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FOOD AND BEVERAGE INNOVATION AND MANAGEMENT

SPRAY-INDUCED GENE SILENCING AGAINST *Botrytis cinerea* IN STRAWBERRY CULTIVATION SYSTEMS: ASSESSMENT OF DISEASE SEVERITY, FRUIT PRODUCTION AND NUTRITIONAL QUALITY

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ABSTRACT

With the ever-increasing demand for nutritious food, advanced technologies emerge rapidly to enhance sustainable plant production and minimize food waste. Boosting the intrinsic plant defense of strawberry to counteract with *Botrytis cinerea*, the most prevalent mold in this Fragaria-genus plant is the most applied technique. Apart from Host-Induced Gene Silencing (HIGS), the external-triggered factor is an alternative method to activate and expand the internal resistance of strawberries and overcome the GMO-related misconceptions of the customers. The compatibility between the host and carrier bacteria is another concern of scientists in studying GMO plants. Therefore, this study sought to evaluate the influence of exogenously applied ds-RNA formulations on productive parameters, Botrytis-resistant feature of strawberry, and nutritional values, performed on two trials, on two strawberry cultivation systems, testing different dsRNA formulations, in comparison with their controls (not treated and standard pesticide).

The obtained outcomes revealed that the dsRNA in both A and B formulations positively affected Botrytis infection with higher than 20% of the disease control value in greenhouse conditions but ineffectively in combating *Botrytis* post-harvest, in which the best performance was observed in treatment no.6 following by no.5. Regarding the C regimes, notably dsRNA-increased content treatments (5 and 6) offered much more significant control issues, 35 and 40% respectively. Regime 7, the same formulation of 5 and 6 but at the highest concentration, declined the effect. This group had a positive effect on protecting fruit from damage caused by grey mold at 3dph. In both environmental conditions, fungicide treatments were still the best choice; however, in the open field, the combination Teldor-Signum-Switch was preferable. After seven days of harvesting, all products were almost spoiled in high humidity surroundings, except for positive treatments with acceptable damage.

In both environment trials of strawberry, different treatments did not influence the sensorial quality however the time of harvesting highly affected these elements. Higher environmental temperature, at the end of the harvesting season, in the open field probably altered sugar content and acidity.

Keywords: Strawberry, Disease severity, Disease control value, ds-RNA, Soluble Solid Content, Acidity, SIGS, HIGS, HPLC, VIGS.

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1. INTRODUCTION AND OBJECTIVE

Background

Strawberry (*Fragaria × ananassa*; *genus: Fragaria*) is a flowering plant belonging to the Rosaceae family, one of the most popular and commonly consumed fruits worldwide, fresh or processed. Strawberries speak to a sound food decision in which are low in complete calories, with a 100 g serving to give just 32kcal (Giampieri *et al.*, 2012), and their sweet flavor makes them a delightful nibble option in contrast to prepared food sources. Simultaneously, they are a significant dietary wellspring of bioactive mixes, the greater part of which are regular cell reinforcements that add to the high nutritional quality of the natural product. Another major healthful pertinence is the incredibly high amount of vitamin C, much higher than citrus organic products. A handful of strawberries are adequate to cover the nutrient C suggested day by day recompense (Carr & Frei, 1999). Along with nutrient C, folate assumes a pivotal part in accentuating the nutritional quality of strawberry while thinking about that, among organic products, it is one of the most extravagant normal wellsprings of this fundamental micronutrient and that folate is a significant factor in wellbeing advancement and infection counteraction (Tulipani, et al., 2009; Tulipani, et al., 2008; Tulipani, Romandini, et al., 2008). Strawberries are discovered to be a great source of manganese, with the goal that a serving of strawberries may give over 20% of the everyday adequate intake for this mineral. With that such quantity, strawberries can give about 5% of the adequate intake for potassium and have been qualified as a decent wellspring of iodine, magnesium, copper, iron, and phosphorus. Strawberries are among the richest dietary sources of phytochemicals, mainly represented by phenolic compounds, a large and heterogeneous group of biologically active non-nutrients, showing many non-essential functions in plants and huge biological potentialities in humans (Hakkinen & Torronen, 2000). Its derived products are rich dietary sources of nutraceutical compounds such as phenolic compounds, flavonoids (mainly anthocyanins, with flavones and flavanols providing a minor contribution), hydrolyzable tannins, and phenolic acids. Nowadays, there is convincing evidence that the combination of antioxidant micronutrients and nonessential phytochemicals, such as polyphenol compounds, present in fruits and vegetables, play a synergistic and cumulative role in health promotion (Johnsen *et al*., 2003; Vauzour, et al., 2010). In the Mediterranean diet, strawberries are a common and important fruit because of their diverse nutritional composition (Tulipani *et al*., 2009).

Grey mold, caused by *Botrytis cinerea*, is the most economically significant pre and postharvest pathogen of strawberry fruits. Strawberry spoilage after harvest, can also occur by

mechanical injury and desiccation. This necrotrophic fungus is particularly harmful in relatively humid environments, in which it can cause significant losses (Nadas et al., 2003). In case the organism contaminates blossoms, it is more harmful to products at pre- and postharvest, particularly beneath favorable conditions, i.e., temperatures around 20°C and drawn-out periods of moist conditions in which are commonly experienced in Italy. Conidia are scattered primarily by wind and creepy crawlies to contaminate blooms. *Botrytis cinerea* has no apparent host specificity and can infect more than 1000 plant species (Elad et al., 2016). The inoculum (e.g., conidia) of the fungus is highly abundant and ubiquitous and usually comes from infected plant tissues and enters the host via wounds or natural openings (Jarvis, 1962; Holz et al., 2007). In the early stages, *B. cinerea* deploys small interfering RNA (sRNAs) and effector proteins to suppress premature host cell death and immune responses, which enables the fungus to establish inside the host and accumulate biomass prior to the necrotrophic phase (Veloso and van Kan, 2018). It was demonstrated that *B. cinerea* Dicer-like proteins DCL1 and DCL2 are able to cleave long dsRNA molecules into sRNAs that are produced from fungal hyphae and translocated to the plant cell where they interfere with the host RNAi mechanisms to silence host immune response genes in Arabidopsis and tomato leaves (Wang *et al*., 2017b; Weiberg *et al*., 2013). *B. cinerea* can also secrete oxalic acid that lowers the pH of the host tissues, stimulating the production and activity of fungal enzymes like pectinases, laccases, and proteases (Fernández-Acero *et al*., 2010; Manteau *et al*., 2003; Prusky and Lichter, 2007; Sharon *et al*., 2007). Furthermore, oxalic acid accumulation leads to Ca^{2+} chelation, which in turn weakens the pectin structures of plant cell walls and inhibits the deposition of callose (Chakraborty *et al*., 2013).

Problem statement

Current strategies used to control grey mold in strawberries rely on preventive applications of fungicides from multiple chemical groups, i.e., succinate de- hydrogenase inhibitors (SdhI), the quinone outside inhibitors (QoI, suppression), hydroxyanilides (Hyd), anilinopyrimidines (AP), and phenylpyrroles (PP), for grey mold control in worldwide. But scientifically contagious persist regarding their safety. The repeated use of pesticides is causing a growing increase in the negative perception on the part of the public opinion regarding their safety of use, environmental contamination, and residuality in the final products.

Many types of research about GMOs have been conducted to find the solution for this issue, typically, Host-Induced Gene Silencing (HIGS) is a dominating approach to combat mold and pests. Alternatively, Spray-Induced Gene Silencing (SIGS), a GMO-free method, gets increasingly significant attention.

Albeit, RNAi has been discovered recently, there is an increasing number of both academic and private research investing this genetic approach to exploit several unique features which offer additional opportunities for food security such as enhancing the nutrient value of food, minimizing waste due to mold, and pest damages. Notwithstanding great quantities of benefits, customer acceptance is still an important hurdle to the application of intrinsic-RNAi gene expression on food (i.e., host-induced gene silencing, HIGS). Topical applications, GMO-free, (e.g., spray-induced gene silencing, SIGS) have exceptionally stood out of the crowd as an innovative technology to sweep this obstacle aside**.**

Objective

The main goal of this thesis is to assess the capacity of new naked and formulated dsRNA sequences in the pre-and postharvest control of *Botrytis cinerea* infection in strawberry plants grown in two cultivation systems: open field in soil conditions and soil-less protected cultivation. Furthermore, from the environmental view, this GMO-free-driven research shall provide some beneficial information that could be applied further in the studies of this field to break down the worldwide applications of chemical fungicide and herbicide in a scenario with the ever-increasing population demanding a tremendous amount of safe and healthy food.

Expected Outcomes

- Identification of the most efficient dsRNA sequences and formulations for the control of *B. cinerea* infection in the two different strawberry cultivation systems.
- Compare the efficacy of dsRNA sequences and formulates with standard chemical and

biological pesticides used for strawberry cultivations.

- Identify the level of protection afforded by dsRNA sequences and formulates on detached strawberry fruits in post-harvest conditions.
- Evaluate the effect of dsRNA treatments on fruit sensorial and quality.

2. LITERATURE REVIEW

2.1 Open field and soil-less cultivation system

Strawberry is a seasonal crop product, hence, in the past, they were commercially traded on a specific period in the year depending on natural environmental conditions. Nevertheless, the European strawberry market is very large and the consumer demand has increased a lot all across the year (Mezzetti and Giampieri, 2018). To cover this demand, many EU also non-EU companies have made an enormous effort to expand the cultivation areas together with appropriate cultivars, fit to different climates (from the north toward the south) and with varied cultivation systems (open field, protected, soil-less). The open-field system is traditionally exploited to grow strawberry plants, provided that the soil disinfestation was applied in advance; nonetheless, chemical pesticide residue in the soil can affect groundwater and rivers nearby and in combination with the presence of soilborne diseases makes the farmer start to find another approach for strawberry cultivation, for example through the application of soilless systems. Suitable mixture and material properties of substrate in soil-less culture within greenhouse systems, extend harvesting duration, out of season strawberry production, and increase in yield, exhibiting direct and indirect effects on plant physiology and production (Takeda, 1999; Cantliffe et al., 2001). To facilitate the growth of strawberry plants, different substrates such as peat moss, coconut coir, perlite, rock wool, and pine bark have been used. However, peat has been the best substrate for hydroponic culture (Lieten, 2001). There are different reports related to the use of zeolite and perlite as substrates in hydroponic culture (Maloupa et al., 1999). Zeolites are crystal alumina silicate that has a negative charge, which is balanced by one or two valences positively charged cations with other properties including high absorption level, water retaining and releasing, high cation exchange capacity (CEC), and high buffering against to change of pH (Allen & Ming, 1995; Mumpton, 1999). Ghazvini in 2014 conducted the mixture of Perlite/zeolite (P/Z) substrates 3:1 and 1:1 ratio (v/v) for the soilless system. The results reported that the mixtures allowed to produce the highest fruit number per plant with 22.23 and 23.05 fruits, respectively, while zeolite alone showed the lowest fruit number.

2.2 Use of fungicides to protect strawberry cultivation against grey mold

Botrytis is one of the most devastating fungi affecting strawberries due to their relatively scarce firmness, facilitating the symptoms mostly at the harvesting state, although the infection happens during the cultivation. Typically, fungicides are applied regularly throughout the flowering stage until harvesting. The best-managed applying technique is integrated disease management (IDM), which involves cultural techniques that reduce canopy humidity and high

synthetic fungicide application during the cropping season (Rosslenbroich & Stuebler, 2000). Even though IDM is suggested, chemical fungicides applications represent the main method for controlling this pathogen, probably the sole choice in some cases.

Until recently, the use of synthetic fungicides for plant protection was thought to be fairly safe. However, it was reported that its continuous use faces three major challenges namely: (1) increased public concern about contamination of fruits and vegetables with residues from synthetic fungicides and its effect on human health; (2) increased resistance development in pathogen populations and (3) environmental pollution (Abbey *et al.*, 2018). Many of the alternative compounds to fungicides are non-toxic for human health and the environment, characterized by antimicrobial activities against the main postharvest pathogens that cause fruit rot, or they are resistance inducers that activate the plant defenses, to reduce the presence or the aggressiveness of a pathogen such as chitosan, laminarin (Feliziani, et al., 2015).

2.3 Sustainable approach for plants protection

Owing to the aforementioned shortage of chemical pesticides on plants and further to long-term environmental surroundings as well as human health. The Green Deal of the European Commission has established a new strategy, Farm-to-Fork, the aim of which is to assure a more sustainable and food-secure society. This Green Deal's pillar aims to include a reduction in agrochemical inputs, such as pesticides, fertilizers, and antimicrobials, to achieve greater sustainability and health, and reduce loss of biodiversity while ensuring continued crop protection. It envisages various practices that promote lesser pesticide usage, such as integrated pest management (IPM), and the use of precision agriculture and artificial intelligence (Nji *et al.*, 2021). Sustainable agricultural practices are those that fulfill these criteria: applicable for long time periods, preserve natural resources, protect the natural environment, and protect human health (Király, 1996). Pesticide utilization apparently is in contrast with these criteria due to their harmfulness to the climate and to human wellbeing. In 2009, the Commission additionally approved a directive on sustainable pesticides, called ‘Sustainable Use Directive’ (SUD). In accordance with SUD, EU countries needed to create and carry out public activity plans for the decrease of pesticide volumes and hazards. Outstandingly, the European pesticide and biopesticide makers' association (European Crop Protection Association; ECPA) committed to help €14 billion interest in the improvement precision agricultural techniques for the more targeted (consequently diminished) use of pesticides, and of natural biopesticides (Nji *et al.*, 2021). Concurrently, the United Nations Environmental Program (UNEP), other global and public associations, and organizations are proactively seeking hazard declining for 'exceptionally dangerous pesticides', encouraging the bio-based, environmental, and health-friendly products with high specificity for the targeted pathogen and affordable costs.

Exceptionally, RNA interference approach has stood out of the crowd recently thanks to its high specificity.

