BcAlgl/BcBegl*, *Cop/αCOP* (negative control)) before being inoculated with 20 µl of a 1 x 10^6 ml^{-1} sporangia. (B) Disease progression of *B. cinerea* expressed as fruit area covered at 6 ,9 and 11 days of post inoculation (dpi) relative to the control group (Water and *Cop/αCOP*). For panel (B) the error bars in the figure indicates standard error between replicates. Means at each dpi (days of post inoculation) followed by a common letter are significantly not different according to Tukey's honestly significant difference test ($P < 0.05$).

3.5. Reverse transcription quantitative PCR (RT-qPCR) of *B. cinerea* genes for grapevine

To confirm that the inhibition of *B. cinerea* growth by *BcCHSI, BcCHSIIIa, BcCHSVI*, *BcAlgl*, *BcBegl, BcCHSI/IIIa/VI and BcAlgl/Begl*–dsRNAs was due to the downregulation of *BcCHSI*, *BcCHSIIIa, BcCHSVI*, *BcAlgl* and *BcBegl* genes, their expression, normalized to *B. cinerea* elongation factor *BcRPL5* was quantified at 3 dpi using qPCR. We found that the relative expression of *BcCHSI, BcCHSIIIa, BcCHSVI*, *BcAlgl* and *BcBegl* was reduced as compared to the controls (Fig.10, Fig. 11). Compared with *Cop/αCOP*-treated grapefruit, the NRQs of *BcCHSI, BcCHSIIIa, BcCHSVI, BcAlgl and BcBegl* transcripts in grapefruits treated with 120 ng/µl concentration of *BcCHSI, BcCHSIIIa, BcCHSVI, BcCHSI/IIIa/VI, BcAlgl, BcBegl and BcAlgl/Begl-dsRNAs* were reduced on average by 81%, 92%, 78%, 67-88%, 82%, 94% and 63-93%, respectively, which is in line with the concept of RNAi-based sequence-specific silencing via spray induced gene silencing. Similarly we found that the relative amounts of *BcCHSI*, *BcCHSIIIa*, *BcCHSVI*, *BcCHSI/IIIa/VI*, *BcAlgl, BcBegl* and *BcAlgl/Begl* transcripts were strongly reduced on average by 82% (*BcCHSI), 92% (BcCHSIIIa), 82% (BcCHSVI), 69-87% (BcCHSI/IIIa/VI), 81% (BcAlgl), 95% (BcBegl), and 69-92% (BcAlgl/Begl)* in grapefruits drop inoculated with *BcCHSI-dsRNA, BcCHSIIIa-dsRNA, BcCHSVI-dsRNA, BcCHSI/IIIa/VI-dsRNA, BcAlgl-dsRNA, BcBegl-dsRNA* and *BcAlgl/Begl-dsRNA,* respectively as compared with water control treatment (Fig. 10 A-C, Fig. 11A-B).

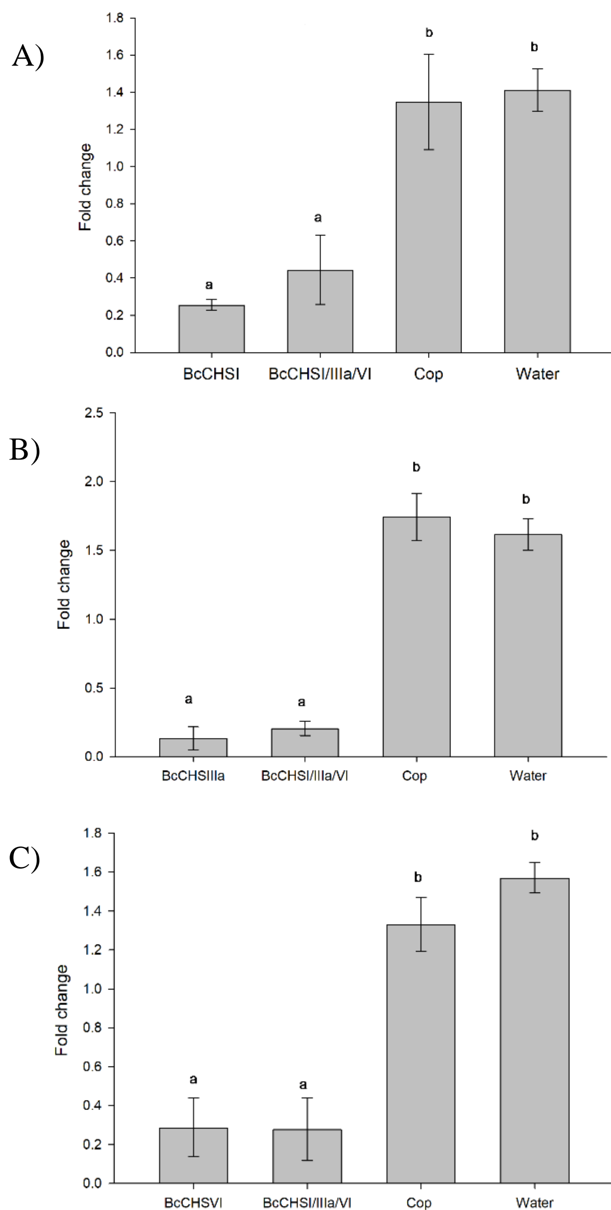


Figure 10. *B. cinerea* treated with dsRNAs has reduced level of *BcCHSI* (A), *BcCHSIIIa* (B), *BcCHSVI* (C), *BcCHSI/IIIa/VI* (A, B, C) transcript as shown by fold change based on RT-qPCR analysis. Expression profiles of *BcCHSI, BcCHSIIIa, BcCHSVI* , *BcCHSI/IIIa/VI*, following *Botrytis cinerea* inoculation on grapes samples treated with 20µl of water (ctrl) or 120 ng μl^{-1} of double-stranded RNA (dsRNA) of *BcCHSI, BcCHSIIIa, BcCHSVI*, *BcCHSI/IIIa/VI*, *αCOP*/*Cop* (negative control). Gene expression level was determined by quantitative PCR (qPCR). Bars represent fold change of dsRNA-treated sample relative to ctrl sample at 3 days post inoculation. Normalization based on the expression levels of elongation factor, *BcRPL5*, was carried out before calculating fold changes. Error bar represents standard error of the mean of three biological replicates. Expression values followed by a common letter are significantly not different among samples, according to Tukey's honestly significant difference test ($P < 0.05$), using one-way ANOVA of normalized relative quantity (NRQ).

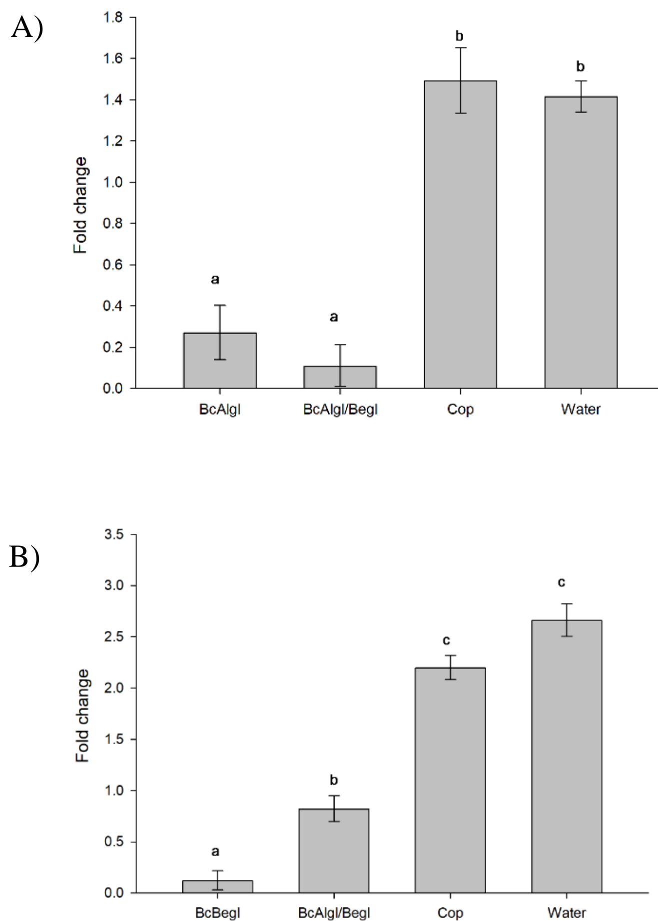


Figure 11. Expression profiles of *BcAlgl* (A), *BcBegl* (B) and *BcAlgl/Begl* (A, B) following *Botrytis cinerea* inoculation on grapefruits samples treated with 20 µl of water (ctrl) or 120 ng µl^{-1} of double-stranded RNA (dsRNA) of *BcAlgl, BcBegl, and BcAlgl/Begl*, *Cop/αCOP* (negative control). Gene expression level was determined by quantitative PCR (qPCR). Bars represent fold change of dsRNA-treated sample relative to ctrl sample at 3 days post inoculation. Normalization based on the expression levels of elongation factor, *BcRPL5*, was carried out before calculating fold changes. Error bar represents standard error of the mean of three biological replicates. Expression values followed by a common letter are significantly not different among samples, according to Tukey's honestly significant difference test (P <0.05), using one-way ANOVA of normalized relative quantity (NRQ).

3.6. Reverse transcription quantitative PCR (RT-qPCR) of *B. cinerea* genes for tomato

To confirm that the inhibition of *B. cinerea* growth by *BcCHSI, BcCHSIIIa, BcCHSVI*, *BcAlgl, BcBegl*, *BcCHSI/IIIa/VI* and *BcAlgl/Begl–dsRNAs* was due to the downregulation of *BcCHSI, BcCHSIIIa, BcCHSVI*, *BcAlgl* and *BcBegl* genes, their expression, normalized to *B. cinerea* elongation factor *BcRPL5* was quantified at 3dpi using qPCR. We found that the relative expression of *BcCHSI, BcCHSIIIa, BcCHSVI, BcAlgl* and *BcBegl* was reduced as compared to the controls (Fig. 12 A-C, Fig. 13A-B). Inline with the concept of spray-induced gene silencing, we found that the relative amounts of *BcCHSI*, *BcCHSIIIa*, *BcCHSVI*, *BcCHSI/IIIa/VI, BcAlgl*, *BcBegl* and *BcAlgl/Begl* transcripts were strongly reduced on average by 96% (*BcCHSI*), 91% (*BcCHSIIIa*), 89% (*BcCHSVI*), 54-75% (*BcCHSI/IIIa/VI),* 84% (*BcAlgl*), 84% (*BcBegl*) and 76-79% (*BcAlgl/Begl)* in tomato fruits drop inoculated with *BcCHSI-dsRNA*, *BcCHSIIIa-dsRNA*, *BcCHSVI-dsRNA*, *BcCHSI/IIIa/VI-dsRNA, BcAlgl-dsRNA*, *BcBegl-dsRNA* and *BcAlgl/Begl* as compared with *Cop/αCOP-dsRNA* control treatment (Fig. 12 A-C, Fig. 13A-B). Likewise compared with water-treated tomato fruits, the NRQs of *BcCHSI, BcCHSIIIa, BcCHSVI, BcCHSI/IIIa/VI, BcAlgl, BcBegl* and *BcAlgl/Begl* transcripts in tomato fruits treated with 120 ng/µl concentration of *BcCHSI, BcCHSIIIa, BcCHSVI, BcCHSI/IIIa/VI, BcAlgl, BcBegl and BcAlgl/Begl-dsRNAs* were reduced on average by 95 %, 90 %, 88%, 52-70%, 83%, 71% and 71-78%, respectively.