2.4 RNA interference (RNAi).

Most of the gene silencing technologies are based on RNA interference principles; RNA interference (RNAi) is a group of mechanisms using small RNA molecules, mediated by double-stranded RNA (dsRNA) that can suppress protein expression through targeted destruction of mRNAs. The description of RNAi in *Caenorhabditis elegans* by Andrew Fire and Craig Mello earned them the Nobel Prize for Medicine in 2006 (Fire et al., 1998; Nobel Media AB, 2017). The discovery of RNAi revolutionized our understanding of gene regulation by revealing an array of related pathways in which small, approximately 20-30 nucleotide noncoding RNAs and their associated proteins control the expression of genetic information (Carthew et al., 2009). RNAi controls vital processes such as cell growth, tissue differentiation, heterochromatin formation, and cell proliferation. The mechanism utilizes short interfering RNAs (siRNAs) to guide the targeted degradation of transcripts using sequence homology (Torres-Martínez & Ruiz-Vázquez, 2017). RNAi was initially discovered in the form of a single microRNA (miRNA) in the *Caenorhabditis elegans* genome (Lee et al., 1993). Eventually, they were found to be widespread: Around 5% of the human genome is dedicated to encoding and producing the >1,000 miRNAs that regulate at least 30% of our genes (Jinek et al 2009; MacFarlane et al., 2009). RNAi has great potential against invading pests and pathogens (Eamens et al., 2008; Martínez de Alba et al., 2013). So far, conventional RNAi applications have been largely based on the use of recombinant viruses (virus-induced gene silencing), *Agrobacterium tumefaciens*-mediated transiently expressed transgenes, and stably transformed transgenic plants that enable the production of dsRNA molecules against selected targets (host-induced gene silencing; Baulcombe, 2004, 2015). The first commercially approved, transgenic plants carrying RNAi constructs against corn rootworm (*Diabrotica virgifera virgifera*) and bean golden mosaic virus were approved for cultivation in the USA and Brazil, respectively (Tollefson, 2011; United States Environmental Protection Agency, 2017). However, despite their demonstrated success, RNAi-based transgenic crops have not been commercialized as much as one might have expected (Dalakouras *et al.*, 2020). The process of RNA interference (RNAi) can be mediated by either siRNA or miRNA, but there is minor variation between them.

MicroRNA is single-stranded and comes from endogenous non-coding RNA, found within the introns of larger RNA molecules. The binding between miRNA with mRNA is imprecise; hence it can target hundreds of endogenous messenger RNAs. Micro RNA is capable of

shutting down genes by inactivating the messenger RNA, which is necessary for transforming the genetic information in protein synthesis that can be carried out either by repressing translation or degrading the mRNA. In contrast with miRNA, small interference RNA (siRNA) derived from longer double-stranded RNAs (dsRNA) that come from experimentally exogenous sources or from the cell itself. It's matching with mRNA is perfect; thus, it can only bind to one specific target.

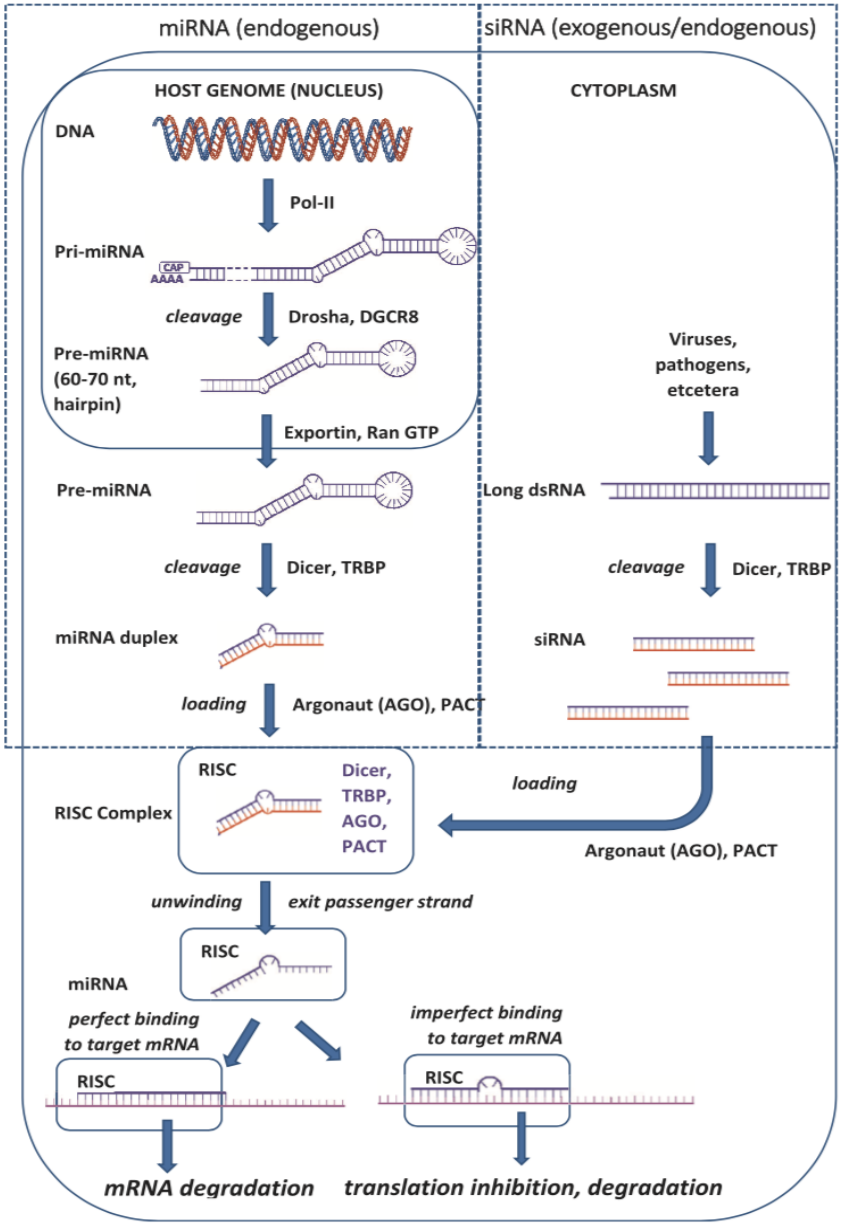

Figure 1:Schematic representation of miRNA and siRNA pathways towards RNAi-based gene silencing in plant cells (Kleter, 2020)

The siRNA pathway (right) begins with Dicer's cleavage of dsRNA of exogenous or nuclear origin. The resulting siRNA duplex is loaded onto Argonaute by the RISC-loading complex, which comprises Dicer, a dsRBP protein such as TRBP, and an Argonaute protein. The

passenger strand is cleaved and ejected. The guide strand remains bound to Argonaute, forming the RISC. The RISC binds to complementary target sequences (black) and silences them via the slicing activity of Argonaute. In the miRNA pathway, a primary miRNA transcript is cropped by the microprocessor complex. The resulting pre-miRNA is exported to the cytoplasm, where dicing and RISC loading takes place. Passenger strand ejection may take place and the guide strand drives silencing activity by the action of the mature RISC; the secondary siRNA following mRNA degradation by endonucleolytic activity can be recycled back to form RISC for further silencing. In the case of miRNA(left), gene silencing can be carried out by either mRNA degradation or preventing ribosomes from binding with mRNA (Wilson and Doudna, 2013). Because this sequence-dependent mode of action depends upon Watson–Crick base-pairing interactions, the RNAi machinery can be both flexible and exquisitely specific, also unique in selectivity and efficiency compared to other conventional agrochemicals (Hannon, 2002; Arpaia *et al.*, 2020). Thus, this regulatory paradigm may have been adapted and adopted for numerous cellular functions. Furthermore, products using the RNAi mode of action can be designed to selectively target the expression of specific genes or groups of similar gene sequences in a targeted species for which they are developed while leaving other non-target organisms unaffected (Arpaia *et al.*, 2020). As such, RNAi has gained remarkable prominence among researchers as a strategy of choice for improving crop yield, for generating plants with novel traits (PNTs), for post-harvest protection, and for managing weeds, other pests and diseases caused by bacteria, fungi, and viruses (Zotti et al., 2018). Being a conserved mechanism in eukaryotes, RNAi has also been harnessed on the animal, i.e., adult mice. In 2002 Essner and co-workers used reporter mice for bioluminescence imaging experiments designed with a luciferase enzyme to examine the influence of RNAi in mice liver. The results indicated that small interfering RNA-mediated led to inhibition of luciferase expression in adult mice. Small interfering RNAs (siRNAs) mimic intermediates in the RNA-interference (RNAi) pathway and can silence genes in somatic cells without activating nonspecific suppression by double-stranded RNA-dependent protein kinase (Elbashir et al., 2001). Given the fact that RNAi is easy to apply, whole-genome screens by RNAi may become a common method of choice in the near future. RNAi may facilitate drug screening and development by identifying genes that can confer drug resistance or genes whose mutant phenotypes are ameliorated by drug treatment, providing information about the modes of action of novel compounds (Agrawal *et al.*, 2003)

2.5 Virus-induced Gene Silencing (VIGS)

A typical breakthrough of Biotechnologies is **V**irus-**i**nduced **G**ene **S**ilencing (VIGS), a plant RNA-silencing technique, that uses viral vectors carrying a fragment of a gene of interest to

generate double-stranded RNA, which initiates the silencing of the target gene (Burch-smith, Schiff and Liu, 2020). In the past, plant biologists relied almost exclusively on forward genetics; that is, the identification of a mutant and the subsequent cloning of the mutated gene to identify the sequence responsible for the process being investigated. According to Sibille and Edgar in 2010, forward genetics is the identification of the genetic cause of an altered or abnormal phenotype introduced by chemical mutagenesis or mutation by irradiation (e.g., phenotype-genotype); in contrast, in reverse genetics, a particular gene is altered, and the phenotype is investigated (e.g., genotype-phenotype). Reverse genetics examines the function of a gene or DNA sequence directly by altering the expression of the sequence of interest and then identifying the produced mutant phenotype. Most reverse genetics approaches described in plants to date rely on posttranscriptional gene silencing (PTGS; Watson et al., 2005). PTGS is a mechanism that degrades specific messenger RNAs and thereby reduces the expression of a specific gene. It is also described as quelling in fungi (Cogoni et al., 1996) and RNA interference in animals (Fire et al., 1998), involving the sequence-specific degradation of RNA. VIGS takes advantage of this defense system to silence endogenous RNA sequences that are homologous to a sequence engineered into the viral genome, which generates the double-stranded RNA that mediates silencing (Burch-smith, Schiff and Liu, 2020). The large part determination for VIGS vectors is dependent on RNA infections that can infect a few plant categories utilized in scientific examinations. The most widely used VIGS vectors are based on the Tobacco rattle virus (TRV; Ratcliff et al., 2001; Liu et al., 2002b), that have been used to silence genes in a number of Solanaceous plant species, usually mediated by *Agrobacterium tumefaciens* with the VIGS vector placed between T-DNA borders, including *Nicotiana benthamiana* (Ratcliff et al., 2001; Liu et al., 2002b), tomato (Liu et al., 2002a), pepper (*Capsicum annuum*; Chung et al., 2004), potato (*Solanum tuberosum*; Brigneti et al., 2004). One distinct advantage of using TRV for VIGS is the ability of the virus to infect the meristem of its hosts (Ratcliff et al., 2001) and it has been used to study flowering in *N. benthamiana* (Liu et al., 2004) and petunia (Chen et al., 2004), in addition to fruit development in tomato (Fu et al., 2005).

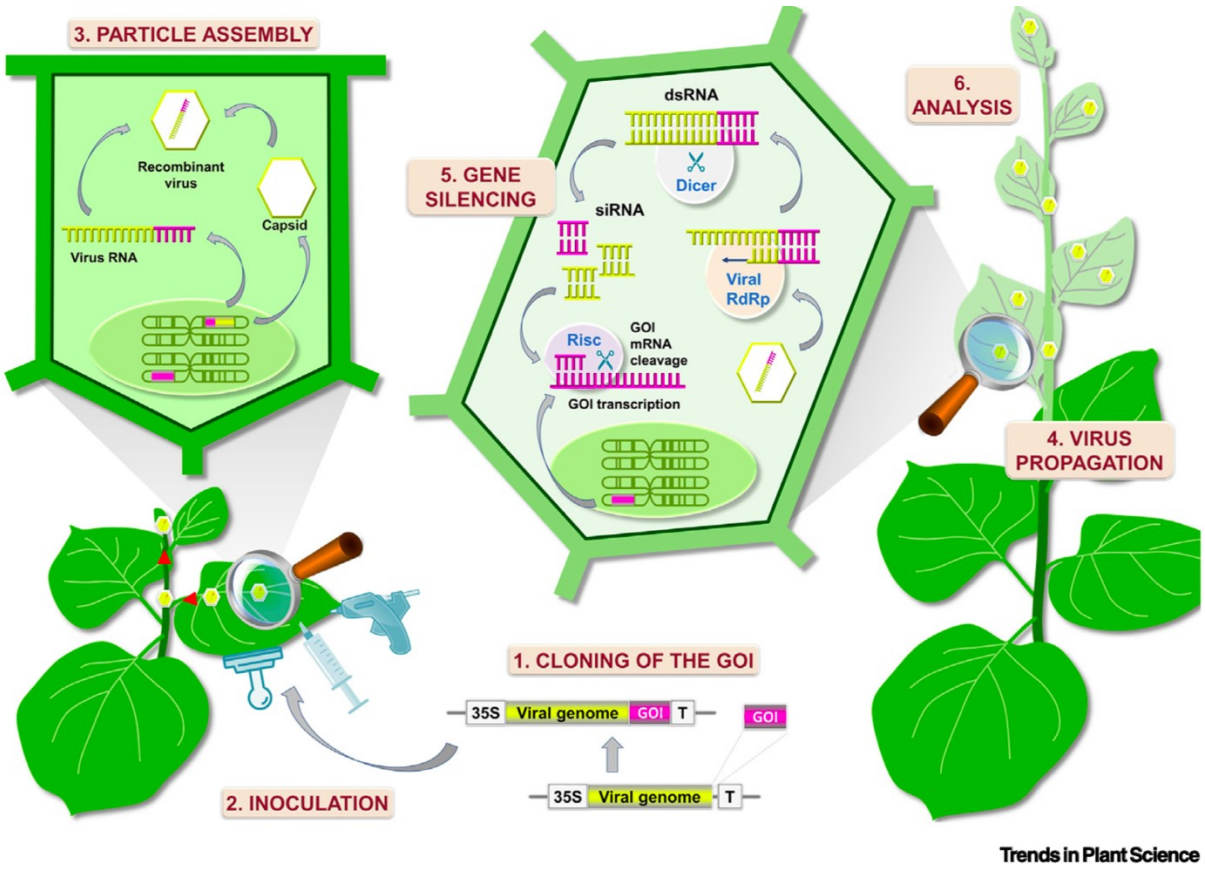

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Figure 2: Virus-induced gene silencing (VIGS) silences a gene of interest (GOI) (Courdavault et al., 2020).