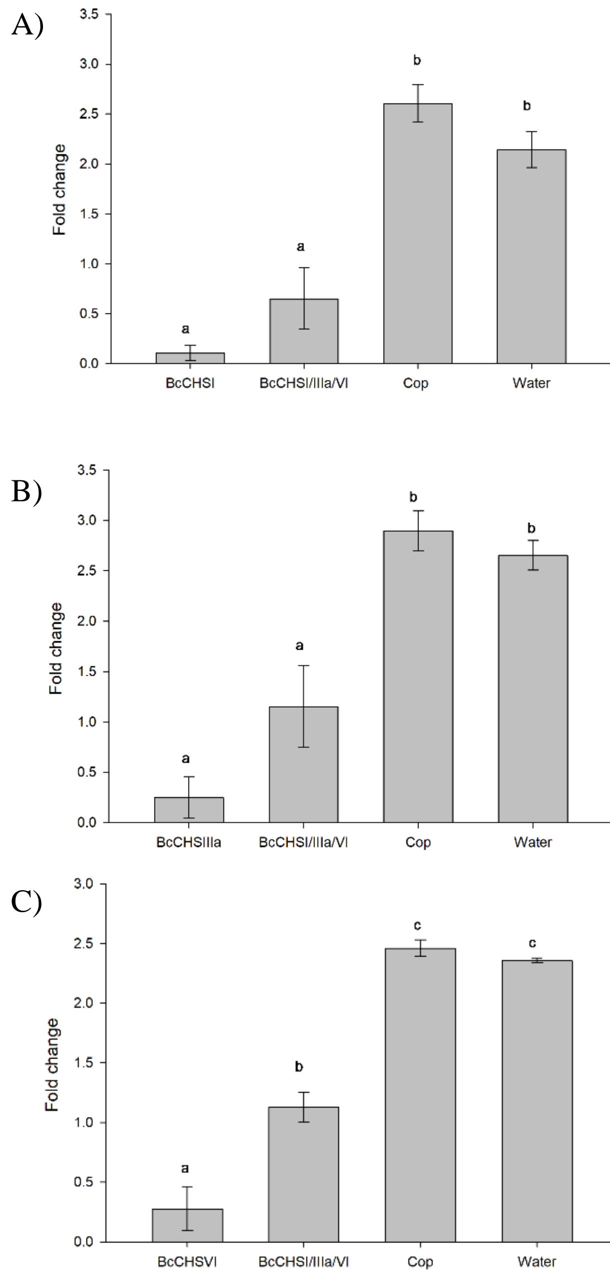


Figure 12 . Expression profiles of *BcCHSI* (A)*, BcCHSIIIa* (B)*, BcCHSVI* (C)*, BcCHSI/IIIa/VI* (A, B, C), following *Botrytis cinerea* inoculation on tomato fruits samples treated with 20µl of water (ctrl) or 120 ng μl^{-1} of double-stranded RNA (dsRNA) of *BcCHSI, BcCHSIIIa, BcCHSVI, BcCHSI/IIIa/VI*, *αCOP*/*Cop* (negative control). Gene expression level was determined by quantitative PCR (qPCR). Bars represent fold change of dsRNA-treated sample relative to ctrl sample at 3 days post inoculation. Normalization based on the expression levels of elongation factor, *BcRPL5*, was carried out before calculating fold changes. Error bar represents standard error of the mean of three biological replicates. Expression values followed by a common letter are significantly not different among samples, according to Tukey's honestly significant difference test ($P < 0.05$), using one-way ANOVA of normalized relative quantity (NRQ).

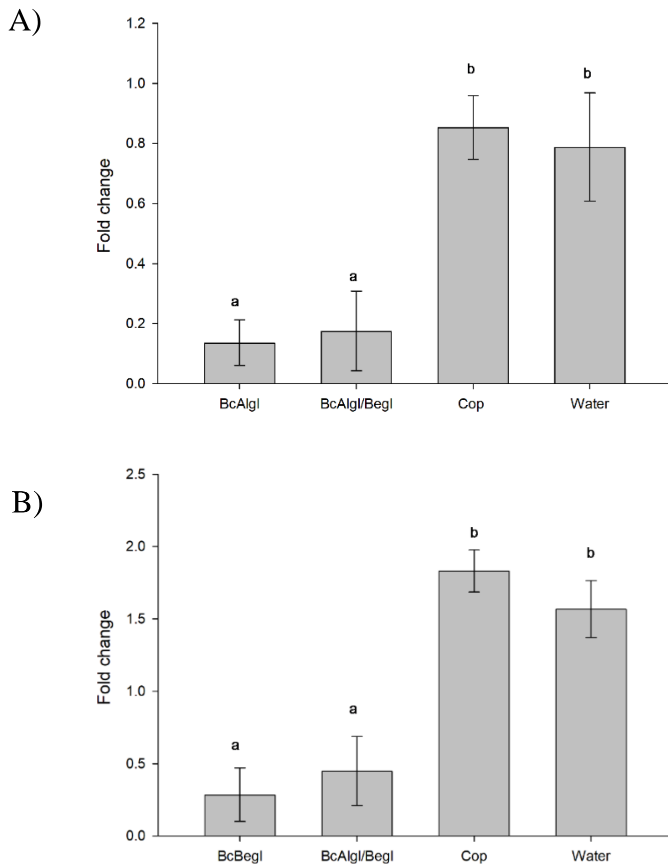


FIGURE 13. Expression profiles of *BcAlgl* (A), *BcBegl* (B), and *BcAlgl/Begl* (A, B) following *Botrytis cinerea* inoculation on tomato fruits samples treated with 20µl of water (ctrl) or 120 ng μl^{-1} of double-stranded RNA (dsRNA) of *BcAlgl*, *BcBegl*, and *BcAlgl/Begl*, *Cop/αCOP* (negative control). Gene expression level was determined by quantitative PCR (qPCR). Bars represent fold change of dsRNA-treated sample relative to ctrl sample at 3 days post inoculation. Normalization based on the expression levels of elongation factor, *BcRPL5*, was carried out before calculating fold changes. Error bar represents standard error of the mean of three biological replicates. Expression values followed by a common letter are significantly not different among samples, according to Tukey's honestly significant difference test ($P < 0.05$), using one-way ANOVA of normalized relative quantity (NRQ).

4. Discussions

In filamentous fungi, various polysaccharides components, such as galactomannan, chitin, β-glucans (β-1,3-glucan), and α-glucans (mainly α-1,3-glucan), are necessary for proper cell wall architecture (Latgè, 2010; Yoshimi et al., 2016). Chitin is biosynthesized by a family of membrane proteins, chitin synthase, which catalyzes the polymerization of N-acetylglucosamine from UDP-N-acetylglucosamine and plays a crucial role in cell wall polymerization. Chitin is unique and common in all fungi, and it is a potential target for the development of selective antifungal drugs (Munro and Gow, 1995; Roncero, 2002; Latge, 2007), and, for this reason, we selected chitin (*BcCHSI, BcCHSIIIa, BcCHSVI*) as the target for our RNAi protocol. It has been already demonstrated that the disruption of *BcCHSI* (Soulie et al., 2003), *BcCHIIIa* (Soulie et al., 2006), and *BcCHSVI* (Morcx et al., 2012; Cui et al., 2013) results in cell wall weakening and reduced virulence of *B. cinerea.* Secondly, we identified two glucan synthase genes namely: alpha-1,3-glucan synthase *(BcAlgl)*, and 1,3-beta-D-glucan synthase *(BcBegl)*. The compound α-1,3-glucan is a major component in the cell wall of filamentous fungi, and it acts as a virulence factor in plant pathogenic fungi: it hides cell wall β-glucan on the fungal cell surface to evade detection by hosts (Yoshimi et al., 2017). A previous study described the loss of a cell wall polysaccharide, α-(1,3) glucan synthase from the cell walls of *Histoplasma capsulatum* led to decreased virulence and pathogenesis of the fungi (Rappleye et al., 2004). On the other hand, β-1,3-glucan is a central and critical component of fungi, often comprising 60–95% of cell wall glucans (Latgé, 2007). Efforts to produce viable glucan synthase deficient mutants in filamentous fungi failed (Latgé, 2007), suggesting that β-1,3-glucan is an essential cell wall component and RNAi experiments have provided evidence for the importance of glucan synthase to growth in two pathogenic fungi (Mouyna et al., 2004; Ha et al., 2006). After the identification of target genes, we proceeded by amplifying a fragment (202bp–399bp) for each with gene-specific primers with restriction enzymes. For chitin synthase and glucan synthase chimeric, gene-specific primers with restriction enzymes were designed to allow overlapping PCR and produce a final PCR amplicon of 643bp and 426bp, respectively. A PCR amplicon of 398bp was obtained from *COP/αCOP* sequence using PCR and used as a negative control for dsRNAs inoculation.

After dsRNAs production we evaluated dsRNAs produced in an *E.coli* bacteria and evaluated the silencing efficiency resulting from exogenous bioassays using these dsRNAs. The potential for using spray-induced gene silencing against *B. cinerea,* was examined through knockdown

of chitin synthase *(BcCHSI, BcCHSIIIa, BcCHSVI, BcCHSI/IIIa/VI),* and glucan synthase *(BcAlgl, BcBegl, BcCHSI/IIIa/VI and BcAlgl/Begl)* genes of *B. cinerea* which were selected based on fungicide sites of action found in the literature (Liu et al., 2018; Fishel and Dewdney, 2021). dsRNAs were applied topically and strong evidence was found for an active RNAi pathway in *B. cinerea* that can be exploited to suppress growth of the fungus. *B. cinerea* treated with *BcCHSI-dsRNAs, BcCHSIIIa-dsRNAs, BcCHSVI-dsRNAs, BcAlgl-dsRNAs, BcBegl-dsRNAs, BcCHSI/IIIa/VI-dsRNAs and BcAlgl/Begl-dsRNAs* showed slower growth (Fig. 6A, Fig. 7A, Fig. 8A, Fig.9A), and this growth suppression correlated well with reduced expression of the target genes (Fig.10A-C, Fig.11A-B, Fig.12A-C, Fig.13A-C), *BcCHSI, BcCHSIIIa, BcCHSVI, BcAlgl, BcBegl, BcCHSI/IIIa/VI and BcAlgl/Begl*, which strongly suggests that exogenous dsRNA application was effective at knocking down *B. cinerea* gene expression to the extent of altering the growth of the fungus. Here, we provide solid evidence that the environmental dsRNA can be taken up by *B. cinerea* with similar uptake efficiencies across grape and tomato fruits which determines the effectiveness of SIGS. The results presented further support the use of SIGS-based strategy for fungal pathogen management (Koch et al., 2016; Wang et al., 2016; Wang and Jin, 2017; Cai et al., 2018; McLoughlin et al., 2018; Nerva et al., 2020).

RNAi machinery has been demonstrated in a wide range of fungal phyla from the *Ascomycota* (*Botrytis* (this study) (Wang et al., 2016) and *Basidiomycota* (e.g., *Cryptococcu*s (Liu et al., 2002) to the *Neurospora* ('quelling'; Romano and Macino, 1992)). The application of RNAi as a tool for reverse genetics, targeted at modification of fungal gene expression, is constantly growing with a large number of fungal species already demonstrated to be responsive (Dang et al., 2011). Additionally, the functionality of absorbed exogenous RNAi molecules allows remarkable adaptability and flexibility in guaranteeing the desired effects on gene expression of fungi, even without the need to genetically modify the targeted pathogen (Nakayashiki and Nguyen, 2008; Wang et al., 2016). Homology-based gene silencing that is induced by transgenes (co-suppression), antisense, or dsRNAs has been shown to function in various plant pathogenic fungi and oomycetes, including blast, blight, and rust fungi, such as *Fusarium asiaticum, Fusarium graminearum, Magnaporthe oryzae*, and *Puccinia striiformis f. sp. tritici* (Nakayashiki, 2005; Koch et al., 2016; Zhu et al., 2017; Gu et al., 2019; Werner et al., 2020); different mold fungi, such as *Botrytis cinerea*, *Neurospora crassa*, and *Sclerotinia sclerotiorum* (Goldoni et al., 2004; Wang et al., 2016; McLoughlin et al., 2018; Nerva et al., 2020); mildew, and others, such as *Blumeria graminis*, *Cochliobolus sativus*, and *Venturia inaequalis*

(Fitzgerald et al., 2004; Leng et al., 2010; Nowara et al., 2010). To date, the number of effective candidate genes examined that led to reduced fungal growth is limited, and includes effectors, cell wall elongation, chitinase, and hexose transporter genes. According to studies, researchers confirmed the existence of RNA silencing machinery in *B. cinerea*, which is active during its interaction with strawberries, lettuce, onion, rose, grapevine, and tomato (Wang et al., 2016; Nerva et al., 2020; Qiao et al., 2021). We show in our study that RNAi is an effective tool for the functional silencing of gene expression in *B. cinerea*. While formally possible that a novel silencing mechanism operates in *B. cinerea*, our results are consistent with the RNAi mechanism demonstrated in other fungal species.