A GOI fragment is cloned into a construct containing viral genome sequence (1) and then inoculated into plants (2), where viral particles are produced (3). Through cell-to-cell movement using plasmodesmata and systemic progression via the phloem (4), viruses infect newly developing leaves and replicate. This involves the formation of dsRNA that is cleaved by plant DICER to release siRNA. siRNAs are loaded in the multiprotein complex RISC and their antisense strand screens RNA in cells. Hybridization of this complex results in specific RNA degradation (or translation repression) of viral RNA and endogenous GOI transcripts causing its silencing (Courdavault *et al.*, 2020).

Besides RNA viruses, DNA viruses have also been applied as VIGS vectors. One of the more interesting of these is derived from the bipartite Cabbage leaf curl geminivirus (CbLCV) to perform VIGS in the model plant species Arabidopsis (Arabidopsis thaliana; Turnage et al., 2002). Nevertheless, this vector has seen limited use for VIGS in Arabidopsis. This may probably be due in part to the difficulty in introducing the VIGS vector into the plant through particle bombardment (Turnage et al., 2002).

2.6 Host-induced Gene Silencing (HIGS)

Plants naturally develop a defense system, based on the RNA silencing machinery, to defend against invading viruses (Csorba et al., 2009; Harvey et al., 2011; Hu et al., 2011). This feature has been utilized to develop **H**ost **I**nduced **G**ene **S**ilencing technology (HIGS) to control other plant pathogens (Guozhong et al., 2006), further development of virus-induced gene silencing (VIGS) (Harvey et al., 2011). HIGS is an innovative concept of RNAi technology for efficient management of plant pathogens, employed to silence one or a few of the important pathogen genes that are necessary for growth, development, and pathogenicity or host genes required for infection (Ghag, 2017). Genomic and biochemical analysis shows that the RNAi protein machinery is conserved in eukaryotes and thus, it is most likely that the trans-gene siRNAs generated and processed in plants can efficiently lead to sequence-specific degradation of the pathogen mRNA in its cytosol (Obbard et al., 2009; Shabalina et al., 2008; Weiberg et al., 2015). HIGS is an RNAi-based process where small RNAs produced in the transgenic plant silence the genes of the pests or pathogens that attack the plant (Fig. 5). The dsRNA can also be experimentally introduced into the plant cells with *Agrobacterium* or viruses that replicate through dsRNA (Qi *et al.*, 2019).

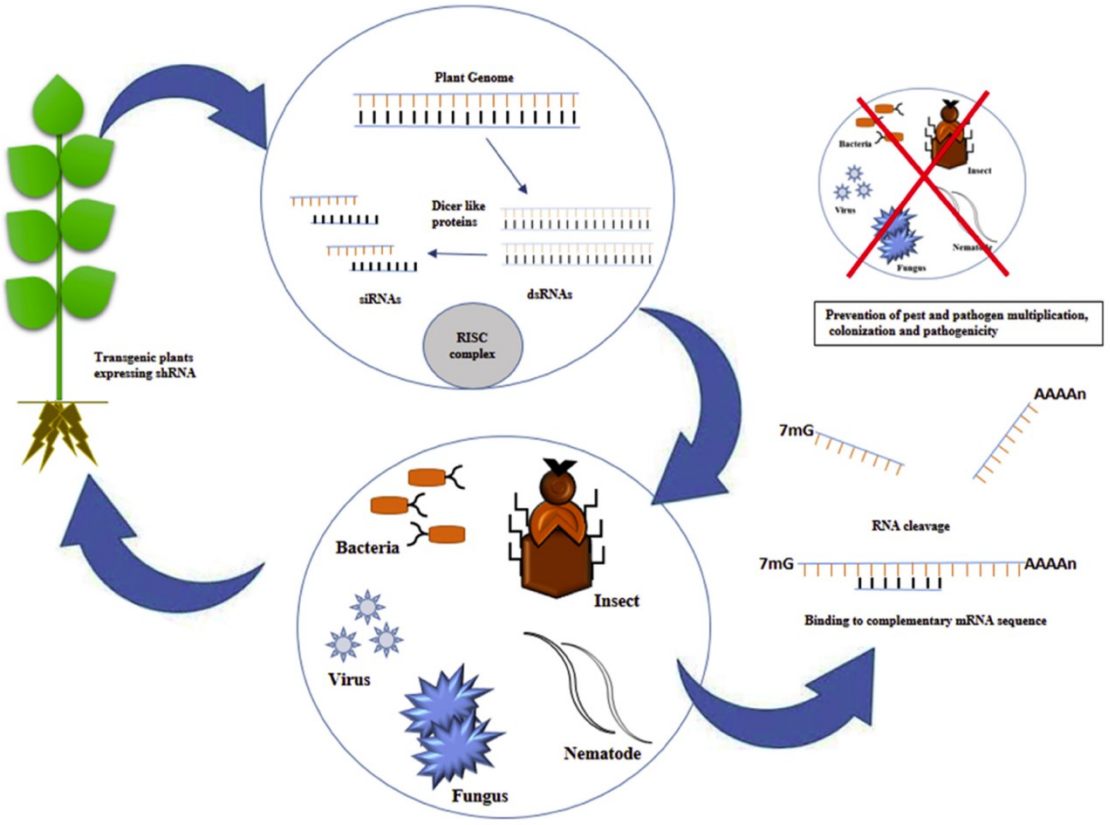

Figure 3: HIGS mechanism in the plant. A transgenic plant produces siRNAs and dsRNAs which specifically target pathogen or pest genes (Ghag, 2017).

HIGS against Pests

Gene silencing effect of siRNA in transgenic plants protect plants from hijacking by targeting the specific gene in aphids and other parasitic pests that threaten crops that have been proven to be effective in some fields such as *Bacillus thuringiensis* (Bt) has been successfully used for the management of cotton insect pests (Baum et al.,2007). Four targets from 66 unigenes were isolated for RNAi against grain aphids in wheat (Wang et al., 2005). In addition, five potential RNAi targets have been identified from 5490 unigenes of grain aphids in wheat (Zhang et al., 2003). The silencing of the chitin synthase gene prevented the insects from hatching (Arakane et al., 2008), and from knocking down the segmentation gene, hairy, which prevented insect feeding (Aranda et al., 2008). The silencing of the essential genes (e.g., ecdysone receptor (EcR) and ultraspiracle protein (USP)) in grain aphids (*Sitobion avenae* F.) reduced its survival and fecundity rate, providing a persistent and transgenerational method for improving wheat resistance (Yan et al.,2016). Transgenic wheat expressing dsRNA to knock down the CarE gene (CbE E4) of *Sitobion avenae* delayed larval growth and reduced resistance to pesticides (Xu et al., 2014). Thanks to the heritability of DNA, dsRNA in transgenic plants are also heritable (Qi *et al.*, 2019), thusly the control of crop diseases would be continuous in many generations until the pests have developed a new mechanism to be against that features.

Additionally, several potential RNAi targets triggered by oral feeding or injection in the grain aphid *Sitobion avenae*, can be: genes encoding catalase, acetylcholinesterase1, cytochrome c oxidase subunit VIIc precursor, zinc finger protein, secreted salivary peptide DSR32, salivary protein DSR33, serine protease 1 DSR48, and olfactory co-receptor (Yu et al., 2016). The genes actively expressed in the gut tissues could be the best targets since dsRNAs are ingested by the insects and are exposed only to gut tissues and associated organs. (Ghag, 2017). Three genes namely, the hexose transporter gene (NlHT1), the carboxypeptidase gene (Nlcar), and the trypsin-like serine protease gene (Nltry) from a Hemipteran insect brown planthopper (*Nilaparvata lugens* Sta^{o}l) were tested for RNAi-based resistance in rice plants. Nymphs fed on transgenic rice targeting the aforementioned genes showed decreased transcript levels in the midgut tissues however no lethal phenotype was observed (Zha et al., 2011). In yet another study, knockdown of Rack-1 and MpC002 reduced aphid fecundity, but not survival; Rack-1 gene in aphid *Myzus persicae* is expressed in the gut, and MpC002 is mostly expressed in the salivary glands. Aphids fed on tobacco and Arabidopsis plants expressing MpC002 and Rack-1 dsRNAs exhibited a 60% reduction in the mRNA levels (Pitino et al., 2011). Since the strong digestion and working in the guts and associated organs, hence dosages of dsRNA must be

considerably bioavailable.

HIGS against Nematodes

The damage caused by nematodes hinges mainly on the migratory or sedentary phases of this species (Qi *et al.*, 2019). The yield losses of wheat in the presence of nematodes are caused mainly by cyst nematodes (*Heterodera* spp.), which also threaten the production of barley (Bernard et al., 2017). RNA interference in several nematode species (e.g., cyst nematodes, root-knot nematodes, root-lesion nematodes, and other ectoparasitic nematodes) has been explored through feeding, soaking, injection, or in planta delivery (Lilley et al., 2007, Tan et al., 2013; Joseph 2012). The knockdown of the expression of pat-10 and unc-87 of *Pratylenchus thornei*, which attacks the wheat roots, reduces reproduction by 77–81% (Tan et al., 2013). The phenotypic effects of these RNA interference experiments were commonly a reduced number of established nematodes or an increase in the male population, which indicates that juveniles experience adverse conditions (Lilley et al., 2007). The first successful application of HIGS in 2006 was used to confer nematode resistance by protecting the host from infection (Yadav et al., 2006). This research offered a firm platform to inhibit nematodes via the RNAi approach.

HIGS against Viruses

Until recently, no technologies have been able to cure virus-infected plants, and the best way to restrict the spread of infections is the chemical preventive control against vector organisms (e.g., insects) and the eradication of the infected hosts. The choice of resistant cultivars has been the best arrangement, but natural resistance is not sufficient. HIGS methodology against plant infections is a significant innovation as pervasive in the previous few years. Effective resistance was proven in transgenic plants against plant viruses having RNA genome as compared to DNA virus (Ghag, 2017). Two intron hairpin RNA (ihpRNA) constructs namely ihpRNA-Rep (containing the partial coding sequence of the viral master replication initiation protein) and ihpRNA-ProRep (containing Rep partial coding sequence along with its 50 upstream regulatory regions) were separately transformed into banana cells. Transgenic banana plants were completely resistant to the banana bunchy top virus (BBTV) infection up to 6 months post-inoculation with viruliferous aphids (Shekhawat et al., 2012) with the Rep gene that can be recognized as the best target for RNAi because of complete BBTV resistance (Elayabalan et al., 2013). Recently, a study demonstrated hpRNA silencing approach in Tomato yellow leaf curl virus-Oman (TYLCV- OM), which is a DNA virus. Around eighty-nine percent of the tomato transgenic lines challenged with TYLCV-OM were symptomless

(Ammara et al., 2015).

HIGS against Fungi

Fungal pathogens are the major cause of the loss of yield throughout the globe. The pervasiveness of RNAi in general, RNAi-based HIGS in particular, is an emerging technology to prevent this nature-based waste as the resistant gene naturally present in the plant is not always efficient working due to the secondary metabolite-toxin and the evolution of strains. This approach has been widely conducted. A study concerning the powdery mildew of barley caused by *Blumeria graminis* f. sp. hordei and wheat caused by *B. graminis* f. sp. tritici has been carried out by Nowara et al. (2010); the result showed that dsRNA targeting the avirulence gene Avra10, which is recognized by the resistance gene Mla10, significantly reduced fungal development in the absence of Mla10, and the silencing of 1,3-b-glucanosyltransferases (BgGTF1 and BgGTF2) reduced the early development of the pathogen. Yin et al. (2010) established a Barley stripe mosaic virus (BSMV)-induced gene silencing system to knock down *Puccinia striiformis* f. sp. tritici (Pst) genes. BSMV-HIGS provide a way to analyze the function and to screen RNAi targets for the control of rust diseases. These experiments provide evidence of the idea that HIGS is a successful methodology for controlling contagious small grain diseases, laying the foundations for trials on the open field.

HIGS against bacterial pathogens

Bacteria do not have complex genome machinery like eukaryotes to counteract external genes but possess a similar pathway for silencing foreign DNA. Successful examples are from silencing transcripts in the host cells, even if they came from the bacteria. Crown gall disease, caused by *Agrobacterium tumefaciens* transferring 2- monooxygenase (iaaM), indole-3-acetamide hydrolase (iaaH), and isopentenyltransferase (ipt) genes into the plant genome result in the formation of gall tumors leading to significant losses in ornamental and horticultural plants (Escobar et al., 2003; Khmel et al., 1998; Morris et al., 1986). The first study demonstrating RNAi-based resistance against plant bacterial pathogen was published by Escobar et al. (2001). In this study, two self-complementary sequences of iaaM and ipt genes were inserted in the RNAi construct driven by CaMV 35S promoter to transform Arabidopsis and tomato plants. Under *in vitro* conditions, when the transgenic plants were treated with pathogenic isolates of *Agrobacterium*, the plants displayed resistance to crown gall disease. Further, this strategy was extended to develop crown gall resistance in English walnut (*Juglans regia* L.) (Escobar et al., 2003). Transgenic walnut plants expressing the same construct

designed by Escobar et al. (2001) showed the presence of specific siRNAs targeting the iaaM and ipt genes of Agrobacterium which suppressed tumor formation in these plants when inoculated with the virulent *Agrobacterium*.