When one member of a multigene family knocks down, there is a chance that the remaining members of the family will compensate for its loss, and functional redundancy has been found within the chitin synthase family in some fungi (Motoyama et al., 1997; Ichinomiya et al., 2005; Takeshita et al., 2006; Kong et al., 2012). However, studies have also shown that a single chitin synthase gene deletion can cause significant phenotypic alterations. A study carried out on *Botrytis cinerea* (Soulie et al., 2003) revealed disruption of *Botrytis cinerea* class I chitin synthase gene cause 30%, reduction in chitin content indicating that class I chitin synthase contributes considerably to cell wall composition. Likewise, disruption of chitin synthase class III gene *(Bcchs3a)* in the phytopathogenic fungus *B. cinerea* showed a reduction of 39% in chitin content in the *Bcchs3a* mutant compared with the wild type (Soulie et al., 2006). A class I chitin synthase mutant in *F. oxysporum* demonstrated a 10% reduction in chitin content (Martín-Udíroz et al., 2004). In *Magnaporthe oryzae*, where class I chitin synthase mutants showed 2-fold reductions in chitin content and impairments in pathogenic capacities, it was discovered that class I chitin synthase proteins are crucial for the development of conidia as well as appressoria (Kong et al., 2012). The deletion mutant of class VI (or VII) CHS gene had been described in numerous filamentous fungi (Takeshita et al., 2006; Larson et al., 2011; Kong et al., 2012). In *Magnaporthe oryzae*, deletion of *CHS6* stops appressorium penetration and invasive growth, and the *chs5chs6* double mutant exhibit more serious flaws than the *chs6* mutant (Kong et al., 2012). In *Botrytis cinerea*, disruption of *CHS6* caused significant decrease in hyphal growth, conidiation and germination in heterokaryotic strains. A strong virulence reduction was also examined. The isolation of no *chs6* homokaryotic strains suggests that *CHS6* may be a crucial enzyme for *B. cinerea* (Morcx et al., 2012). Therefore, our work, together with other studies, indicates that *BcCHSI, BcCHSIIIa, BcCHSVI* and Chimeric *BcCHSI/IIIa/VI* genes may be effective targets for RNAi in *Botrytis cinerea*.

Although our study was centered on *B. cinerea*, α-(1,3)-glucan is also a cell wall component of many fungal pathogens, including *Aspergillus fumigatus* (Bernard and Latge, 2001), *Blastomyces dermatitidis* (Hogan and Klein, 1994), *Coccidioides immitis* (Cole and Hung, 2001), *Cryptococcus neoformans* (Bacon et al., 1968), *Histoplasma capsulatum* (Rappleye et al., 2004), and *Paracoccidioides brasiliensis* (Moreno et al., 1969). In many of these instances, the importance of α-(1,3)-glucan has been inferred from analysis of avirulent mutants that no longer produce this polysaccharide (Klimpel and Goldman, 1988; Hogan and Klein, 1994; Silva et al., 1994). Our RNAi-based gene silencing of α-(1,3)-glucan suggests that fungicides or treatments that target the biosynthesis of α-(1,3)-glucan could constitute effective antifungal treatments for *B. cinerea* as well as many other fungal diseases. Our experiment to silence α-(1,3)-glucan synthase by RNAi provides may be the first direct evidence causally linking α-(1,3)-glucan to *B. cinerea* virulence.

Studies with the human pathogenic fungus *Aspergillus fumigatus* employing RNA interference (RNAi) showed that the β-1,3-glucan synthase gene *FKS1* (for FK506 Sensitivity) is indispensable for vegetative growth and viability (Mouyna et al., 2004), and similar results have been obtained from RNAi studies with *Fusarium solani* (Ha et al., 2006). Likewise, transgenic wheat plants carrying an RNAi hairpin construct against the β-1, 3-glucan synthase gene of *Fusarium culmorum* showed enhanced *Fusarium* head blight (FHB) resistance in leaf and spike inoculation of wheat (Chen et al., 2016). In another study, exogenous siRNAs were applied to target a *β-1,3-glucan synthase* gene in *Macrophomina phaseolina, a* causal agent of charcoal rot disease. The fungal growth was suppressed under various testing conditions due to exogenous siRNA treatments (Forster and Shuai, 2020). In our experiment, the topical application of *BcBegl* (β-1,3-glucan synthase) on grape and tomato fruits inhibited *B. cinerea* growth either. In addition, to our knowledge, this is the first trial in which *BcBegl* (β-1,3-glucan synthase) gene was targeted by Spray Induced Gene Silencing to control *B. cinerea* using grape and tomato fruits.

Production of dsRNAs can be achievable by employing either *in-vivo* (Yin et al., 2009; Huang et al., 2013) or *in-vitro* synthesis (Sohail et al., 2003; Koch et al., 2016; Wang et al., 2016; Nwokeoji et al., 2019). *In-vivo* production of dsRNA using genetically engineered bacteria (*Escherichia coli* and *Pseudomonas syringae*) and yeast (*Yarrowia lipolytica*) (Voloudakis et al., 2015; Álvarez-Sánchez et al., 2018) produce large quantities of dsRNAs at low cost. The application of recombinant bacteria to produce dsRNA is an efficient technique due to their

ability to maintain plasmid, ease of handling and the fast growth rate of bacteria (Terpe, 2006). There are many advantages in using genetically engineered bacteria to produce and deliver dsRNA in fungal disease control when compared with using *in vitro* synthesized dsRNA. The most significant advantage is the lower cost per application of bacteria-expressed dsRNA when compared with that of *in vitro* synthesized dsRNA. Genetically engineered bacteria able to produce large amounts of dsRNA molecules needed for field trial applications. Since RNAi is not a knockout, but a knockdown method that is generally transient, to maximize the potential use of RNAi in crop protection, continuous and large-scale delivery of dsRNA for target gene silencing might be necessary to kill the pathogen (Huvenne and Smagghe, 2010). With the ease of manufacturing large quantities, the bacteria-expressed dsRNA could be used whenever necessary. Different researchers revealed that bacterially expressed dsRNAs can be used to induce RNAi in fungus (Nerva et al., 2020), insect pests (Tian et al., 2009; Ghag et al., 2014), virus (Pliego et al., 2013) and worms (Newmark et al., 2003). Here in our study, we also confirmed that bacterially produced dsRNA can be used to induce RNAi in *Botrytis cinerea*.

In this study, we demonstrated that dsRNA specifically designed to silence *BcCHSI, BcCHSIIIa, BcCHSVI, BcCHSI/IIIaVI, BcAlgl, BcBegl,* and *BcAlgl/Begl*, genes efficiently control gray mold disease caused by *B. cinerea* on grape and tomato fruits. Although the mechanism behind the uptake and transport of the externally applied dsRNA is still unknown, the presented data give important scientific information on such new-generation RNA-based fungicides, which are environmentally safe and sustainable. So far, externally applied RNAi-based disease suppression data for *B. cinerea* was limited but with our findings, we demonstrated the possibility of using externally applied dsRNAs for managing *B. cinerea*. Future SIGS commercial uses will likely be possible. Though our study provides strong evidence that dsRNAs are effective at suppressing *B. cinerea* growth through knockdown of *BcCHSI, BcCHSIIIa, BcCHSVI, BcAlgl, BcBegl, BcCHSI/IIIa/VI and BcAlgl/Begl* genes, there are practical limitations to using exogenous dsRNAs in a real-world scenario. Concerning the delivery of RNAi products against *B. cinerea* in the field, one of the biggest challenges is the degradability of dsRNA in the field. This shortcoming can be overcome through use of host induced RNAi and complexation of dsRNA with carrier molecules (Numata et al., 2014; Lichtenberg et al., 2019; Christiaens, et al., 2020). Considering that *B. cinerea* will constantly be exposed to the target gene dsRNAs expressed in the transgenic plant, this might result in optimal levels of dsRNA being taken up by the fungus to induce RNAi effects. Such transgenic plants have been developed and shown effective control against fungal pathogens (Nowara et

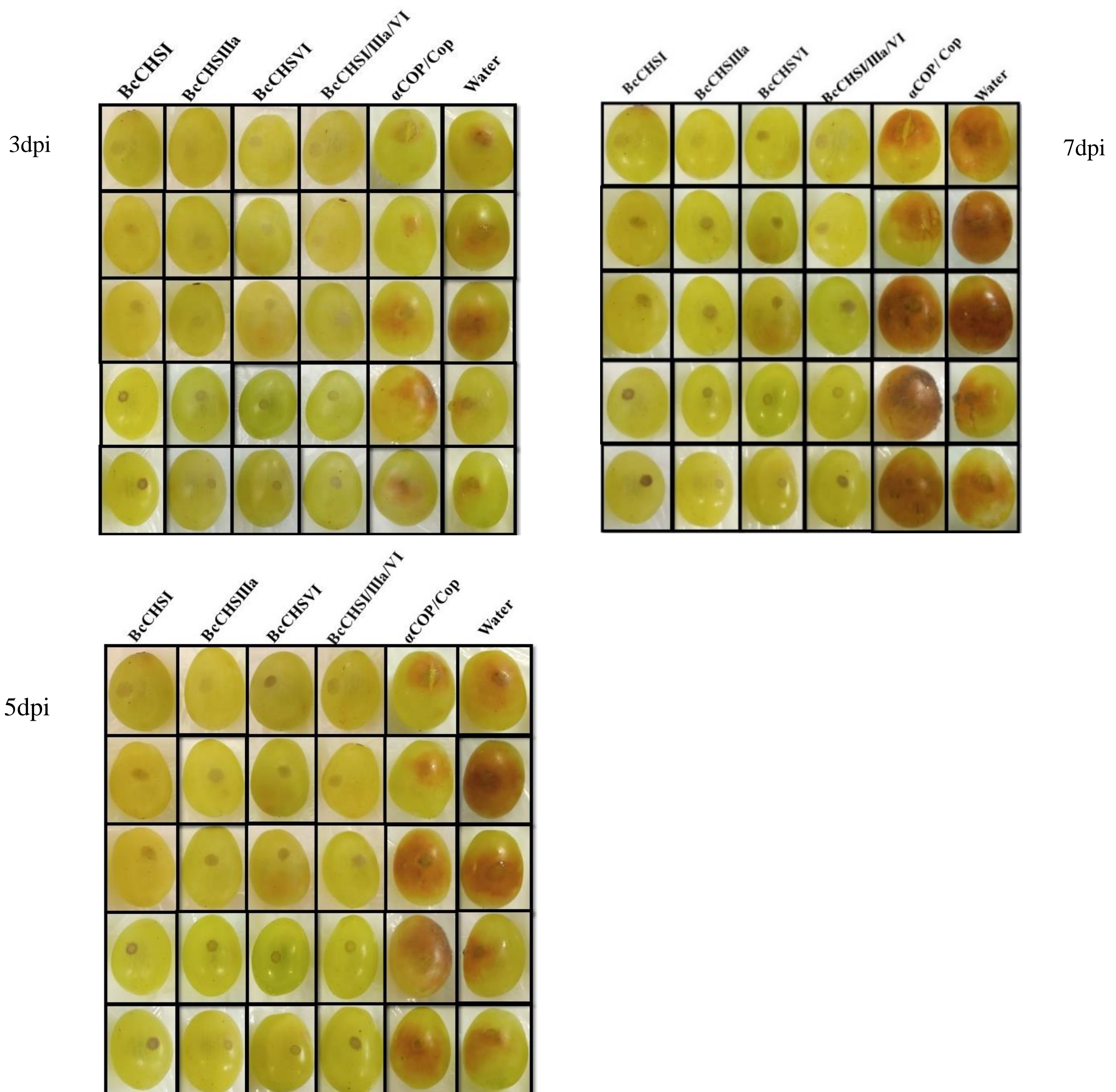
al., 2010; Mumbanza et al., 2013; Cheng et al., 2015; Hu et al., 2015; Chen et.al., 2016; Song and Thomma, 2016; Tiwari et al., 2017; Johnson et al., 2018; Qi et al., 2018). However, in many cases, the use of transgenic crops is not realistic. This can be due to political or legislative reasons, or because the crop in question is technically difficult or unable to be transformed. In order to improve stability and uptake efficiency, dsRNA can be incorporated into nanoparticles. Since nanoparticles shield the dsRNA/siRNA from degradation, they are the most popular choice when delivering unstable naked dsRNA/siRNA to the targeted sites (Young et al., 2016). Chitosan is one of the most popular polymers used to produce nanoparticles to shield and deliver dsRNA/siRNA to target cells (Lichtenberg et al., 2020). It has been demonstrated that endonuclease formulations based on chitosan increase the stability and absorption in a range of insect species (Zhang et al., 2010; Wang et al., 2020). Layered double hydroxide clay nanosheets is another way to boost RNAi effectiveness. According to Mitter et al. 2017, persistent release of the dsRNA for up to 30 days was made possible by loading RNAi-inducing dsRNAs into layered double hydroxide clay nanosheets and applying to plant surfaces. Schwartz et al., 2020 reported that siRNA can be delivered to the *Nicotiana benthamiana* and tomato plants using a class of very small nanoparticles known as carbon dots. Additionally, a liposome-based delivery technique has been used successfully to change gene expression and/or mortality in insects, fungus, and worms (Lin et al., 2016; Nami et al., 2017; Adams et al., 2019).