HIGS against parasitic plants

It has been more than 10 years since the discovery of the ability of small mobile RNAs could be transmitted from silenced rootstocks to non-silenced scions (Brosnan et al., 2007; Molnar et al., 2011; Palauqui et al., 1997). These molecules are transported via the vascular tissue which conjoint after a successful grafting procedure, indicating that mobile RNAs can also traffic between the two divergent plant species (Westwood et al., 2007, 2009). Parasitic plants directly attack the host plant vasculature to draw out water, minerals, carbohydrates, and other vital nutrients. HIGS strategy can be an effective strategy that can kill the parasitic weed population by targeting the genes essentially required for parasitism. Attempts have been made in the past to employ HIGS to control major parasitic species from the Orobanchaceae and dodder species (Runo et al., 2011). One of these is the HIGS approach for controlling maize parasitic *Striga asiatica*. Some growth anomalies have been observed in Striga-parasite and almost no resistance was seen in transgenic maize plants. The authors speculated that the selected gene targets were not appropriate for RNAi or the interaction of maize and Striga is comparatively different from other host-parasite interactions (Framond et al., 2007). Mannose 6-phosphate reductase (M6PR) is a key enzyme required for *Orobanche* development. HIGS-based transgenic tomato showed a significant reduction in mannitol levels, reduced M6PR transcripts, and increased percentage of dead *O. aegyptiaca* tubercles (Aly et al., 2009). These trials show the accomplishment of HIGS innovation can defend hosts from parasitic plants, although further studies are needed.

Current Challenges of HIGS

The effectiveness of HIGS depends on sufficient supply and the perfect transportation of siRNA between the two host-pathogen/pest organisms. HIGS can't be utilized inappropriately against necrotrophic organisms since they uptake substances and different metabolites from dead host tissue, which couldn't supply adequate measures of siRNAs. Off-target effects are likewise significant issues requiring avoidance when transgenic plants are constructed. Also, customer acceptance is a far most considerable factor hindering the industry-scaled advancement.

2.7 SPRAY INDUCED GENE SILENCING (SIGS)

As the ever-increasing biotechnology applied in food is accompanied by the poor-consumer acceptability on GM crops, Spray-Induced Gene Silencing (SIGS) strategy is GM-free, having the potential to control pathogen and other kinds of threat on plants. Since as a GM-free approach, it does not depend on the transgene or recombinant virus still being capable of activating RNAi through the exogenous application of dsRNA molecules targeting the gene of the pathogenic organism (Fig. 6). The fungal pathogen directly takes the dsRNAs up and induces the fungal RNAi machinery, and/ or the host plant takes dsRNAs up first, induces the plant RNAi machinery, and then dsRNAs or siRNAs are transferred into fungal cells and induce the fungal RNAi machinery (Sang and Kim, 2020). Thus, this approach silences the pathogen's gene without introducing heritable modifications into the plant genome (Koch et al. 2016; Wang and Jin 2017; Cagliari et al. 2018).

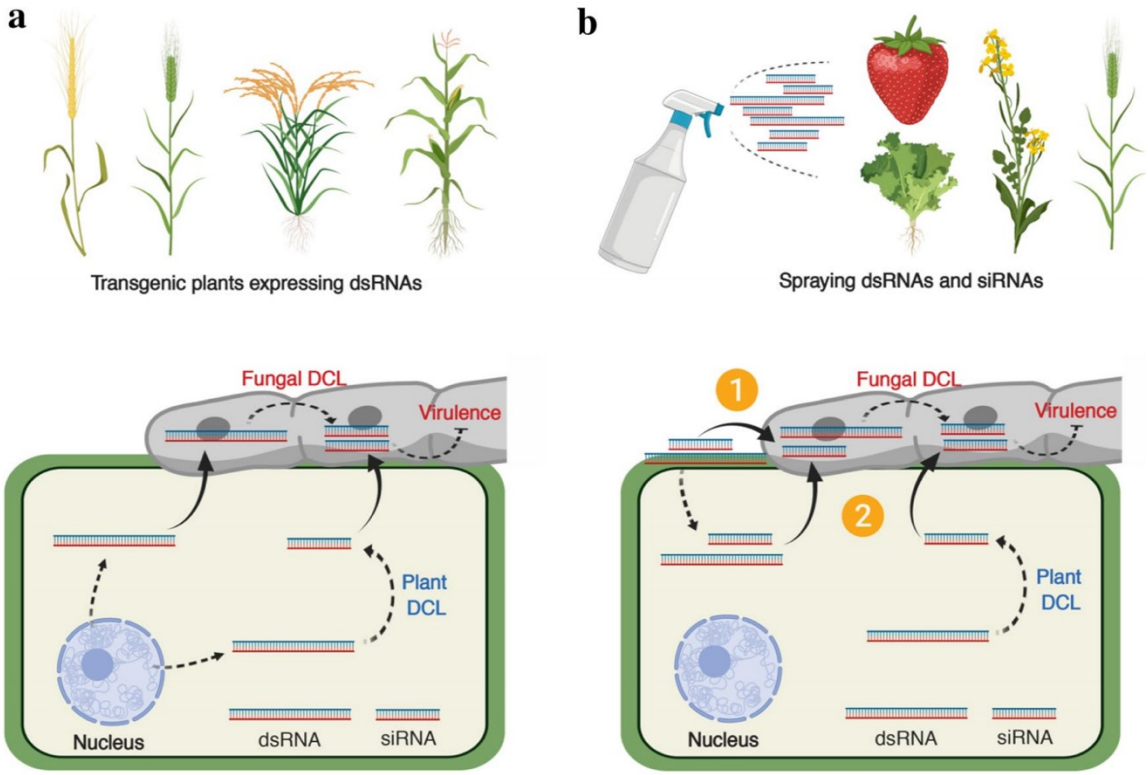

Figure 4: HIGS & SIGS. a, Host-induced gene silencing (HIGS) and b, spray-induced gene silencing (SIGS) for crop protection against fungal pathogens (Sang and Kim, 2020

The most critical factors that could affect the efficiency of exogenous dsRNAs are the delivery method used and the absorption capacity of different plant organs, e.g., leaves, petioles, buds, roots, stems, and seeds. According to Dalakouras et al. (2018), exogenously applied siRNAs by high-pressure spraying onto plant leaves and buds triggered local and systemic RNAi, whereas delivery of siRNAs by petiole absorption and hpRNA by trunk injection failed to induce RNAi. The author also included that (i) In terms of delivery method, high-pressure

spraying is an efficient approach to deliver exogenous siRNAs into plant cells to induce RNAi; and (ii) When the absorption rate is concerned, leaf and bud spraying efficiently induce RNAi compared to petiole absorption or trunk injection. Another observation was obtained by Song et al. (2018) that dsRNA uptake was more efficient through the wounded surface than the healthy surface. The first report wherein exogenous RNA application into plants triggered RNAi of a plant gene was described in 2011 on *Nicotiana benthamiana* plants pretreated with the surfactant Silwet L-77 and sprayed (2.5 bar) with *in vitro*-transcribed 685-bp dsRNA and/or chemically synthesized 21-nt sRNAs targeting the endogenous phytoene desaturase mRNA which displayed extensive phytoene desaturase RNAi (Sammons et al., 2011). After this very first path, several other successors also went deeper into this field using diverse methods of RNA application. Numata et al. (2014) worked on *Arabidopsis* leaves infiltrated with 21-nt sRNAs fused to a positively charged carrier peptide that combined a copolymer of His and Lys, (KH)9 (18 amino acids), RNAi of the yellow fluorescent protein (YFP) transgene and the chalcone synthase endogene was recorded. Following studies indicated that RNA molecules can be absorbed by the roots and display biological activity throughout the plant. In 2014, Jiang et al. worked with SHOOT MERISTEMLESS (STM) which is a protein required for shoot apical meristem formation, whereas WEREWOLF (WER) is an R2R3-type MyB-related transcription factor expressed in the root epidermal cells. When STM or WER dsRNA conjugated to cationic fluorescent nanoparticle was applied to Arabidopsis seedling roots for five consecutive days, expression of STM and WER was suppressed and resulted in phenotypic defects (Jiang et al., 2014). In another study performed by Lau et al., in two consecutive years 2014 & 2015, instead of using *in vitro*-transcribed dsRNA, crude extracts of *Escherichia coli* HT115 expressing a 430-bp dsRNA targeting MYB1 were mechanically inoculated into the hybrid orchid (*Dendrobium hybrida*) flower buds. The results revealed that the application of MYB1 dsRNA changed the phenotype of floral cells, an outcome of great interest for floriculture biotechnology.

The production of dsRNAs utilizing *in vitro* transcription systems requires the utilization of a commercial kit which is quite expensive, only applicable for small-scale production. Additionally, plant cells contain an intense cellulose-rich cell wall ranging from 0.1 mm to a few micrometers in thickness representing an actual obstruction to biomolecule conveyance. To facilitate RNA delivery inside the plant cell, RNA molecules are usually conjugated to carrier compounds (Jiang et al., 2014; Numata et al., 2014). Recently, DNA nanostructures have been widely applied, instead of remaining in the mesophyllic apoplast, the nanostructure-conjugated sRNAs entered the symplast and silenced gene expression. Although carrier compounds significantly encourage RNA conveyance, they are quite costly and difficult to

synthesize. "RNAgri" agriculture industry created microbial fermentation innovation to fabricate dsRNAs at a bigger scope using a protein to tie the ideal RNAs, henceforth securing them against debasement. The final dsRNA items are viewed as more protected and stable to utilize than bare dsRNAs. It is assumed that approximately 2 to 10 g of dsRNAs are required per hectare of arable land, varying accordingly the target species' sensitivity to RNAi, systemic silencing capacity, and application method as well as delivery techniques (Das and Sherif, 2020). The *in vitro* dsRNAs production cost using nucleoside triphosphate (NPT) synthesis was nearly $12,500/g in 2008, but then decreased to $100 in 2016, and $60 today (Andrade and Hunter, 2016; Zotti et al., 2018; Dalakouras et al., 2020). More recently, to meet the high market demand, several industrial companies are now shifting to microbial-based production systems to manufacture dsRNAs at a larger scale and nearly at $ 2/g (Zotti et al., 2018; Dalakouras et al., 2020).

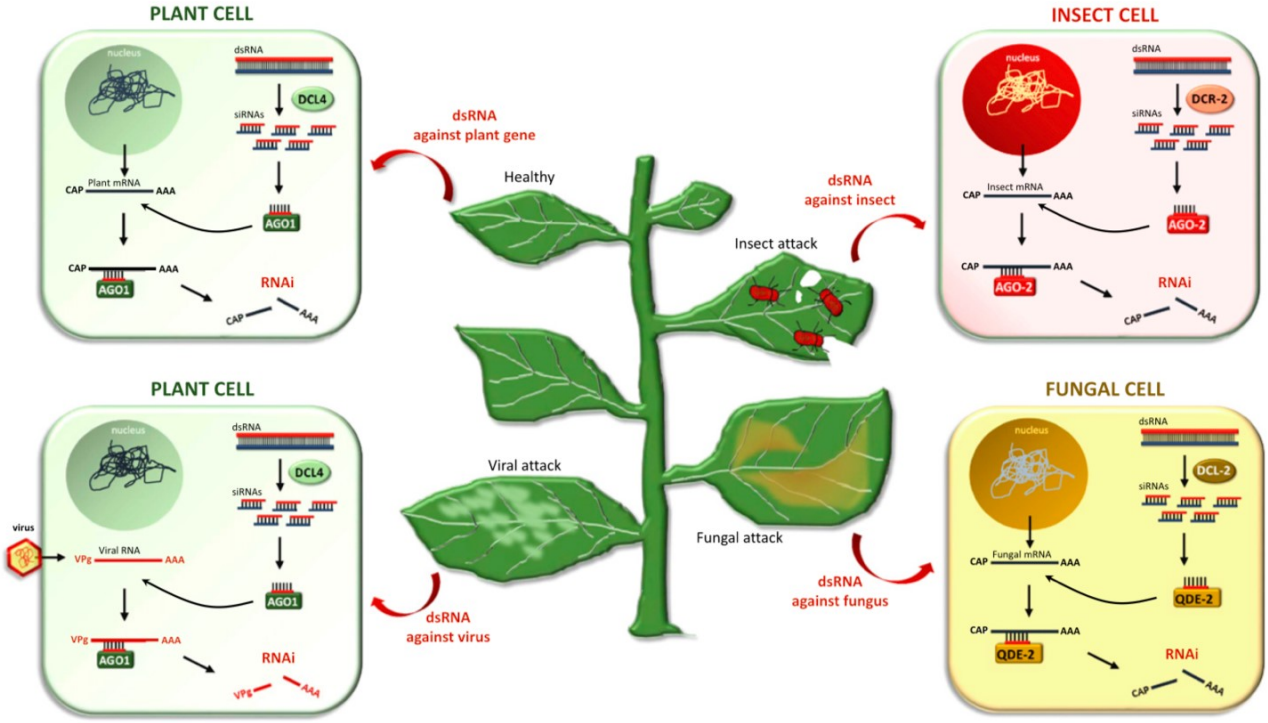

Figure 5: Exogenous application of RNA molecules into plants against various targets, such as endogenous plant genes, viruses, insects, and fungi. (Dalakouras et al., 2020)

In the case of plant genes and viruses, RNAi should take place inside the plant cell. Thus, the most suitable application method is high-pressure spraying, which allows symplastic RNA delivery. In contrast, in the cases of insects and fungi, RNAi takes place inside the insect and fungal cells, which thus need to uptake intact dsRNA (unprocessed by the plant DCLs) to achieve efficient RNAi. Hence, in these cases, trunk injection, petiole absorption, and/or low-pressure spraying (wherein RNA stays on the leaf surface) are the most suitable methods, because these methods do not result in symplastic RNA delivery (Dalakouras et al., 2020)

SIGS against viruses

Two common methods against the virus are a crude extract from *E. coli* and *Pseudomonas syringae*. In comparison between E. coli-expressed dsRNA, which may result in relatively low yields of fully duplexed dsRNA, the *P. syringae* system seems advantageous because the RNA-dependent RNA polymerase of phage phi6 converts single-stranded RNA templates into dsRNA with high processivity using a de novo, primer-independent initiation mechanism (Dalakouras *et al.*, 2020). This statement has been proved with wide arrays of applications i.e. in maize, upon spraying of crude extract from *E. coli* HT115 (DE3) expressing dsRNA targeting the sugarcane mosaic virus CP (Gan et al., 2010); in papaya (*Carica papaya*), upon mechanical inoculation of crude extract from *E. coli* M-JM109lacY expressing 279-bp dsRNA targeting the papaya ringspot virus CP (Shen et al., 2014); in orchid (*Brassolaeliocattleya hybrida*), upon mechanical inoculation of crude extract from *E. coli* HT115 (DE3) expressing dsRNA targeting the cymbidium mosaic virus CP (Lau et al., 2014); A significant issue involving these approaches is that dsRNA can only retain in the shortly protective period from 5–10 days, due to the eventual degradation.