5. Conclusion

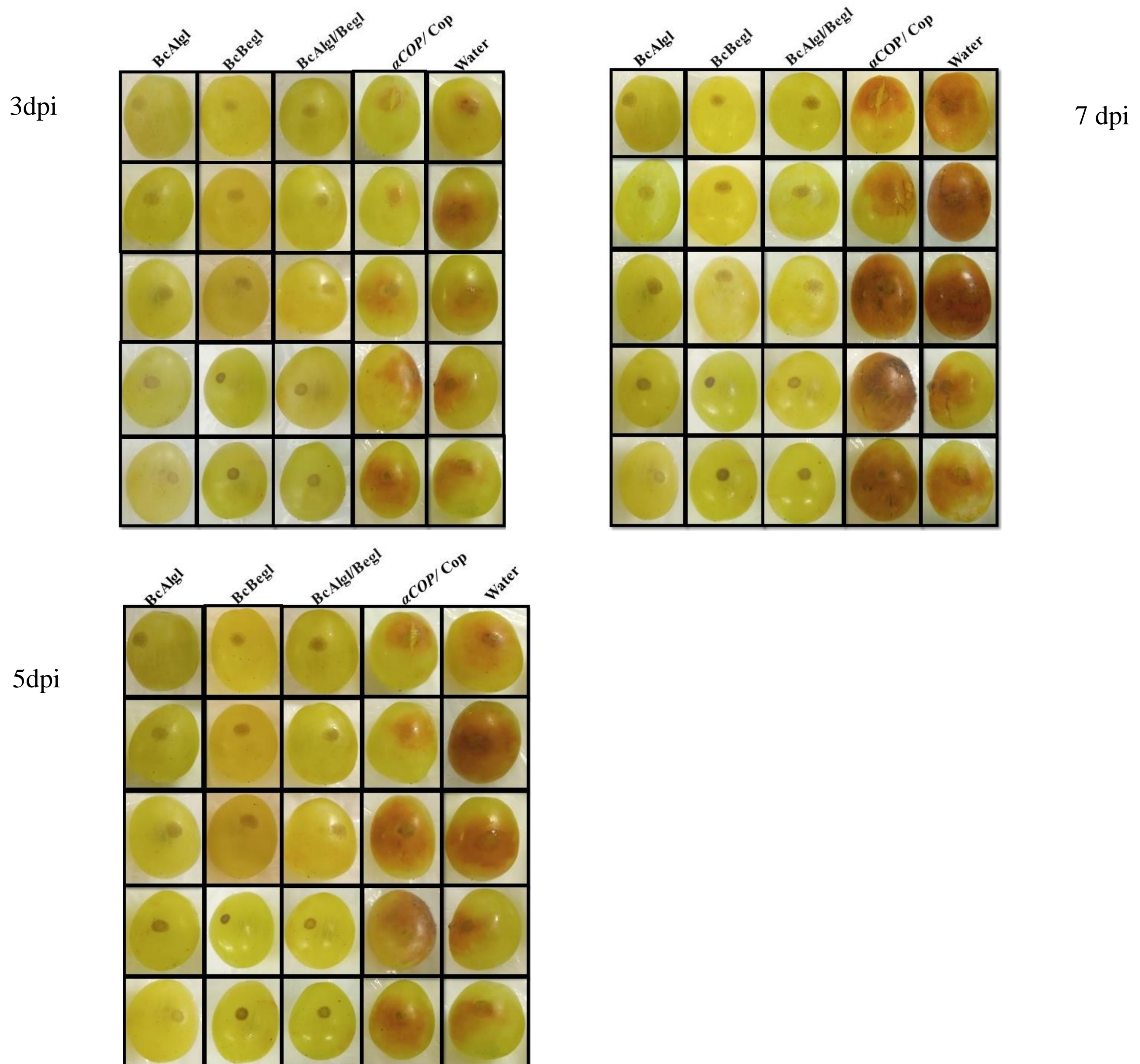
The external application of *BcCHSI-dsRNA, BcCHSIIIa-dsRNA, BcCHSVI-dsRNA, BcCHSI/IIIa/VI-dsRNA, BcAlgl-dsRNA, BcBegl-dsRNA* and *BcAlgl/Begl-dsRNA* to *B. cinerea,* a fungus with the largest host range, resulted in significant *BcCHSI, BcCHSIIIa, BcCHSVI, BcCHSI/IIIa/VI, BcAlgl, BcBegl, BcAlgl/Begl* gene silencing. Our results indicate that by silencing *BcCHSI, BcCHSIIIa, BcCHSVI, BcCHSI/IIIa/VI, BcAlgl, BcBegl* and *BcAlgl/Begl* genes, it is possible to protect grape and tomato fruits from gray mold contaminations and to reduce the virulence of *B. cinerea*. Future efforts should be directed towards identifying the optimal amount of *BcCHSI-dsRNA, BcCHSIIIa-dsRNA, BcCHSVI-dsRNA, BcCHSI/IIIa/VI-dsRNA, BcAlgl-dsRNA, BcBegl-dsRNA* and *BcAlgl/Begl-dsRNA* used in SIGS and formulations to keep dsRNA stable. Thus, our findings can contribute to RNAi-based control strategies against *B. cinerea*, which can be both environmentally friendly and cost efficient. Although a lot remains to be explored and understood about the molecular process of RNAi in plants and their pathogens, the current knowledge available and the results of our study have proved that exogenous RNAi technology is an essential tool to control *B. cinerea*.

6. Supplemental materials

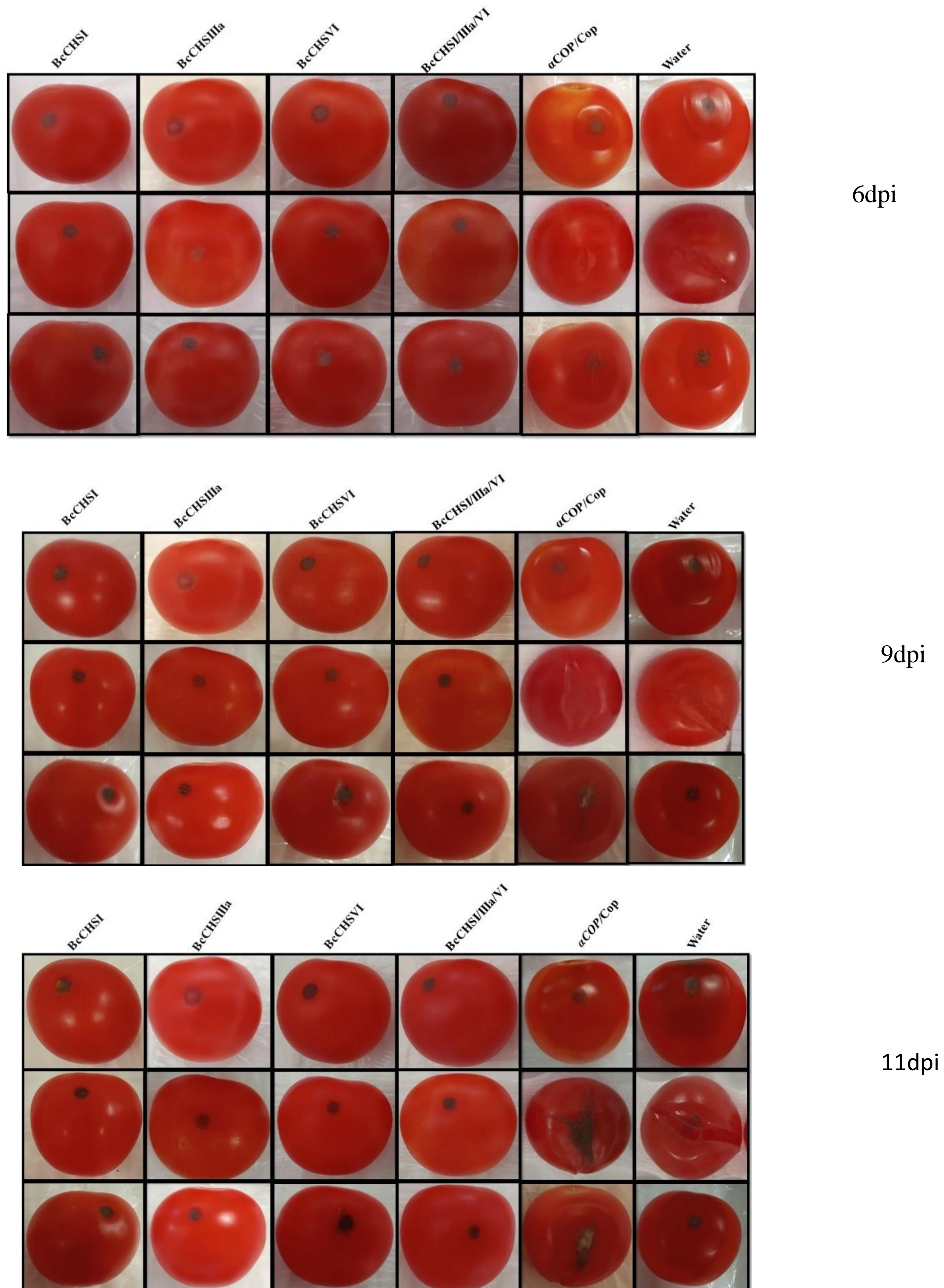
6.1. Supplemental figure 1. Progress of *B. cin*erea infection on grapefruits at 3, 5, and 7-days post inoculation (dpi). Fruits were treated with 20µl of water (ctrl) or *BcCHSI-dsRNA*, *BcCHSIIIa-dsRNA*, *BcCHSVI-dsRNA*, *BcCHSI/IIIa/VI-dsRNA*, *αCOP/Cop* (negative control)-*dsRNA* (120 ng/µl dsRNA) before being inoculated with 20 µl of a 1 x 10^6 ml^{-1} sporangia.



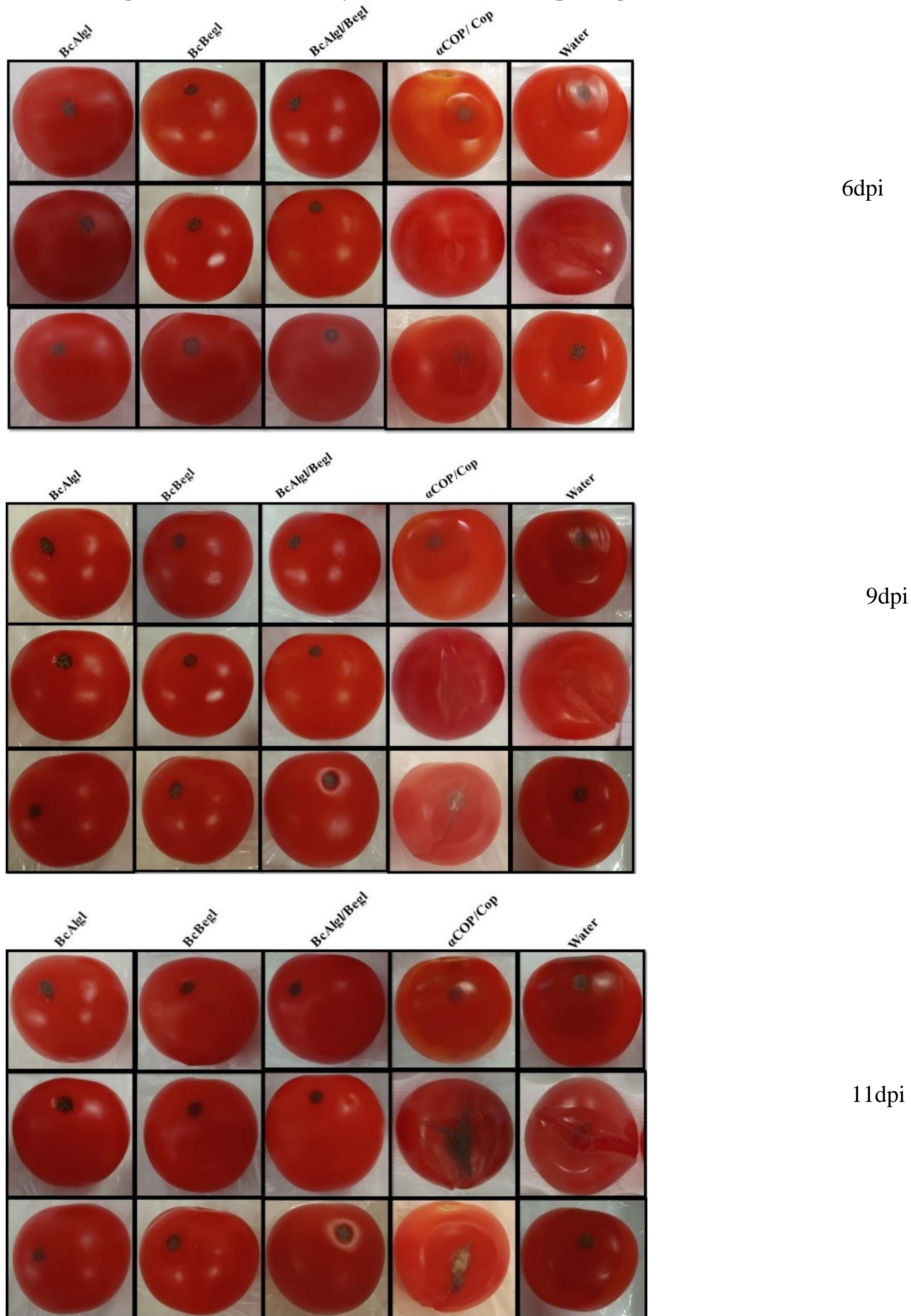
6.2. Suplemental figure 2. Progress of *B. cinerea* infection on grapefruits at 3, 5, and 7-days post inoculation (dpi). Fruits were treated with 20µl of water (ctrl) or *BcAlgl-dsRNA, BcBegl-dsRNA, BcAlgl/Begl-dsRNA, BcAlgl/Begl-dsRNA*, *αCOP/Cop* (negative control)-*dsRNA* (120 ng/µl dsRNA) before being inoculated with 20 µl of a 1 x 10^{6} ml^{-1} sporangia.



6.3. Supplemental figure 3. Progress of *B. cinerea* infection on tomato fruits at 6, 9, and 11-days post inoculation (dpi). Fruits were treated with 20µl of water (ctrl) or *BcCHSI-dsRNA, BcCHSIIIa-dsRNA, BcCHSVI-dsRNA, BcCHSI/IIIa/VI-dsRNA*, *αCOP/Cop* (negative control)-*dsRNA* (120 ng/µl dsRNA) before being inoculated with 20 µl of a 1 x 10^6 ml^{-1} sporangia.



6.4. Supplemental figure 4. Progress of *B. cinerea* infection on tomato fruits at 6, 9, and 11-days post inoculation (dpi). Fruits were treated with 20µl of water (ctrl) or *BcAlgl-dsRNA, BcBegl-dsRNA, BcAlgl/Begl-dsRNA, αCOP/*Cop (negative control)-*dsRNA* (120 ng/µl dsRNA) before being inoculated with 20 µl of a 1 x 10^6 ml^{-1} sporangia.



6.5. Supplementary tables

6.5.1. Supplemental table 1. Quantifiction of the final cDNA used for qPCR assessed using a Nanodrop 2000 Spectrophotometer. cDNA synthesis from all samples (Tomato fruit) were performed with three biological replicates.

No.	**cDNA**	**Conc. (ng/µL)**	**260/280**	**260/230**
	Group 1			
1	BcCHSI	166.6	2.02	1.91
2	BcCHSIIIa	174.4	2.01	1.96
3	BcCHSVI	206.3	2.08	1.96
4	BcCHSI/IIIa/VI	219	2.07	2.03
5	BcAlgl	214.5	2.06	2.05
6	BcBegl	259.6	2.02	1.95
7	BcAlgl/Begl	214.9	2.06	1.98
8	Cop	240.9	2.05	1.98
9	Water	192.5	2.07	2.08
	Group 2			
1	BcCHSI	176.1	2.05	1.97
2	BcCHSIIIa	171.3	2.05	2.01
3	BcCHSVI	285.5	2.04	1.96
4	BcCHSI/IIIa/VI	255	2.03	1.98
5	BcAlgl	287.3	2.04	2.03
6	BcBegl	256.5	2.05	2.02
7	BcAlgl/Begl	189.5	2.05	1.96
8	Cop	251.1	2.05	1.98
9	Water	342.1	2.04	2.04
	Group 3			
1	BcCHSI	329.8	2.04	2.06
2	BcCHSIIIa	340.0	2.04	2.08
3	BcCHSVI	304.6	2.03	2.06
4	BcCHSI/IIIa/VI	306.9	2.05	2.00
5	BcAlgl	203.8	2.12	2.30
6	BcBegl	339.1	2.04	2.08
7	BcAlgl/Begl	179.1	2.05	1.96
8	Cop	398.3	2.04	2.05
9	Water	333.2	2.06	2.05

6.5.2. Supplemental table 2. Quantification of the cDNA used for qPCR assessed using a Nanodrop 2000 Spectrophotometer. cDNA synthesis from all samples (Grape fruits) were performed with three biological replicates.

No.	cDNA	Conc. (ng/µL)	260/280	260/230
	Group 1			
1	BcCHSI	319.1	2.01	1.98
2	BcCHSIIIa	185.2	2.01	1.97
3	BcCHSVI	290.6	2.00	2.01
4	BcCHSI/IIIa/VI	119.9	2.00	1.95
5	BcAlgl	185.3	2.02	1.96
6	BcBegl	136.1	2.05	2.00
7	BcAlgl/Begl	162.9	2.00	1.98
8	Cop	218.2	2.02	1.97
9	Water	185.2	2.09	2.00
	Group 2			
1	BcCHSI	143.1	2.00	2.12
2	BcCHSIIIa	163.8	2.01	2.09
3	BcCHSVI	168.5	2.02	1.95
4	BcCHSI/IIIa/VI	269.6	2.03	2.01
5	BcAlgl	196.3	2.08	1.98
6	BcBegl	226.8	2.02	1.98
7	BcAlgl/Begl	244.3	2.00	1.97
8	Cop	161.2	2.05	2.01
9	Water	129.4	2.00	2.17
	Group 3			
1	BcCHSI	202.5	2.02	1.94
2	BcCHSIIIa	158.9	2.08	2.10
3	BcCHSVI	259.7	2.05	2.10
4	BcCHSI/IIIa/VI	177.3	2.01	1.98
5	BcAlgl	171.3	2.06	1.98
6	BcBegl	143.9	2.03	1.93
7	BcAlgl/Begl	178.6	2.03	2.04
8	Cop	190.2	2.03	2.03
9	Water	167.8	2.01	2.01

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Chapter 5: Double-Stranded RNA Targeting Dicer-Like Genes Compromises the Pathogenicity of *Plasmopara viticola* on Grapevine

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Abstract

Downy mildew caused by *Plasmopara viticola* is one of the most devastating diseases of grapevine, attacking all green parts of the plant. The damage is severe when the infection at flowering stage is left uncontrolled. *P. viticola* management consumes a significant amount of classical pesticides applied in vineyards, requiring efficient and environmentally safe disease management options. Spray-induced gene silencing (SIGS), through the application of exogenous double-stranded RNA (dsRNA), has shown promising results for the management of diseases in crops. Here, we developed and tested the potential of dsRNA targeting *P. viticola* Dicer-like (*DCL*) genes for SIGS based crop protection strategy. The exogenous application of *PvDCL1/2 dsRNA*, a chimera of *PvDCL1* and *PvDCL2*, highly affected the virulence of *P. viticola*. The reduced expression level of *PvDCL1* and *PvDCL2* transcripts in infected leaves, treated with *PvDCL1/2 dsRNA*, was an indication of an active RNA interference mechanism inside the pathogen compromising its virulence. Besides the protective property, the *PvDCL1/2* dsRNA also exhibited a curative role by reducing the disease progress rate of already established infection. Our data provide a promising future for *PvDCL1/2 dsRNA* as a new generation of RNA-based resistant plants or RNA-based agrochemical for the management of downy mildew disease in grapevine.

Keywords: *Dicer-like genes*, double-stranded RNA (dsRNA), *Plasmopara viticola*, spray-induced gene silencing, *Vitis vinifera*

1. Introduction

As we mentioned in the previous chapters some of the economically important diseases of grapevine includes gray mold, powdery mildew, and downy mildew caused by *Botrytis cinerea*, *Erysiphe necator*, and *Plasmopara viticola*, respectively. The obligate biotrophic *oomycete P. viticol*a attacks all green parts of grapevine, and the damage is severe if the infection occurring during flowering is not managed. Surprisingly, all cultivated European *V. vinifera* cultivars are susceptible to the pathogen (Armijo et al., 2016), which makes the management of downy mildews in vineyard and other crops rely on synthetic fungicides. As a result, its management, together with powdery mildew, consumes about two-thirds of all synthetic fungicides sprayed for disease management of crops in the European Union (Eurostat., 2007). With such heavy reliance on agrochemicals to control *P. viticola*, not only pathogen strains have developed resistance to several fungicides (Gisi and Sierotzki, 2008), but there also exist social concerns about environment and human health, which makes it urgent to find alternative control strategies.

The findings that exogenous small RNAs (sRNA) and doublestranded RNA (dsRNA) trigger posttranscriptional gene silencing (Fire et al., 1998; Hamilton and Baulcombe, 1999) have opened new avenues to exploit the gene silencing mechanism as a new class of regulatory molecules during plant–pathogen interaction. The gene silencing occurs *via* RNA interference (RNAi) machinery, a natural biological process conserved in most eukaryotes where sRNA molecules regulate gene expression by targeting specific endogenous messenger RNA molecules in a sequence-specific manner (Vaucheret and Fagard, 2001; Castel and Martienssen, 2013). The silencing signals of sRNA are bidirectional cross-kingdom, moving from the host to its interacting organism, and *vice versa* (Tomilov et al., 2008; Weiberg et al., 2015; Wang et al., 2016; Cai et al., 2018). The involvement of sRNAs in the crosstalk between plant hosts and their fungal and oomycete pathogens has also been suggested (Weiberg et al., 2013; Brilli et al., 2018), implying that exploiting the RNAi mechanisms of both the hosts and the pathogens can represent a new strategy in fungal and oomycete disease management. Transgene-derived artificial sRNAs inducing gene silencing, called host-induced gene silencing (HIGS), have been observed providing resistance to plants against fungi (Nowara et al., 2010; Koch et al., 2013; Zhu et al., 2017) and oomycetes (Vega-Arreguin et al., 2014; Jahan et al., 2015). Interestingly, recent findings revealed that the external application of dsRNA also conferred host plant resistance to fungal pathogens by silencing targeted genes (Koch et al.,

2016; Wang et al., 2016; McLoughlin et al., 2018; Nerva et al., 2020), an approach referred to as spray-induced gene silencing (SIGS).