SIGS against fungi

Many studies have been done to prove the effectiveness of SIGS on Fungi. Koch and coworkers demonstrated that spraying of barley (*Hordeum vulgare*) with *in vitro*-transcribed 791-bp dsRNA simultaneously targeting three genes (CYP51A, CYP51B, and CYP51C) strongly inhibited fungal growth (Koch et al., 2016), wherein dsRNA molecules were taken up directly rather than processing into siRNA. In another study, foliar application in *Brassica napus* of *in vitro*-transcribed dsRNA targeting various fungal genes conferred plant protection against *Sclerotinia sclerotium* and *Botrytis cinerea* (McLoughlin et al., 2018).

SIGS against insects

Activating RNAi in insects upon exogenous utilization of dsRNA in plants is a difficult undertaking. After the take-up by oral feeding, the dsRNA should endure the salivary nucleases in the midgut or potentially hemolymph which rapidly debases it. Next, the dsRNA must be taken up from the epithelial cells through either the endocytic pathway or the transmembrane Sid-1 channel protein-mediated pathway and processed by Dicer-2 into siRNAs, which will be loaded onto Ago-2 and trigger local RNAi (Dalakouras *et al.*, 2020). Several others following studies, exhibiting significant degrees of pest management upon exogenous application of *in vitro*-transcribed/synthesized RNAs: in tomato plants, upon application of dsRNA targeting the *D. virgifera* vacuolar ATPase (Ivashuta et al., 2015); in rice, upon root absorption of dsRNA targeting the brown planthopper *Nilaparyata lugens* P450

(Li et al., 2015); in maize, upon root absorption of dsRNA targeting the *Ostrinia furnacalis* KTI (Li et al., 2015); in potato (*Solanum tuberosum*), upon spraying of dsRNA targeting the Colorado potato beetle (*Leptinotarsa decemlineata)* actin; in tomato, upon petiole uptake of dsRNA targeting the tomato leaf-miner (*Tuta absoluta*) vacuolar ATPase (Camargo et al., 2016). Of note, and to improve dsRNA stability, uptake, and overall RNAi response, various insecticidal dsRNA formulations have been explored, such as liposomes, chitosan nanoparticles, cationic core-shell nano-particles, and guanylated polymers (Joga et al., 2016; Vélez and Fishilevich, 2018). Mites belong to the subphylum Chelicerata, a sub-group in the phylum Arthropoda together with the insects. Until recently, the only study wherein mites took up dsRNA that was exogenously applied in plants involved mechanical inoculation of tomato leaves with *in vitro*-transcribed dsRNA (Gogoi et al., 2017). However, in that case, it was not possible to determine whether the mite absorbed the applied dsRNA or the plant-produced siRNAs. In addition, no conclusions could be drawn concerning the RNAi action of the detected siRNAs, because the applied dsRNA had no mite target (Dalakouras *et al.*, 2020).

2.8 dsRNA formulation

For the reason of being highly perishable, hence, dsRNA is quickly degraded during performing activity, especially the highly alkaline environment of most insects such as *Lepidoptera* in general, exhibiting a very alkaline pH in the gut environment, are notorious for their strong and fast dsRNA-degrading capacity (Terenius et al., 2011; Garbutt et al., 2013). Many types of research have been investigated exhibiting that unmodified dsRNAs are very labile and won't aggregate or persist in the environment, due to the action of biotic (enzymatic degradation) and abiotic agents (UV rays, rainfall). A comprehensive series of environmental fate and degradation studies were performed in the soil, surface water, and sediment for the insecticidal DvSnf7 dsRNA expressed in MON 87411 maize (Dubelman et al., 2014; Fischer et al., 2017) with the results indicating that nucleic acids are rapidly degraded in soil and half-lives of less than 3 days in aquatic environments. However, the environmental fate of the active biomolecules, double-stranded RNA (dsRNA), is thought to be short-lived since dsRNAs are shown to be degraded in the soil in a matter of days not depositing in the groundwater, considered as big merit for using as a topical application (Dubelman et al., 2014). To create an efficient RNAi-based technique to counteract these economically very important pests or molds, formulations or complexions are needed to ensure the dsRNA against nucleolytic and environmental degradation and prolong the stability of the dsRNA long enough to allow sufficient uptake by the organisms.

Guanidine-containing polymers have previously been investigated in DNA delivery studies

with mammalian systems and have proved to be not only able to complex the nucleotides but also to improve the transfection efficiency due to the guanidine functionalities present (Funhoff et al., 2004; Choi et al., 2015; Guo et al., 2015). To enhance the stability of dsRNA and enhance cellular uptake, transfection reagents have been used to encapsulate dsRNA in cationic liposome complexes that slow down the degradation and increase the effectiveness of RNAi silence in fruit flies (Whyard et al., 2009; Deng F & Zhao Z., 2014) and in the German cockroach (Lin et al., 2017). Another alternative method of dsRNA delivery is the use of the chelating agent EDTA that can act as a protein inhibitor of the nucleases present in the saliva, as recent studies have been reported that EDTA may inhibit nuclease digestion of DNA in blood samples (Barra et al., 2015). Liposomes can be a protective vehicle of dsRNA, conducted by Yu-Hsien Lin, against the degradation that takes place in midgut juice when it is administrated orally in a German cockroach. This coating material would be taken up by gut epithelial cells because of the biocompatibility of liposomes. Probably, liposome-encapsulated dsRNA can meet the challenges of stabilizing dsRNA and allowing more exposure time in the field, and it appears to be a promising approach for pest control, at least for insect pests in the semi-protected (e.g. net houses) farms with limited space. (Lin, Huang and Belles, 2017). Yang Zheng and colleagues, considering the limitations of injection-, ingestion- and soaking-based delivery methods, introduced a fluorescent nanocarrier to establish a transdermal delivery system on aphids, which improved the dsRNA penetration within 4 h and RNAi-induced gene silencing through the topical application (Zheng *et al.*, 2019). To increase the dsRNA stability and thus prolong antiviral protection, Mitter and coworkers protected dsRNA molecules among layered double hydroxide clay nanosheets having an average particle size of 80–300 nm (BioClay; Mitter et al., 2017) to protect dsRNA significantly from nucleases, while the dsRNA/BioClay complex did not wash off, even after rigorous rinsing.

2.9 Cross-kingdom

Small RNAs (sRNAs), a class of regulatory non-coding RNAs around 20~30-nt long, including small interfering RNAs (siRNAs) and microRNAs (miRNAs), are critical regulators of gene silencing. Recently, accumulating evidence has indicated that sRNAs can be transferred not only within cells and tissues of individual organisms, but also across different eukaryotic species, serving as a bond connecting the animal, plant, and microbial worlds (Zeng et al., 2019). While RNAi has been discovered for more than 20 years, *cross-kingdom* sRNAs have only been reported quite recently (LaMonte et al., 2012; Buck et al., 2014; Zhang et al., 2016; Wang et al., 2016; Shahid et al., 2018; Hua et al., 2018). The movement of sRNAs inside the cell is critical for gene silencing regardless of the fact that they are exogenous injected into

or endogenous physiological response. Once triggered within a single-cell type, the RNA-silencing signal can move from cell to cell and over long distances to alter gene expression in cells/tissues (Sarkies et al., 2014; Brosnan et at., 2011; Palauqui et al., 1997). Cell-to-cell movement of sRNAs has been previously studied in plants, and it is likely that siRNA duplexes function as mobile silencing signals between plant cells (Dunoyer et al., 2010). There is a result, based on the studies of mammalian cells, of a transfer of siRNAs and miRNAs between cells and tissues. Moreover, sRNA transfer appears to be a process of active selection for potentially functional sRNAs, since mobile sRNA profile is usually distinguished from the total sRNA population within the cells. (Hoen et al., 2012; Colombo et al., 2014; Guduric et al., 2012). It has been recently noted that sRNA signals can be transmitted between different species, revealing a new form of communication between distantly related organisms that interact, which is also called 'cross-kingdom RNAi' (Knip et al., 2014).

Cross-Kingdom sRNAs across Pathogens/Parasites and Host Animals

The sRNA traffic was firstly reported in 1998 when *Caenorhabditis elegans* were fed a dsRNA-expressing bacterial strain; siRNAs were ingested by the nematode and blocked its endogenous gene expression (Timmons et al., 1998). In addition to double-stranded siRNA, single-stranded miRNA has also been found to transfer between the host and the invasive species. For example, miRNAs deriving from parasites, such as *Schistosoma Japonicum* and *Litomosoides sigmodontis*, have been found in the body fluids of infected individuals (Buck et al., 2014; Cheng et al., 2013). Conversely, miRNA-mediated silencing signals can be transmitted in the opposite direction. Many studies have proved such finding as to the role of fecal miRNAs in regulating and affecting the growth of gut microbiome in mice; Loss-of-miRNA function in mice exhibited uncontrolled gut microbiota and exacerbated colitis (Liu et al., 2016). With the same mechanism, the resistance of sickle erythrocytes to malaria was partly enabled by miRNAs that could translocate into the parasite *Plasmodium falciparum* and interfere with its mRNA transcript, resulting in translational inhibition via impaired ribosomal loading (LaMonte et al., 2012).

Cross-Kingdom sRNAs from Pathogens/Parasites to Host Plants

Pathogenic fungi on plants are the major factors that cause crop yield loss and affect global food security since plants are susceptible to a broad spectrum of fungal pathogens. It has been reported that fungal sRNA molecules of *Botrytis cinerea* were transferred into host plant cells, acting as sRNA effectors to suppress host immunity and achieve infection (Weiberg et al., 2013). Similar to the fungus-derived siRNAs, virus-derived dsRNAs can also be processed by

Dicer-like (DCL) proteins into virus-derived siRNAs (vsiRNAs), which then guide AGO proteins to target host genes to mediate disease symptoms in plants (Smith et al., 2011; Ruiz-Ferrer et al., 2009). Gene expression can be suppressed in a sequence-specific manner by infection with virus vectors carrying fragments from the exons of host plant genes (Baulcombe et al., 1999). Other studies have been conducted in regard to this pathogens-sRNA machinery, i.e., vsiRNAs from the Y-satellite of Cucumber mosaic virus specifically downregulated the mRNA of tobacco ChII gene, which induced a bright yellow mosaic symptom (Shimura et al., 2011). Silencing phytoene desaturase (PDS) and squalene synthase (SQS) leading to reduced phytosterols, withanolides, and stress tolerance in *Withania somnifera* (Singh et al., 2015). These research works have led to a statement that both virus and fungi can make use of cross-kingdom RNAi strategy to restrict the inherent defense system of the host plants and ensure the success of their infection.

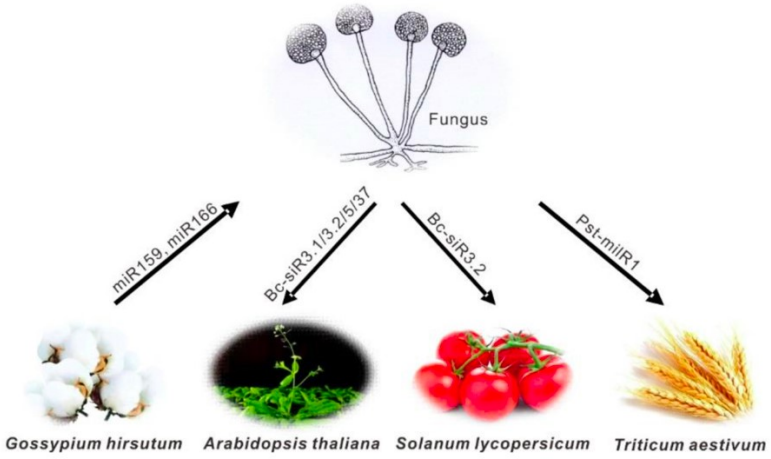

Figure 6: Cross-kingdom interactions. The arrows indicate the direction of the sRNA transfer (Zeng et al., 2019)

In contrast with the tremendous data reported on the transfer of sRNA from pathogen to hosts, the functional movement of sRNAs from parasitic plant to host plants are rarely reported, until recently the parasitic plant *Cuscuta campestris* was found to deliver specific 22-nt miRNAs to suppress host messenger RNAs and trigger endogenous secondary siRNA production, probably as a universal strategy for plant parasitism (Shahid et al., 2018).

Cross-Kingdom sRNAs from Host Plants to Pathogens/Parasites

Likewise, virus, fungi, parasitic plants are able to transfer sRNA into host plants to sure the successful infection, host plant can also do the same mechanism with sRNA plant-origin to counteract with these pathogens and reduce pathogenicity as well as virulence (Deleris et al., 2006; Waterhouse et al., 2006). As the movement of sRNA parasite-to-host, host-to-parasite movement is less reported, likely due to the limited host range and insufficient silencing

efficacy (Tomilov et al., 2008). A study about cotton upon infection with *Verticillium dahliae* in which cotton accumulated miR166 and miR159 targets *V. dahliae* genes encoding a Ca2+-dependent cysteine protease (Clp-1) an isotrichodermin C-15 hydroxylase (HiC-15), respectively. These two miRNAs were exported to fungal hyphae for specific silencing. More importantly, both Clp-1 and HiC-15 transcripts were reduced in the hyphae recovered from *V. dahliae*-infected cotton, and the fungus mutants with targeted genes knocked out indeed displayed reduced virulence (Zhang et al., 2016). Meanwhile, it was noted that the Sequences of Clp-1 and HiC-15 targeted respectively by miR166 and miR159 were highly conserved among different strains of *V. dahliae*, especially within the miRNA-binding regions (Zhang et al., 2016). These findings indicated that a fungal pathogen might have preserved or evolved this miRNA-dependent regulation to prevent host plant hypersensitive responses and to keep them alive during the biotrophic phase of the infection (Zeng et al., 2019). This study also described a conserved host plant defense strategy against fungal pathogens by specifically downregulating virulence genes expression (Zhang et al., 2016).