The exogenous application of dsRNAs targeting *Dicerlike (DCL), lanosterol 14a-demethylase, chitin synthase*, and *elongation factor* genes of *B. cinerea* negatively affected its pathogenicity in multiple hosts (Wang et al., 2016; Nerva et al., 2020). Similarly, spraying of dsRNA targeting three cytochrome P450 genes of *Fusarium graminearum* inhibited fungal growth at sprayed and distal parts of detached barley leaves (Koch et al., 2016). While these research findings provided proof that SIGS-based plant protection is effective against targeted pathogens, there is also indication that the effects of dsRNA can be reproduced on closely related pathogens based on sequence homology (McLoughlin et al., 2018). According to McLoughlin et al. (2018), dsRNA targeting *SS1G_05899* and *SS1G_02495* genes of *Scelerotinia sclerotiorum*, both involved in redox reaction, restricted the progress of the pathogen on a susceptible *Brassica napus* cultivar. Remarkably, the cultivar was also resistant to *B. cinerea* when treated with dsRNA targeting *BC1G_01592* and *BC1G_04955*, the *B. cinerea* homologs to *SS1G_05899* and *SS1G_02495*, respectively. Such results provide compelling evidence about the adaptability and flexibility of SIGS technology in crop disease management. In this study, we investigate the potential of dsRNA targeting *P. viticola DCL* genes for SIGS-based crop protection strategy. We show that the application of dsRNA targeting *PvDCL1/2* extremely reduces the pathogenicity of *P. viticola* and the expression level of the targeted genes, indicating that RNAi-based control strategy can indeed represent a promising alternative to hazardous agrochemical application to manage downy mildew disease of grapevine.

2. Materials and Methods

2.1. Design and Production of dsRNA and Rate of Application

Plasmopara viticola genes encoding two Dicer-like proteins, as defined by the presence of a Dicer dimerization domain, corresponding to *PVITv1_T038441* and *PVITv1_T003331*, hereafter referred to as *PvDCL1* and *PvDCL2*, respectively (Brilli et al., 2018), were selected. For RNAi, 258- and 257-bp fragments of *PvDCL1* and *PvDCL2* sequences, respectively (Table 1), were chosen as target, and the corresponding chimeric dsRNA molecule (*PvDCL1/*2, 515 bp) was chemically synthesized by AgroRNA (Genolution Inc., Seoul, Republic of Korea;

Figure 1). DsRNA targeting *B. cinerea DCL 1* and *2* genes, *BcDCL1/2* (490 bp; Wang et al., 2016), produced in the same way, was used as the negative control. After assaying different dsRNA concentrations, 75, 100, or 125 ng μ^{-1} concentrations of dsRNA were used for spot inoculation in a total volume of 50 µl.

2.2. Plant Material and *Plasmopara viticola* Inoculation

Seedlings of *V. vinifera* cv. Trebbiano were raised in growth chamber at 22 ^{0}C ± 1 ^{0}C and 12/12 h light cycle. *P. Viticola* (strain 465, belonging to University of Bologna collection) was maintained on grapevine leaves at 22 0C ± 1 ^{0}C and 12/12 h of photoperiod. Sporangia were harvested in distilled water and filtered through cheesecloth. Sporangia concentration was determined using hemocytometer. Fully expanded third and fourth leaves from 6–8-week-old grapevine seedlings were detached and immediately placed on wet absorbing paper in a plastic box. Detached leaves were surface sterilized for 1 min with 70% ethanol and then rinsed three times with sterile water. For assaying dsRNA as preventive treatment, the abaxial side of each leaf was treated with three droplets of 50 µl of either dsRNA or water. After 2 h, 7.5 µl of a 1 x 10^5 ml $^{-1}$ sporangia solution was placed on top of the droplets. Disease progress was evaluated until 14 days post inoculation (dpi) in five biological replicates. A single leaf was considered a biological replicate. For assaying dsRNA as curative treatment, each leaf was first challenged by the pathogen by applying four droplets of 7.5 µl of a 1 x 10^5 ml^{-1} sporangia solution, and after 7 dpi, when a visible sign of *P. viticola* was observed, 50 µl of either dsRNA or water was placed on top of each spot of the progressing pathogen. Disease progress was evaluated until 14 dpi, i.e., 7 days post treatment (dpt) of either dsRNA or water, in three biological replicates. To assess the progress of the pathogen, leaf area covered by *P. viticola* (in square millimeters) was measured from the digital images using the free software ImageJ program. Leaf area covered by the pathogen, area under disease progress curve (AUDPC), and disease progress rate data were analyzed using analysis of variance. Means were separated by Tukey's honestly significant difference test.

2.3. RNA Extraction and Quantitative PCR Analysis

Leaves that were treated in the preventive assay were collected at 7 dpi, in three replicates, immediately frozen in liquid nitrogen, and kept at -80 ^{0}C until use. RNA was extracted using a rapid cetyltrimethylammonium bromide (CTAB) method (Gambino et al., 2008). First-strand cDNA was synthesized from 1 µg of total RNA, pretreated with TURBO DNA-free Kit^{TM}

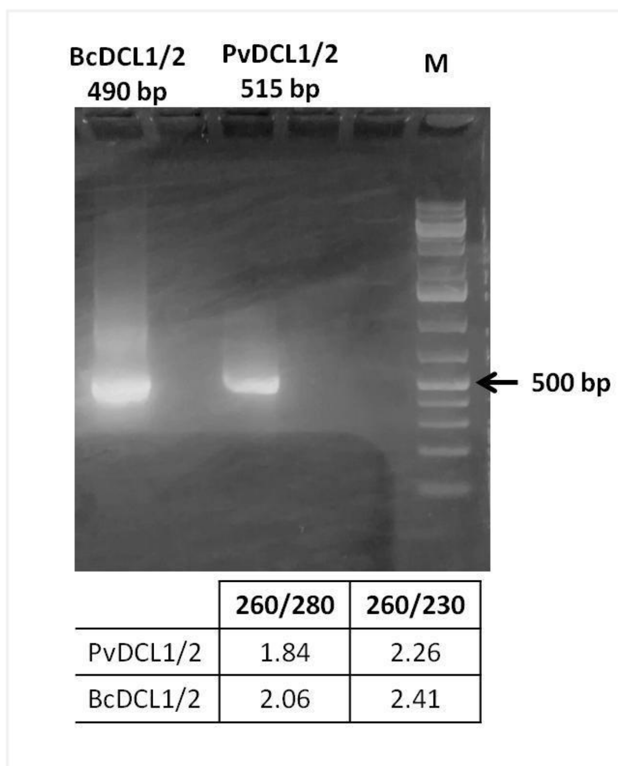
(Invitrogen, CA, United States), using ImProm-II Reverse Transcriptase (Promega), following the manufacturer's guide. Quantitative PCR (qPCR) was performed in an MX3000 thermocycler (Stratagene, CA, United States) using 0.25 µl of cDNA and 200 nM of specific forward and reverse primers (Table 2) in a total volume of 12.5 µl using MaximaR SYBR Green/ROX qPCR Master Mix (Fermentas). Each amplification reaction was run in duplicate. The cycling parameters were as follows: 5 min at 95 ^{0}C, 40 cycles of 15s at 95 ^{0}C, 25s at 61 ^{0}C, and 30 s at 72 ^{0}C. A melting curve was established from 55 ^{0}C to 90 ^{0}C by changing 0.5 ^{0}C every 10s. For normalization, *P. viticola* elongation factor eIF1b was used. Each primer pair's amplification efficiency was calculated using LinReg (Ruijter et al., 2009). The amplification efficiency value obtained was used to calculate the relative quantity (RQ) and normalized RQ (NRQ) according to Hellemans et al. (2007). Statistical analyses of the qPCR results were made after log2(NRQ) transformation (Rieu and Powers, 2009). Statistical significance was calculated by Tukey's honestly significant difference test.

3. Results

3.1. Spray-Induced Gene Silencing of *Plasmopara viticola DCL* Genes Hampers Disease Development

Preliminary inoculation assay was conducted to determine a baseline concentration of *PvDCL1/2 dsRNA* that could affect *P. viticola DCL1* and *DCL2* genes and consequently inhibit its germination and/or colonization of grapevine leaves. After the treatment with 10 and 50 ng µl^{-1} *PvDCL1/2 dsRNA* and water, as control, detached grapevine leaves were challenged with *P. viticola* sporangia. Inoculated leaves were monitored for 2 weeks. Sign of *P. viticola* infection was conspicuous around the inoculation spot starting from the 5dpi, mostly on control and on leaves treated with 10 ng µl^{-1} *PvDCL1/2 dsRNA*, where white fluffy growth of sporangiophores and sporangia appeared. At 14 dpi, the rate of disease progress was relatively lower in leaves that received 50 ng µl^{-1} of *PvDCL1/2 dsRNA* (Figure 2), indicating that pathogen control efficiency can increase with higher concentrations. Therefore, the ability of *PvDCL1/2 dsRNA* to control *P. viticola* growth in preventive treatment was assessed using higher concentrations (i.e., 75, 100, and 125 ng µl^{-1}). Treatments with *BcDCL1/2* targeting *B. cinerea DCL1* and *DCL2* genes and water were used as controls. As shown in Figure 3A, the fluffy growth of sporangiophores was quite visible on control leaves treated with either water or *BcDCL1/2 dsRNA* at the three different concentrations. On the contrary, the pathogen progress was substantially low or null on leaves that received *PvDCL1/2 dsRNA*. As a

consequence, the area covered by *P. viticola* and the AUDPC values at 7, 10, and 14 dpi were significantly and consistently lower in leaves treated with *PvDCL1/2* dsRNA than in those treated with *BcDCL1/2 dsRNA* or water (Figure 3B), confirming that *PvDCL1/2* dsRNA hampered *P. viticola* growth. To confirm that the inhibition of *P. viticola* growth by *PvDCL1/2* dsRNA was due to the downregulation of *PvDCL1* and *PvDCL2* genes, their expression, normalized to *P. viticola* elongation factor *eIF1b*, was quantified at 7 dpi using qPCR. We found that the relative expression of both *PvDCL1* and *PvDCL2* was reduced as compared to the controls (Figure 4). Compared with water and *BcDCL1/2*-treated leaves, the NRQs of *PvDCL1* and *PvDCL2* transcripts in leaves treated with 100 ng μl^{-1} concentration of *PvDCL1/2* dsRNA were reduced on average by 48 and 44%, respectively, which is in line with the concept of RNAi-based sequence-specific silencing via SIGS.



	260/280	260/230
PvDCL1/2	1.84	2.26
BcDCL1/2	2.06	2.41

Figure 1. Agarose gel electrophoretic analysis *of PvDCL1/2* and *BcDCL1/2 dsRNA* chemically synthesized by AgroRNA (Genolution Inc., Seoul, Republic of Korea). Samples, diluted 40x were loaded as 5 µl. M: size molecular marker. The quality of the dsRNA, as measured by 260/280 and 260/230 absorbance, was quantified by NanoDrop 1000 Spectrophotometer (Thermo scientific, Waltham, USA).

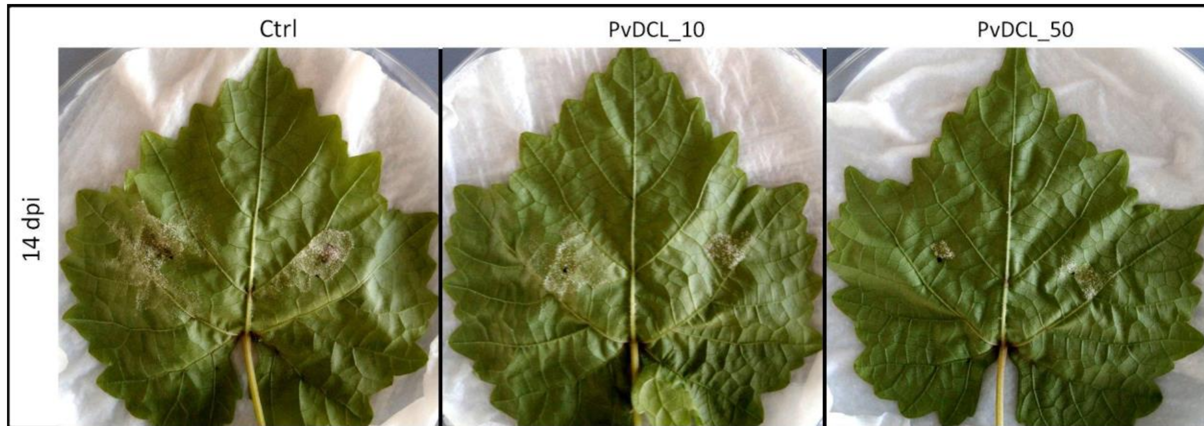


Figure 2. Externally applied *PvDCL1/2* dsRNA on detached grapevine leaves inhibited *Plasmopara viticola* infection. Leaves were treated with 50 µl of water (ctrl) or dsRNA before inoculated with 7.5 µl of a 1 X10^5 mL^{-1} sporangia. *PvDCL*-10: *PvDCL*1/2 dsRNA at 10 ng μl^{-1} concentration ; *PvDCL*-50: *PvDCL1/2* dsRNA at 50 ng μl^{-1} concentration; dpi: days post inoculation.

Table 1. Sequences used for double strand RNA synthesis

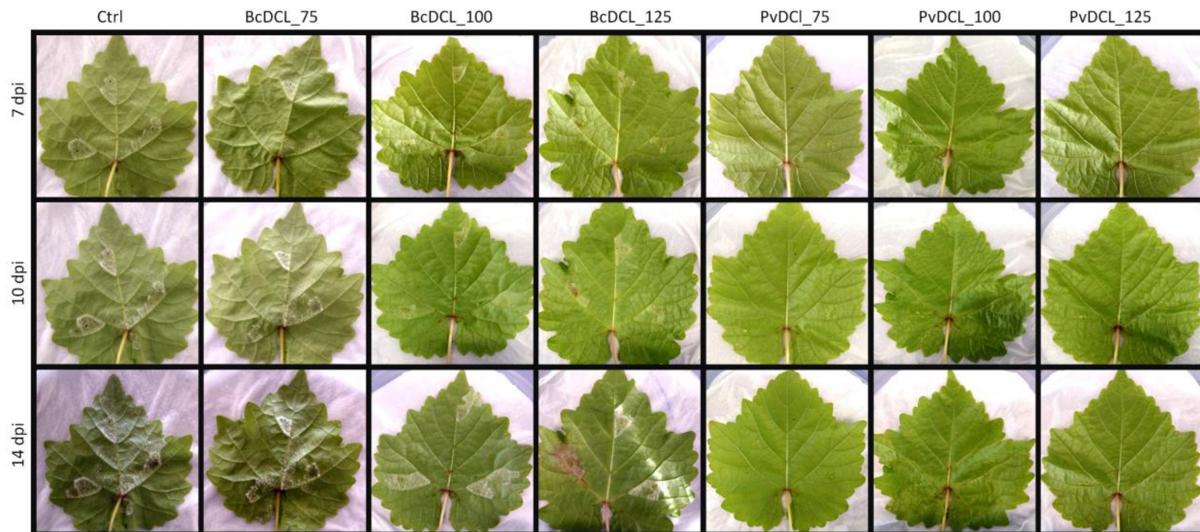
Gene ID	Gene name	Sequence
PVITv1_T038441	*PvDCL1*	ATGATGGACACCTCGTTGTGGGAGCACCAACGGGAGATCGTGGCT GTGGCGCGACATCGCAGCGTGTTAGTGAGTAGTTCGCAGTCTGTA GGAAAGACGCATGTAAGCTGTGCACTGCTGTGCGAGGCCGCTGCC TCTAGTCCGAAGCTACACGCATTGGCGATTGCTGCATCGCCTGTGG GCCGATCGGCTCTACAGACGCAGCTAGCGAGACTGTGTGGACTTC GCGTGCTCTGTAGCGATTCAGACAATGCAAGA
PVITv1_T003331	*PvDCL2*	TAGGCGATACGGGAATCGGCAAAACCTTTCTTGCCATAGCATTATT GTCCGAGCAAGACTACTCGGGCGACCGACGTGCGTTCTTTATGGCT CCGACCCGCCAGTTGGTGGTGCAGATTACGGCCAAGATTCGCCAG ACGAGCACGTTGCGCGTCAATTCGTATTGCGGACGGACAGCTGAT TTGTGGGACGCCACACAGTGGGAACGGGAGCTGCAGCTCACGCGC GTGTTTGTGTGCACACCCGAGATTGTACGC

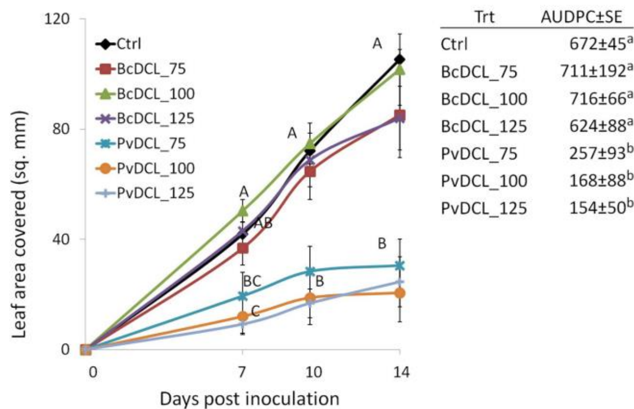
Table 2. List of qPCR primers used. Gene identification, gene name, primer name, and primer sequence are provided.

Gene ID	Gene name	Primer name	Primer sequence
PVITv1_T004162	PveIF1b	PveIF1b_F	ACAACGGTGCAAGGCTTAGC
		PveIF1b_R	ACTCGCGAATGTTAGTCCGC
PVITv1_T038441	PvDCL1	PvDCL1_F	AGCGAGACTGTGTGGACTTC
		PvDCL1_R	GCCTTTTCGCAGCATCTCTT
PVITv1_T003331	PvDCL2	PvDCL2_F	CGGACAGCTGATTTGTGGGA
		PvDCL2_R	GGCACTCGTCAAACACTAGC

3.2. Spray-Induced Gene Silencing of *PvDCLs* Shows a Curative Effect Against *Plasmopara viticola*

The observed protective effect of *PvDCL1/2 dsRNA* prompted us to check whether the dsRNA also has a curative effect against *P. viticola*. Detached leaves were initially inoculated with *P. viticola* sporangia, and then, once the infection has been established (i.e., 7 dpi), dsRNA was applied [i.e., the time of either dsRNA or water application is marked as 0 day post treatment (dpt)]. At each inoculation spot, 50 µl of dsRNA or water was added on top of the growing mycelia. At 4 dpt, the progress of the pathogen stagnated in most of the treatments, with more pronounced effect on leaves that received 100 and 125 ng μl^{-1} of *PvDCL1/2 dsRNA* (Figure 5A). After 4 dpt, recovering of pathogen growth was more apparent on all leaves. At 7 dpt, the disease advanced more on leaves treated with *BcDCL1/2* and water than on those treated with *PvDCL1/2*, especially at the highest concentration (Figure 5A). Computing the rate of disease progress, taking diseased area at 7 dpi (0 dpt) as a reference, the disease progress rate was relatively slower on leaves treated with *PvDCL1/2*, with more pronounced effect at 7 dpt (Figure 5B). The result shows that the *PvDCL1/2 dsRNA* can also hamper the expansion of already established downy mildew disease. Compared to the preventive application, where all the three concentrations of *PvDCL1/2* inhibited the growth of the pathogen significantly, when used as curative treatment, the rate of the pathogen growth was reduced significantly only at the highest concentration of *PvDCL1/2 dsRNA*. These data show that the exogenously applied dsRNA targeting *PvDCL1/2* has both promising protective and curative effects.





Trt	AUDPC±SE
Ctrl	672±45[a]
BcDCL_75	711±192[a]
BcDCL_100	716±66[a]
BcDCL_125	624±88[a]
PvDCL_75	257±93[b]
PvDCL_100	168±88[b]
PvDCL_125	154±50[b]

Figure 3. Externally applied *PvDCL1/2* double-stranded RNA (dsRNA) on detached grapevine leaves and *Plasmopara viticola* infection. (A) Progress of *P. viticola* on grapevine leaves at 7, 10, and 14 days post inoculation (dpi). Leaves were treated with 50 µL of water (ctrl) or dsRNA (75, 100, or 125 ng μl^{-1} of dsRNA of *BcDCL1/2* (*BcDCL*_75/100/125) and *PvDCL*1/2 (*PvDCL*_75/100/125)) before being inoculated with 7.5µl of a $1x10^5$ ml^{-1} sporangia. (B) Disease progression of *P. viticola* expressed as leaf area covered and as area under the disease progress curve (AUDPC ± SE, mm^2 x day) through 14 dpi. Error bars indicate standard error. Means at each dpi and AUDPC followed by a common letter are significantly not different according to Tukey's honestly significant difference test (P < 0.05).

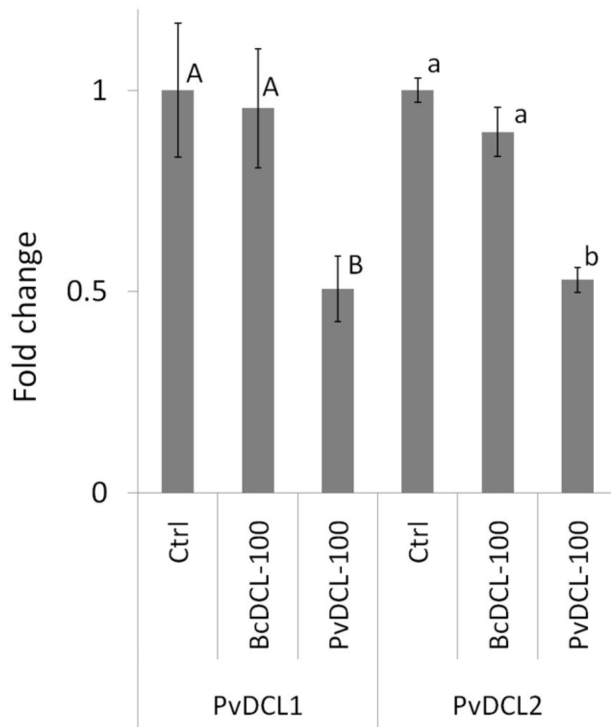
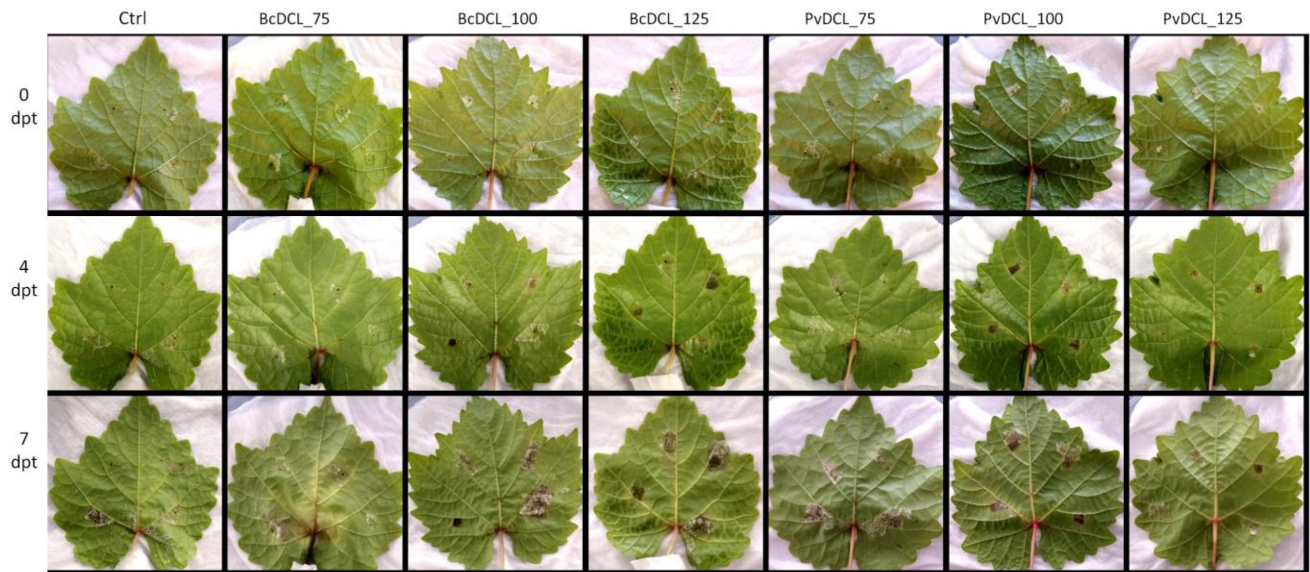


Figure 4. Expression profiles of *PvDCL1* and *PvDCL2* following *Plasmopara viticola* inoculation on leaf samples treated with 50 µl of water (ctrl) or 100 ng μl^{-1} of double-stranded RNA (dsRNA) of either *BcDCL1/2* (*BcDCL*-100) or *PvDCL1/2* (*PvDCL*-100). Gene expression level was determined by quantitative PCR (qPCR). Bars represent fold change of dsRNA-treated sample relative to ctrl sample at 7 days post inoculation. Normalization based on the expression levels of elongation factor, *PveIF1b*, was carried out before calculating fold changes. Error bar represents standard error of the mean of three biological replicates. Expression values followed by a common letter are significantly not different among samples, according to Tukey's honestly significant difference test ($P < 0.05$), using one-way ANOVA of log2.