Cross-Kingdom sRNAs across Plants and Animals (Insects/Mammals)

Plants have also been found to transfer double-stranded siRNAs to closely interacting insects to silence their transcripts and suppress their growth, also known as plant-mediated RNA interference (PM-RNAi) (Baum et al., 2007). RNAi has been conducted as a choice to control insect pests, yet the trafficking of sRNA into and between bug cells is not yet illuminative. However, two types of RNAi response have been recognized in different insect orders (Velez and Fishilevich, 2018), one is systemic RNAi, which means the silencing effect is transported from the cell in which the dsRNA is applied or expressed to other cells, also to other tissues, in which the silencing will then take places, such as in the insect's western corn rootworm (Velez and Fishilevich, 2016) and Colorado Potato Beetle (Palli, 2014). Another one is cell-autonomous, in which RNAi effects are limited to the cell in which dsRNA is expressed or introduced, such as the insect *Drosophila melanogaster* (Belles, 2010). Furthermore, the transferring of plant miRNAs from plant to the animal has also been reported. Jia and co-workers conducted multiple assays and confirmed that mulberry-derived miRNAs could enter silkworm hemolymph and multiple tissues (Jia et al., 2015). Other studies focusing on miR162a highlighted that this miRNA could directly bind to the target gene *Apis mellifera* TOR (amTOR), essential for honeybee caste differentiation, thereby inhibiting larval ovary growth and inducing development into worker bees (Zhu et al., 2017). Since the cross-linking of miRNA from plant to insect are mainly by orally feeding raising the wonder if there is a passage of short-single-strand-sequence from plant to mammals. In 2012, Zhang et al., first

demonstrated the accumulation and biological function of dietary miRNAs in animal tissues (Zhang et al., 2012). Several following studies generated either similar or contradictory results, and the focus of the debate is whether the dietary uptake of plant miRNAs into the mammal tissues is stable and biologically functional. A typical report came from Dickinson et al., who found that insignificant levels of rice miR168a did not result in a cross-kingdom modulation of low-density lipoprotein receptor adapter protein 1 (LDLRAP1) levels in mouse liver (Dickinson et al., 2013). Similarly, plant miRNAs could hardly be detected in the plasma of healthy athletes and mice after ingestion of commonly consumed miRNA-rich food (Snow et al., 2013). In addition, the apparent uptake of dietary plant miRNAs was not observed in the macaque blood by droplet digital PCR (Witwer et al., 2013). In contrast, much experimental evidence has demonstrated the absorption and bioavailability of cross-species plant miRNAs (Luo et al., 2017; Philip et al., 2015; Liang et al., 2014). More recently, it has been reported that plant-derived exosome-like nanoparticles (ELNs) containing sRNAs could alter microbiome composition and host physiology in mice, linked to improving mouse colitis (Teng et al., 2018). Scientists also have started to specifically assess the function of dietary miRNAs for cancer therapy (Chin et al., 2016). However, a recent study showed that transgenic miRNAs did not have any bioavailability, even though they were highly expressed and displayed digestive stability (Yang et al., 2017). In a nutshell, whether the food containing miRNA becomes a 'rising star' in medical therapy is still an open question that needs further scientific explanation and experimentation.

2.10 The effect of pre-and post-harvest treatments on fruit sensorial and nutritional quality

The sensory quality of strawberries is the combination of a complex balance among sweetness, aroma, texture, and appearance. Sugar and volatile contents were found to be important biochemical components that influence consumer acceptance. In fact, consumer quality acceptance is generally related to specific perceived organoleptic traits such as fruit color, shape, acidity, and sweetness, combined with flavor and aroma determined by volatile compounds (Vittori *et al.*, 2018). Esters are known to be important contributors to the typical strawberry aroma. Among them, methyl and ethyl butanoates, methyl and ethyl hexanoates, and ethyl 2-methyl butanoate are often mentioned as active aroma compounds in strawberries. Furanones (furaneol and mesifurane) are also contributors to aroma providing sweet caramel-like notes (Larsen and Poll, 1992; Pérez et al., 1996; Sanz et al., 1994). The nutritional value of berries gained significant care by customers, described as the content of bioactive compounds with healthy effects. These parameters belong to the so-called nutritional quality

and they are of essential significance, along with the wide range of various quality attributes. Fruit sensorial and nutritional quality most likely drive consumer attention and together forming the market trend; however, there are multitudes of factors changing these two parameters. The pre-harvest factors such as cultivar, cultivation practices, plant age, and environment have a significant effect on attributes associated with the sensorial, commercial, and nutritional quality of various fruits, including berries (Alvarez-Suarez et al., 2014). The genotype is the main factor that influences quality traits, notably genetic background is the first factor defining the capacity of fruit to accumulate bioactive compounds, even if it is strictly related to the environmental and agronomic factors (Vittori *et al.*, 2018). A significant difference was found in soluble sugars and total acidity values in different genotypes of wild strawberry (Caracciolo et al., 2013); moreover, also the ratio between these two parameters was found to be influenced by genotypes, but only in particular environments (Doumett et al., 2011).

The pre-harvest factors influencing fruit quality that breeders vigorously take into account is growing location, in particular in open field conditions. Krüger et al. (2012) demonstrated that the same strawberry cultivars grown in northern Europe can produce fruit with higher commercial and organoleptic traits, such as increased dry matter, total acidity, and soluble solids content compared to those grown in southern Europe. A study by Ulrich and Olbricht (2014) on strawberry fruit volatile compounds concentrations demonstrated that three strawberry genotypes harvested in three different years had a variation in aroma concentrations depending on the year of harvest, decreasing from the first to the third year, when weather conditions were untypical for strawberry growing and as a consequence, demonstrating that harvest season also plays a role to the quality of strawberry.

Plant age could also be considered another genetic factor that might influence the fruit quality, whereby higher sugar concentrations in the second year of cultivation were found in the biennial crop of *F. x ananassa* fruits, as well as citric and malic acids (Vittori *et al.*, 2018). The third crop cycle showed a dramatic decrease in fruit production, underlining the need of replacing the plants after the second year. Apart from that, light exposure, and radiation (Anttonen et al., 2006; Kadomura-Ishikawa et al., 2013) also affect the berry quality and many other fruits such as cranberry (Zhou and Singh, 2004), raspberry (Wang et al., 2009), bilberry (Uleberg et al., 2012); temperature is another vital factor (Diamanti et al., 2009) for strawberry.

As far as sensorial factors, fruit shape and color are highly dependent on variety-specific fruit. Fruit color is not only plainly dictated by the genotype but also exceptionally influenced by the ecological conditions and development stage. The genotype is the main factor that

influences quality traits, fruit soluble solids and titratable acidity contents (Galletta et al., 1995). Notably, genetic background is the first factor defining the capacity of fruit to accumulate bioactive compounds, even if it is strictly related to environmental and agronomic factors. Sugar content is especially appealing to the consumer, measured as Total Soluble Solids (TSS), the unit expressed as °Brix. Also, the acidity of berries is a vital element to detect for the assessment of organoleptic quality: the Total Acidity (TA) is utilized to show the acidity of the fruit. Sugar/Acid (S/A) proportion is used as a means to evaluate the sweet-acid balance of fruit, a balanced of which most appreciated by the consumer is TSS of 7% and maximum level TA 0.8% (Manning, 1996). Wild species have a higher level of nutritional attributes when compared with their respective cultivated species, but at the same time, they may have a loss of some other important organoleptic traits (e.g., fruit size and firmness). Thus, wild germplasm has an important role as a genetic source for improving fruit nutritional quality (Diamanti et al., 2014; Wang and Lewers, 2007). Micronutrients and phenolic compounds concentration in berries, as well as commercial and organoleptic attributes, have been reported to change according to many pre-harvest conditions, such as genotype, environment, and cultivation techniques (Cordenunsi et al., 2002; Kafkas et al., 2007). Some authors underlined how the genetic background of a berry species is the most important factor that affect the fruit quality (Milošević et al., 2016), in particular regarding the sensorial and the nutritional quality.

Clearly, also harvest and storage conditions influence the composition of the fresh fruit. The ripening stage of the fruit at the harvest time is an important factor because many chemical and compositional modifications occur when the fruit is still attached to the mother plant (Park and Yoon, 2013). In case berries are collected at the completely matured stage when anthocyanins are at the most extreme content and aroma is fully peaked, postharvest life might be decreased because of diminished firmness and expanded affectability to mechanical damage incurred during handling. This behavior is due to the fact that some phenolic compounds, in contrast to anthocyanins, have been found to be higher in unripe than in ripe fruits (Kosar et al., 2004; Tulipani et al., 2011; Wang and Lin, 2000). Other organoleptic parameters were investigated in raspberries, with titratable acidity increasing significantly with enhanced ripening, while the concentrations of soluble sugars remained relatively unaltered (Krüger et al., 2011).

2.11 Sugar and acid contents in strawberry

Strawberry quality is largely determined by the relative amounts of sugars and organic acids present. The metabolic composition of strawberries has been the focus of many studies, given the economic importance of nutritional and flavor traits (sugar and organic acid content,

especially ascorbic acid content), berry color (anthocyanins), and softening (Basson et al., 2010). It is fundamental to know the best stage at which to harvest the fruit due to the fact that acids can affect flavor directly and are also important in processing since they affect the formation of off-flavors, the gelling properties of pectin and regulate cellular pH and may influence the appearance of fruit pigments within the tissue (Montero et al., 1996). Few studies have been made on the changes in acid content in soft fruits during ripening. The main acids in strawberries are citric and malic acids; glycolic and shikimic acids are also present but in lesser quantities (Woodward, 1972). This fruit is an excellent source of ascorbic acid (Lundergan and Moore, 1975). For strawberry, sucrose, glucose, and fructose account for more than 99% of the total sugars in ripe fruit, with sorbitol, xylitol, and xylose occurring in trace amounts (Maniken and Siiderling, 1980). Few data are available on changes in sugar content during the ripening of strawberries previous of which indicate only that the total content of sugars increases rapidly until the fruit is fully ripe (Montero et al., 1996). On the other hand, the total acidity declines during ripening, but ascorbic acid increases (Avigdori-Avidov, 1986). Although not many studies are available documenting the effect of pesticides/biostimulants treatments on fruit quality, this type of study is important to determine the absence of effects on fruit quality by the application of new molecules for protecting plants from diseases.

3. METHODOLOGY

This section addresses the standardized procedure for performing Spray Induced Gene Silencing protocol and nutritional analysis methods.

3.1 SIGS on strawberry soil-less cultivation system

This experiment was conducted on a standard soil-less cultivation system, in a plastic greenhouse located in Mazzanti's farm – Trecastrelli Senigallia, Ancona.

Figure 7: Google maps top view of the experimental greenhouse in Mazzanti farm

The selected strawberry cultivar VIVARA were cultivated starting from frigo plant A, planted in April 2020. The experimental trial was set up in one row, plots were organized following a Randomized Complete Block design (Tab. 1), following the scheme defined thanks to the collaboration established by UNIVPM and GreenLight Bioscience company.

Block 1	Block 2	Block 3	Block 4
7	4	1	5
1	6	7	7
8	7	5	3
2	8	4	1
4	3	6	6
3	5	2	2
6	2	3	8
5	1	8	4

*Table 1: Randomized Complete Block (RCB) in soil-less system, designed with four block - Each treatment was composed by 2 meters * 0,3 meters = 0,6 m2 * 4 = 2,4 m2*

Treatment:

The topical treatments were applied to four biological replicates (plots), each plot was

composed of two linear meters of twenty strawberry plants. There were 8 treatments including an untreated control, a typical fungicide regime, and two dsRNA-based (A and B) formulations applied at different concentrations, which details are reported below:

1. Non-treatment

2. Normal Fungicide regime

Product	**rate**	Rate in 1 L
Fontelis	900 ml/Ha	0,216 mL
Signum + Silwet L-77	1.8 kg/Ha 0,025%	0.432 g 0,25 mL
Fontelis	900 ml/Ha	0,216 mL
Signum + Silwet L-77	1.8 kg/Ha 0,025%	0.432 g 0,25 mL
Captan	3.38 kg/Ha	0.811 g
Captan	3.38 kg/Ha	0.811 g
Switch + Silwet L-77	0,8kg/Ha 0,025%	0,192 g 0,25 mL
Silwet + L-77Switch	0,8kg/Ha 0,025%	0,192 g 0,25 mL

3. dsRNA A + Silwet L-77: 1,25 L/Ha

4. dsRNA A + Silwet L-77: 9,24 L/Ha

5. dsRNA A formulated + Silwet L-77: 100 L/Ha

6. dsRNA A formulated + Silwet L-77: 750 L/Ha

7. dsRNA B formulated + Silwet L-77: 100 L/Ha

8. dsRNA B formulated + Silwet L-77: 750 L/Ha

Treatment application recommendation*:*

The first treatment was the control. In treatment no.2, strawberries were sprayed with fungicide every 3-4 days beginning at approximately 100% bloom after the removal of all fruits (fig. 11): Fontelis has been alternated with Signum for the first 4 applications, followed by two applications of Captan, then Switch was applied at the last two applications. The other treatments were applied every 3-4 days beginning at approximately 100% bloom after stripping all fruit.

Figure 8: Strawberries and the tunnel system

Cropping Considerations:

Strawberries were healthy and growing well and were not applied by any fungicides within 2 weeks prior to the first application of the treatments. Developed and ripe strawberries were completely discarded prior to making the first application. In terms of inoculation, *Botrytis cinerea* conidia-containing suspension was sprayed (using a medium-high level of inoculum) on the strawberry plants within one day following the first fungicide application (but after sprays dry) to ensure consistent *Botrytis* pressure. At the application of first (A) fungicide treatments plants had 100% bloom and flowers fist the preliminary fruit set and visible infection was observed on flowers. Strawberries were harvested once a week and were firstly performed after 3 weeks of the first treatment. The disease evolution in post-harvest was monitored by storing fruit in a plastic box. A total of ten treatment applications and five harvests have been done during the trials (Table 2).