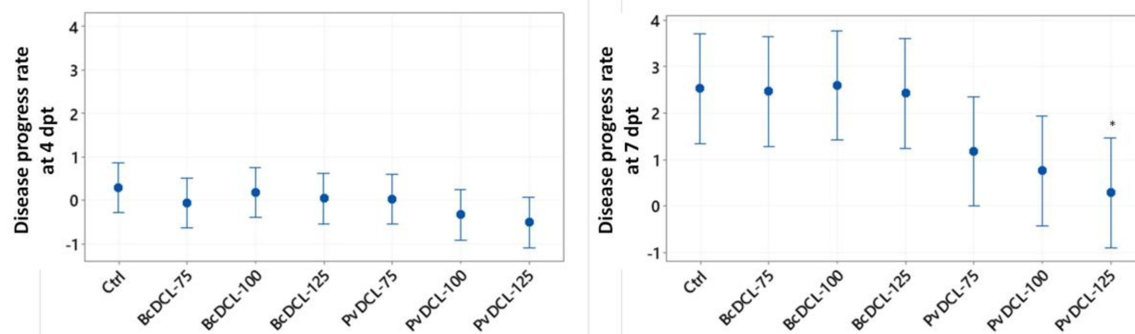


Figure 5. Progress of *Plasmopara viticola* on grapevine leaves after being treated with *PvDCL1/2* double-stranded RNA (dsRNA). (A) Progress of already established *P. viticola* infection after receiving dsRNA treatments. Leaves were treated with 50 of water (ctrl) or dsRNA [75, 100, or 125 ng μl^{-1} of dsRNA of *BcDCL1/2* (*BcDCL*_75/100/125) or *PvDCL1/2* (*PvDCL*_75/100/125)] 7 days after being inoculated with 7.5 µl of a 1 x 10^5 ml^{-1} sporangia [i.e., 0 days post treatment (dpt) of dsRNA]. (B) Disease progress rate at 4 and 7 dpt, computed by taking leaf area covered by *P. viticola* at 7 dpi (0 dpt) as a reference. Bars are 95% confidence interval, and asterisks (*) indicate statistically significant differences according to Tukey's honestly significant difference test (P <0.05).

4. Discussions

In grapevine cultivation, downy mildew, caused by *P. viticola*, is among the major diseases requiring repeated applications of pesticides within a growing season. In this study, we show that external application of long non-coding dsRNA, 515 bp long, targeting the two *DCL* genes of *P. viticola*, reduced the progress of the pathogen on grapevine leaves. Transcript level reduction of the target genes, *PvDCL1* and *PvDCL2*, suggests a specific RNA silencing effect triggered by *PvDCL1/2 dsRNA*. To our knowledge, this is the first report showing the potential of exogenously applied RNAi molecules as an effective strategy for oomycete management in crops. The results presented further support the use of SIGS-based strategy for fungal pathogen management (Koch et al., 2016; Wang et al., 2016; McLoughlin et al., 2018; Nerva et al., 2020). Non-coding sRNA molecules derived from plant pathogens could play a role in suppressing host immunity (Weiberg et al., 2013; Brilli et al., 2018) and hence could be regarded as additional classes of effectors, besides protein coding effector genes studied so far. It has been demonstrated that *B. cinerea* sRNAs (*BcsRNAs*) triggered the silencing of Arabidopsis and tomato targets involved in host immunity, such as *mitogen-activated protein kinase 1 (MPK1), MPK2, peroxiredoxin*, and cell wall-associated kinase genes. Once they have entered the plant cell, *Bc*-sRNAs hijack the host's RNAi machinery, binding to Argonaute 1 (AGO1) protein and directing the silencing of host immunity genes (Weiberg et al., 2013). Accordingly, the ago1 mutant Arabidopsis exhibited reduced susceptibility to *B. cinerea*, and the expression of sRNAs that target *B. cinerea DCL1 and DCL2* in Arabidopsis and tomato led to the silencing of the *BcDCL* genes and affected the fungal pathogenicity and growth, also when exogenously applied on different organs and tissues (Weiberg et al., 2013; Wang et al., 2016). In addition, *dcl1 dcl2 B. cinerea* double mutant, which is unable to produce sRNAs, displayed a stunted pathogenicity on several hosts (Weiberg et al., 2013; Wang et al., 2016). In a recent study, it was observed that during *V. vinifera–P. viticola* interaction, the sRNA profile of *P. viticola* showed enrichment in 21- and 25-nt sRNAs, which were also abundantly expressed in sporangia (Brilli et al., 2018). According to the study, the presence of *DCLs*, AGOs, and RNA dependent RNA polymerase confirms the existence of RNA silencing machinery in *P. viticola*, which is active during its interaction with grapevine (Brilli et al., 2018). The fact that the external application of *PvDCL1/2* dsRNA extremely reduced the pathogenicity of *P. viticola*, coupled with the observed reduction in *PvDCL1* and *PvDCL2* transcript levels, might suggest that the pathogen can take up external dsRNA and that the RNAi machinery is active during the infection process. Similarly, reduced disease symptoms

and sequence specific silencing of target genes were also observed in *B. cinerea*, *F. graminearum*, and *S. sclerotiorum* (Koch et al., 2016; Wang et al., 2016; McLoughlin et al., 2018), following the external application of dsRNA.

Reduced pathogenicity of plant pathogens due to sRNA and dsRNA has put forward the considerations of RNAi-based technology as a new plant protection method, at least for those pathogens having bona fide RNA silencing machinery. In planta gene silencing of pathogen target genes, a mechanism known as HIGS, has also been reported (Nowara et al., 2010; Koch et al., 2013; Vega-Arreguin et al., 2014; Jahan et al., 2015; Zhu et al., 2017). Furthermore, for vegetatively propagated crops like grapevine, HIGS can be exploited to obtain RNAibased rootstocks, which can produce sRNA able to move to a grafted untransformed scion and protect it from pathogen infection, as sRNAs have high mobility between shoot and root (Gouil and Lewsey, 2021; Li et al., 2021). In addition, in planta expressed RNAi sequences do not encode for protein products and are designed against specific genes of target pathogens or susceptibility factor without affecting other nontarget organisms. All these features together could reduce data requirements for risk assessment of such RNAi-based plants (Limera et al., 2017; Arpaia et al., 2020). In addition to the HIGS potential application, the results of this research confirm the potential of the gene silencing technology also to develop new RNAi-based fungicides, known as SIGS. To ensure sustainable food production, European Union and global sustainability policies emphasize the need to replace contentious pesticides with safe, efficient, and costeffective alternatives (Taning et al., 2020). The high selectivity of RNAi-based products, due to sequence-specific modes of action, compared with other conventional pesticides, makes them a promising solution to substitute or reduce reliance on contentious pesticides. Yet there are still relevant aspects to be clarified, such as local and remote translocation and environmental stability of applied sRNAs, before pushing forward SIGS as an alternative solution to toxic pesticides. Despite many solutions reported to stabilize the RNA molecules and make their administration in the field easy and effective, more effort should be taken on the risk assessment studies in order to clarify the risks associated with the use of these molecule for the farmers, consumers, and environment and proceed with the necessary regulatory protocols in order for them to reach the market.

In this study, we demonstrated that dsRNA specifically designed to silence *PvDCL1* and *PvDCL2* genes efficiently controls downy mildew disease caused by *P. viticola* on grapevine, a disease that forces to consume significant amounts of pesticides that are applied every year

on vineyards. Although the mechanism behind the uptake and transport of the externally applied dsRNA needs further studies, the presented data give important scientific information on such new-generation RNA based fungicides, which are environmentally safe and sustainable. So far, externally applied RNAi-based disease suppression data are limited on plant pathogens from Ascomycetes, but with our findings, we extended the possibility of using externally applied dsRNA for managing devastating plant pathogen oomycetes like *Phytophthora* and *Pythium* species.

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Chapter 6: General discussions and perspectives for future research

In our spray-induced gene silencing RNAi experiments in *Botrytis cinerea* and *Plasmopara viticola*, we observed that topical administration of dsRNA causes a strong enough silencing response for RNAi to be useful for future disease control applications. For RNAi to be used in disease control in the field, the target pathogen must ideally uptake external RNA molecules from the environment. In many pests, it has been observed that RNAi through external uptake of dsRNA works efficiently in controlling the disease caused by the pathogens (Gan et al., 2010; Wang et al., 2016; Mitter et al., 2017; Ahsan and Yuanhua, 2021; Patil et al., 2021; Qiao et al., 2021; Sarkar and Subhankar, 2021). Naked dsRNA demonstrated effectiveness under lab conditions but when sprayed in fields, it can become unstable, where it could be degraded by UV radiation or washed off by rain. To avoid this, various studies, encouraged by human RNAi therapy research (Swaminathan et al., 2021), tried to improve stability and durability by establishing protective envelopes around dsRNAs/siRNAs. Liposome encapsulation facilitates the administration of siRNA therapies in mammals (Liu and Huang, 2021; Zabel et al., 2021). Nanoparticles and other carrier-based delivery of dsRNA/siRNA have been used extensively with amazing success, in start-up firms like "RNAissance1" which concentrates on broad, economical topical RNAi uses in agriculture. Their finished dsRNA products are marketed as being more stable than naked dsRNAs and safe for use. This development offers a step in reducing the amount of applied RNA biopesticides since it is projected that 2–10 g of dsRNA are required to protect 1 ha (Das and Sherif, 2020). However, several largely unknown parameters that affect dsRNA persistence, distribution, and dilutional and degradational processes in both host plants and target species affect how much and how frequently dsRNA is applied. It is also undeniable that mechanistic insights will be necessary to optimize and further develop RNA sprays as well as to foresee challenges that will come up when transferring RNA sprays to field environments. Mechanistic insights determine strengths and limitations in a pathosystem-specific manner. In conclusion, in this PhD thesis, we confirmed the functionality of the RNAi machinery in *B. cinerea* and *P. viticola*. The findings all confirmed the potential of RNAi technology as a possible tool in the development of a management strategy for *B. cinerea* and *P. viticola*. However, as discussed above, several more factors will have to be evaluated before an RNAi-based product targeting *B. cinerea and P. viticola* is available for field application.

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Dedication

I would like to dedicate this dissertation to my Lord and Savior Jesus Christ. I have witnessed Him realize countless dreams in wholly unanticipated ways, and I am eager to see what He has for me in the future.

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