Number of treatment & harvest	**Date of treatment & harvest**	**Note**
-	21/09/2020	Removing fruits of the plants
1st	22/09/2020	
Inoculation	23/09/2020	Inoculation with Botrytis
2nd	25/09/2020	
3rd	28/09/2020	

4th	01/10/2020	
5th	05/10/2020	
6th	08/10/2020	
7th & 1st harvest	12/10/2020	
8th	15/10/2020	
9th & 2nd harvest	19/10/2020	
10th	22/10/2020	Last treatment
3rd harvest	26/10/2020	4 days after the last treatment application
4th harvest	02/10/2020	11 days after the last treatment application
5th harvest	11/09/2020	18 days after the last treatment application

Table 2: Treatment and harvest activity distribution of soil-less system

Fungal culture and inoculation

Botrytis cinerea B05.10 strain was cultured and propagated on PDA (potato dextrose agar) and MEA (malt extract agar) medium. Conidia were collected on sporulating-Petri dishes 10 days following the culture. 15 mL of distilled sterile water was spread on the surface of the medium, collecting them with the aid of a sterile inoculating loop. A nylon filter mesh of 70 µm has been used to remove mycelium selecting only conidia. The final concentration was measured by a hemocytometer in order to reach the concentration of 10^5 conidia/mL in distilled deionized water, supplemented with 0,05 g/ L of PDB (Potato Dextrose Broth, PDB).

Strawberry Harvesting

The temperature was recorded continuously during the fruit maturation, and the relative humidity has been kept constant to around 60%. Considering the optimal condition of the photoperiod and term-period during the autumn season in central Italy, fruits were harvested once a week to evaluate the disease progression, removing rotten or overripe fruits in time, and better detect the total and commercial yield from the different treatments. The first strawberry harvest has been performed four weeks after the application of the first treatment, based on the degree of maturation reached by the fruits under the greenhouse and soilless cultivation system. The production of each treatment was harvested independently, in order to have four

replications of each treatment.

3.2 SIGS on strawberry open field cultivation system

This experiment was conducted on a standard open cultivation system located in Gianni Malavolta farm – 63823 Lapedona, Fermo, (IT), organized in twin rows with mulching cloth and drip irrigation in the open field.

Figure 9: Google maps top view of the experimental greenhouse

The selected strawberry cultivar was TEA, a short day cultivar (intermediate harvesting period) that was planted as Frigo plants A at the end of July 2020. Strawberries have been grown under normal commercial production conditions in the open field, managed with adequate fertilization and irrigation regime to produce good healthy strawberry plants during the period from mid-May to mid-June. The experimental trial was set up in two mulched rows, each containing two rows of plants, with a density of 5.5 plants/meter. Each row had 140 strawberry plants, organized following a Randomized Complete Block design (table 3), with four repetitions of each treatment of 10 plants per plot. Four blocks were distributed along the two rows. Each treatment involved 10 well-formed plants, delimited by yellow identification poles.

Block 1	Block 2	Block 3	Block 4
6	2	6	6
12	3	1	12
10	5	11	11
9	6	4	3
1	1	7	2
5	12	12	8
11	10	3	9
3	8	5	5
2	7	2	4
7	11	10	1
8	4	9	10

4	9	8	7

Table 3: Randomized Complete Block (RCB) in open field, designed with 4 plots, 10 plants/plot/12 treatments.

Treatments

The topical application experiment included twelve treatments composed by an untreated control, a typical fungicide regime, a fungicide regime used in an organic cultivation system, and two dsRNA-based formulations (C and D) applied at different concentrations and time of applications, which details are reported below:

For the treatment the recommended application amount was 500 L/Ha

1. Untreated check
2. Normal Fungicide regime:

Product	**rate**	Rate in 1 L
Teldor Plus	1,5 L/Ha	3 mL
Teldor Plus	1,5 L/Ha	3 Ml
Signum	1.8 kg/Ha	3,6 g
Signum	1.8 kg/Ha	3,6 g
Switch	0,8kg/Ha	1,68g
Switch	0,8kg/Ha	1,68 g

Two application for each fungicide, for a total of six application, once a week.

3. Fungicide regime based on the application of Serenade ASO (6 L/Ha) (12 mL in 1 L) for six applications, once a week
4. dsRNA C + Silwet L-77: 186,30 L/Ha
5. dsRNA C + Silwet L-77: 745,16 L/Ha
6. dsRNA C + Silwet L-77: 1487,37 L/Ha
7. dsRNA C + Silwet L-77: 2986,57 L/Ha
8. dsRNA D + Silwet L-77: 195,16 L/Ha
9. dsRNA D + Silwet L-77: 783,61,16 L/Ha
10. dsRNA D + Silwet L-77: 1567.21 L/Ha
11. dsRNA D + Silwet L-77: 3134,42 L/Ha
12. dsRNA D + Silwet L-77: 1567,21 L/Ha

Treatment 4, 5, 6, 7, 8, 9, 10, 11 were applied each 3-4 days, whereas treatment 12 was sprayed every 7 days for a total of six applications instead of twelve.

At the execution of each treatment, each parcel was isolated both longitudinally and sideways by anti-drift barriers, applied before treatment, and manually removed afterward.

Cropping Considerations:

Strawberries were healthy and growing well and were not applied by any fungicides within 2 weeks prior to beginning applications of the treatments and were at the flowering stage before making the first application. In this trial, *Botrytis cinerea* was not inoculated on the strawberry plants, due to the fact that open field is an ideal environment for grey mold thanks to an initial rainy season wetting the surface of the plants, especially from the late afternoon. At the very first application of fungicide treatments, plants were at an early flowering stage with about 50% of flowers not already open. Flower infections and plant phytotoxicity were not observed. Strawberries were harvested twice a week after 2 weeks from the first treatment application and the disease evolution in post-harvest was monitored by storing first harvested fruits once a week in plastic boxes at room temperature for 7 days and detecting the infections at 3 and 7 days of storing. The second harvest fruits were sampled and frozen at -20°C for further quality analyses.

Treatments and harvest plan

27/04/21 1° treatment

30/04/21 2° treatment

04/05/21 3° treatment

07/05/21 4° treatment

11/05/21 5° treatment and first harvest

14/05/21 6° treatment and second harvest

18/05/21 7° treatment and third harvest

21/05/21 8° treatment and fourth harvest

25/05/21 9° treatment and fifth harvest

28/05/21 10° treatment and sixth harvest

01/06/21 11° treatment and seventh harvest

04/06/21 12° last treatment and eighth harvest

08/06/21 ninth harvest

12/06/21 tenth harvest

15/06/21 eleventh harvest

Figure 10: Strawberries in different stages

Strawberry Harvesting

Considering the optimal condition of the photoperiod and term-period during the spring season in central Italy, it was necessary to harvest fruits two times a week, to better evaluate the disease progression, removing rotten or overripe fruits in time, and better detect the total and commercial yield from the different treatments. The first strawberry harvest was performed on the 11th of May, two weeks after the first treatment application, determined by the first number of ripening fruits available on the plants. The production of each treatment/plot was harvested independently, in order to have four replications for each treatment.

3.3. Analysis of production parameters

For each experimental trial, composed of a various number of strawberry plants, fruits were harvested every 3-5 days beginning at fruit maturity till the end of the season. At each harvest, all ripe fruits were picked taking into account these classifications: **commercial products**: fruits accepted by the consumer; **total products** including marketable, small, and deformed fruits; **waste products** composed of biotic- or abiotic-damaged products. At the time of harvesting, all strawberry classes were weighted using a field dynamometer, each of which was counted and calculated, dividing into:

-average fruit weight (g)= total weight/total fruit numbers;

-consumer-accepted fruits per plant,

-waste fruits per plant,

-total products per plant.

3.4. Analysis of disease severity and disease control value:

The **disease severity index** was calculated at each observation as the % of infected area of each fruit compared to the total fruit area. Relying on the percentage ratio among infected area and

total area, each fruit was rated in a class, following this class division: 0 = no disease symptoms; 1 = 0.1-5%; 2 = 5.1-20%; 3 = 20.1-40%; 4 = 40.1-100% (Sabbadini et al., 2021).

The **disease severity value** at each time was calculated using the following formula: Disease severity (%)=((Σ (the number of diseased fruits × disease severity index)) / (4 x the number of fruits rated)) x 100 (Sabbadini et al., 2021).

The **disease control value (%)** was calculated as reported below (Choi et al., 2007):

$$\text{Disease control value} = \left(\frac{treatment\ disease\ severity - negative\ control\ disease\ severity}{negative\ control\ disease\ severity}\right) \times 100$$

These parameters have been measured at each harvest time, in order to have a clear picture of the evolution of gray mold at the time of harvest of each treatment. Post-Harvest detection of disease severity and disease control value has been also conducted to investigate whether treatments could have a protective effect also after 3, 4, or 7 days post-harvest.

3.5 Analysis of quality parameters:

a) **Solid Soluble (SS)**: Five full red strawberries from all five harvest were sampled from each treatment and frozen at −20 °C, defrost then extracted to get the juice. Drop 1-2 drops the extract on the surface of hand-held refractometer prism (ATAGO, Tokyo, Japan); the results were refractometrically measured as total solid soluble content expressed as °Brix. This number represents approximately the amount of total sugar contents in fruit extract. (One degree Brix is 1 gram of sucrose in 100 grams of solution).

b) **Titratable Acidity (TA)**: Titratable Acidity measures the total acid concentration in extract, which is determined by diluting 5 mL of juice diluted with distilled water (1/2 v/v) and titrating/neutralizing with 0.1 N NaOH solution until pH 8.2 - the equivalence point at which the amount of titrant (NaOH 0.1 N) added is just enough to completely neutralize the analyte solution (acid in the extract) and expressed as mEQ of NaOH per 100 g Fresh Weight (FW).

3.6 Statistical analyses

All acquired data from each trial of the **high tunnel system** were analyzed by one-way ANOVA, and the **Newman-Keuls** test ($p < 0.05$) was used to identify significant differences. The bars depicted in the graphs represent the standard error derived from the standard deviation of the values.

All acquired data from each trial of the **open field system** were analyzed by one-way ANOVA, and the **Duncan** test ($p < 0.05$) was used to identify significant differences. The bars depicted in the graphs represent the standard error derived from the standard deviation of the values.

Regarding **sensorial analysis**, all acquired data from each trial of open field system were

analyzed by one-way ANOVA, and the **Fisher LSD** test, $p<0.05$, was used to identify significant differences. The bars depicted in the graphs represent the standard error derived from the standard deviation of the values.

4. RESULTS AND DISCUSSION

4.1 High tunnel soil-less production system

Strawberry Productive parameters

Strawberry is a highly perishable non-climacteric fruit; hence it must be harvested at full maturity to achieve maximum quality in terms of visual appearance, texture. Among many considerations, visual appearance, particularly the average fruit weight, is the first factor in contact with the consumer, which mainly dictates their option. From the farmer's point, they care most about the productivity per plant and mass of marketable fruit. For these reasons, the project was carried out, taking into account the most considered parameter.

All treatments with different formulate applied in strawberry soil-less cultivation, mostly have not affected the average fruit weight (12 or 13 grams) (Fig. 11). The same for the total fruit yield per plant, not showing significative difference among treatments (Fig. 11). On the contrary, the incidence of commercial production per plant was significantly higher when fungicide was applied to protect crops from fungal diseases when compared with the production collected from the treatment 8 and non-treated experimental units, while the other treatments had an intermediate level of commercial production (about 40 g/plants), so showing a different rate of capacity to protect plants depending to the type of treatment.

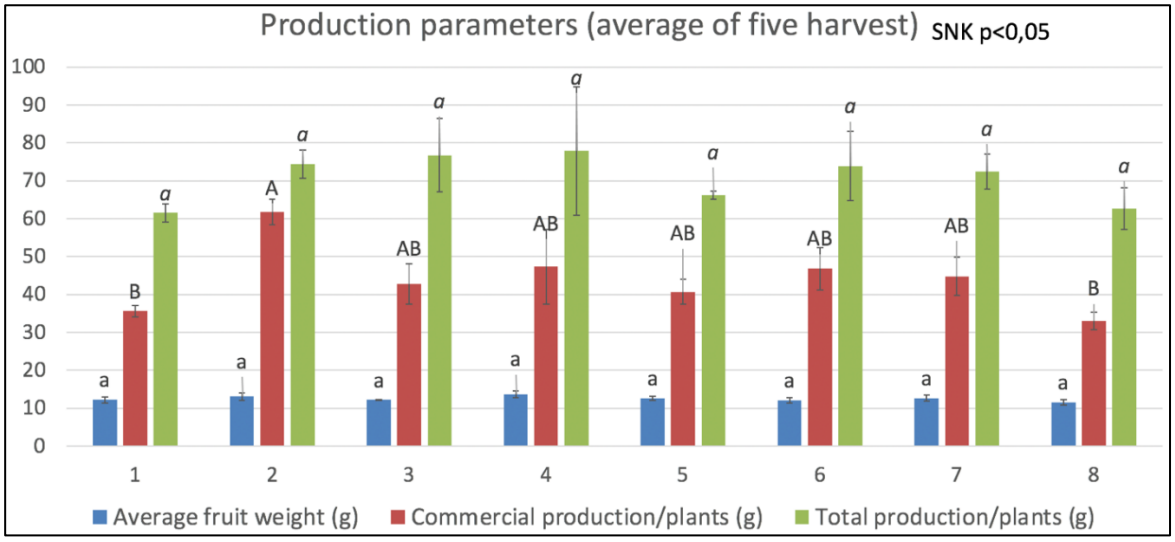

Figure 11: Productive parameters (average of five harvests) of 8 different treatments. Different letters and the lowercase italics letters indicate significant differences at $p < 0.05$

Disease severity at the time of harvest

Disease severity and disease control value (expressed as %) for five harvests in each treatment were assessed to examine the effect of the different treatments to control the infection of *B. cinerea* on fruits.

In terms of the *Botrytis*-caused damage, at the first harvest, albeit the fungicide applications appear to manage a slightly superior control with the other applications, no critical contrasts have been established among the treatments. Different results were observed at the second harvest. The untreated exploratory plot had the most infected products, measurably unique in relation to those fungicide treated. Nonetheless, the dsRNA-treated application had no significant difference among the 6 types with intermediate values. dsRNA handling appears to have satisfactory control against grey mold. On the other hand, disease control value has a dramatic increase experienced in the eighth treatment. Standard fungicide has the highest control, not reached by the dsRNA formulates which had an intermediate capacity to control the disease (Fig. 12).

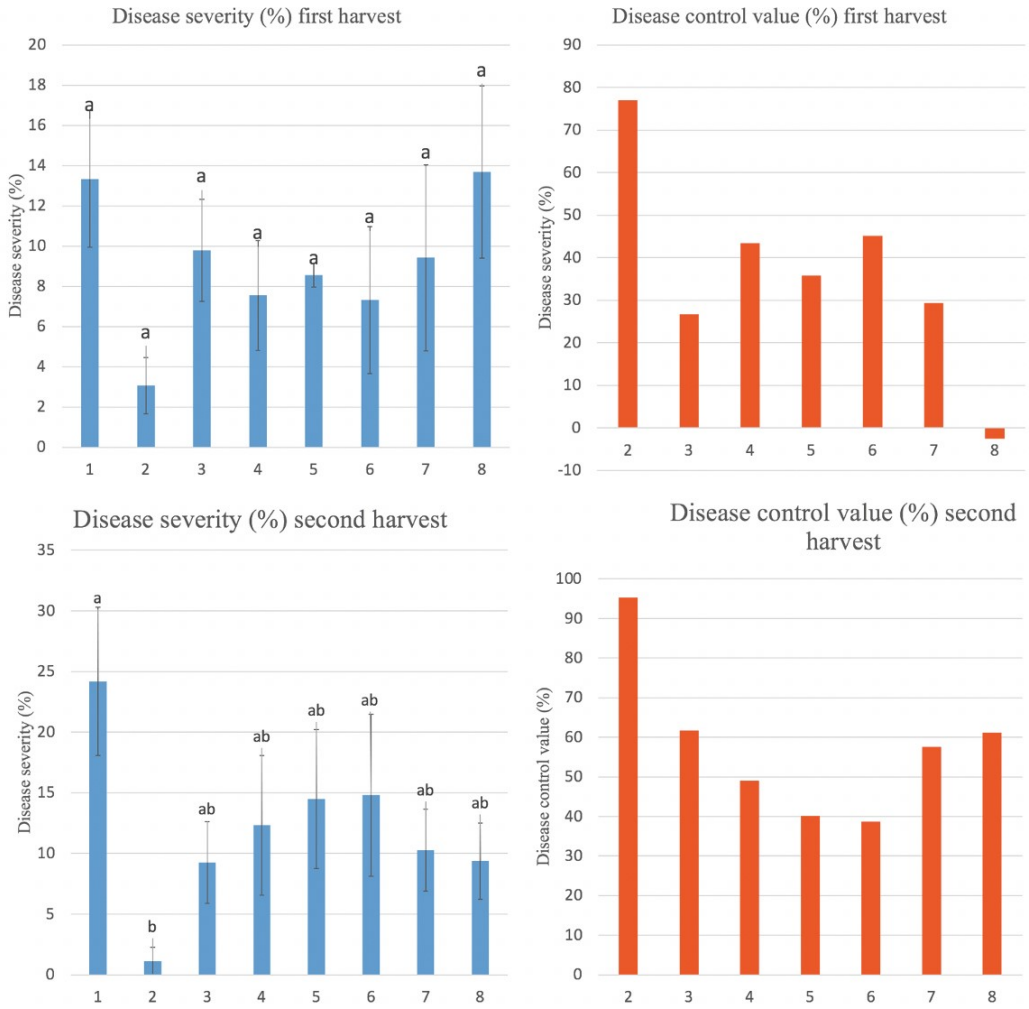

Figure 12: Disease severity & Disease control value of the first and second harvest. Different letters indicate a significant difference, Newman-Keuls test, p<0.05Figure 10: Disease severity & Disease control value of the first and second harvest. Different letters indicate a significant difference, Newman-Keuls test, p<0.05

As indicated in Fig. 13, there was still a noticeable divergence in terms of the influence caused

by the pathogen in untreated and fungicide-treated plants corresponding with the highest and lowest performance among eight plots in both 3rd and 4th harvest, not taking into account the naked dsRNA treatment. A considerable notice was recorded in treatment no.3 in the third harvest, the second most damaged plant, which had almost the same value with others five dsRNA-treated designs, whereas the lowest severity was recorded in 5 and 6. Similar to the two previous harvests, the second treatment was still the best option for resisting grey mold growth. Treatments 3 and 4 had the lowest efficiency in controlling the pathogen in these harvests (Fig. 13); wherein there was a small difference in terms of protective effect in the third harvest while no significant difference was witnessed of these two formulae in the fourth harvest.

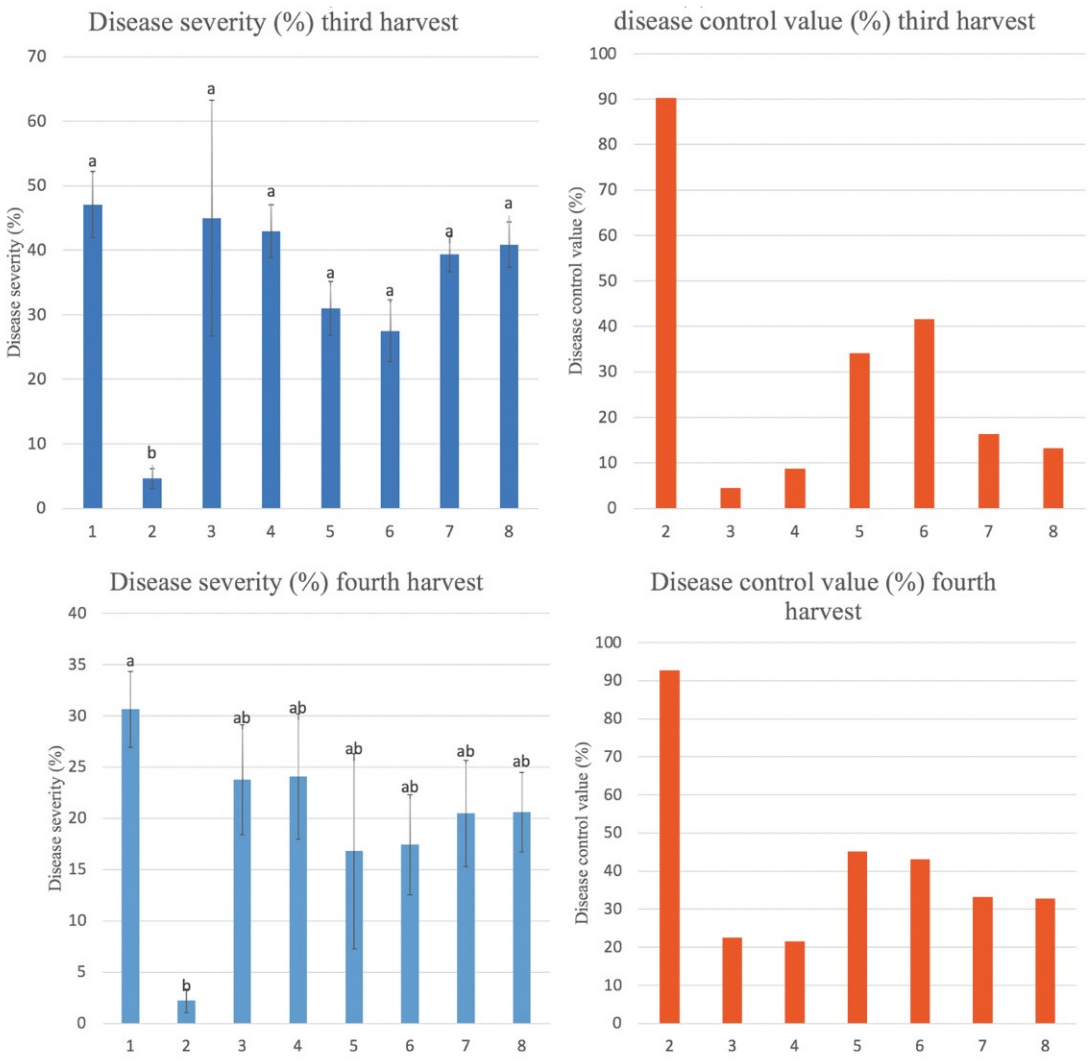

Figure 13: Disease severity & Disease control value of the third and fourth harvest. Different letters indicate a significant difference, Newman-Keuls test, $p<0.05$

At the end of the season, the fifth treatment, not so many fruits were harvested, so this harvest cannot be representative. The spoilage incidence was similar to no significant difference except the tiniest damage plot because it was treated with fungicide. As expected, fungicide has the

best counteracting effect against pathogenicity. The eighth treatment did not affect the control of grey mold (Fig. 14).

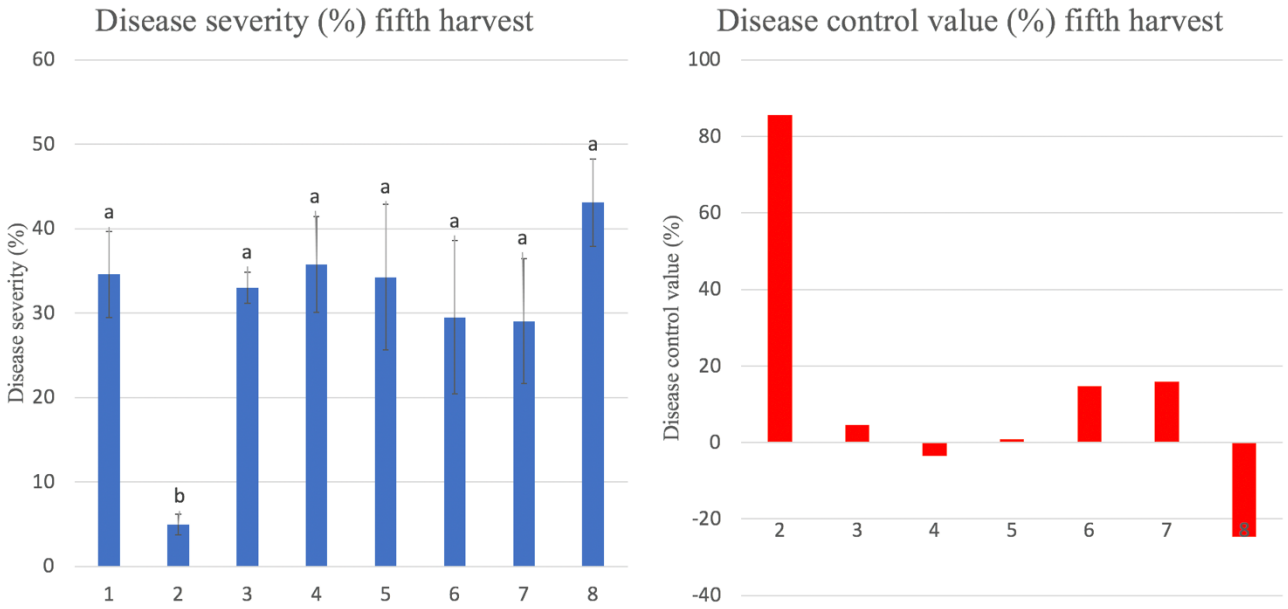

Figure 14: Disease severity & Disease control value of the fifth harvest. Different letters indicate a significant difference, Newman-Keuls test, p<0.05

Average of the disease control value (%) in four harvests

As indicated in the graph, fungicide-applied plots were far most effective to counteract *Botrytis cinerea*, triple times to treatment no.3, double time to treatment 6. Apart from chemical treatment, treatments no. 5, 6 of A-formulated groups were most effective in terms of using dsRNA to resist grey mold, although there was no statistical difference among the other dsRNA-based treatments (Fig. 15)

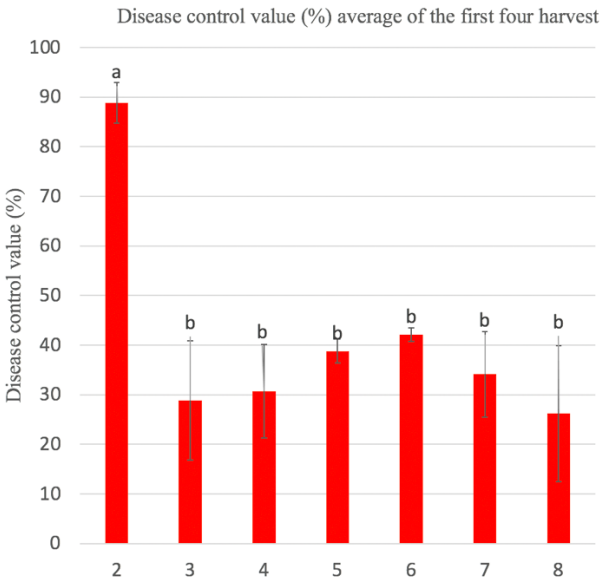

Figure 15: Average disease control value (%) in four harvests. Different letters indicate a significant difference, Newman-Keuls test, p<0.05.

Disease severity during post-harvest

At 4dph dsRNA-treated fruits were noticeably attacked by grey mold (more than 60%) but incomparable to standard fungicides-treated fruits in which disease severity was around 10% in all 4 harvests; however, nearly all treatments were completely ineffective after 7dph (disease severity values that average around 100 %), with the exception of fungicide treatment in the second harvest, where fruits were significantly more infected compares to other three harvest times (Fig. 14, 15 and 16). At the second harvest date, in addition to the positive control, also treatment 8 showed adequate protection at 4 days of post-harvest, while at 7 days almost all the fruits were totally infected by *B. cinerea*. On the next harvest, fungicide-treated products were protected most effectively both in 4dph and 7dph and statistically different from other plots. On the other hand, treatment 8 at 4dph showed the intermediate protection between no.6 and remaining treatments, including negative control treatment. At the last harvest, except for positive control with 10% of infection, all fruits were seriously infected by grey mold with more than 80% in terms of 4dph.

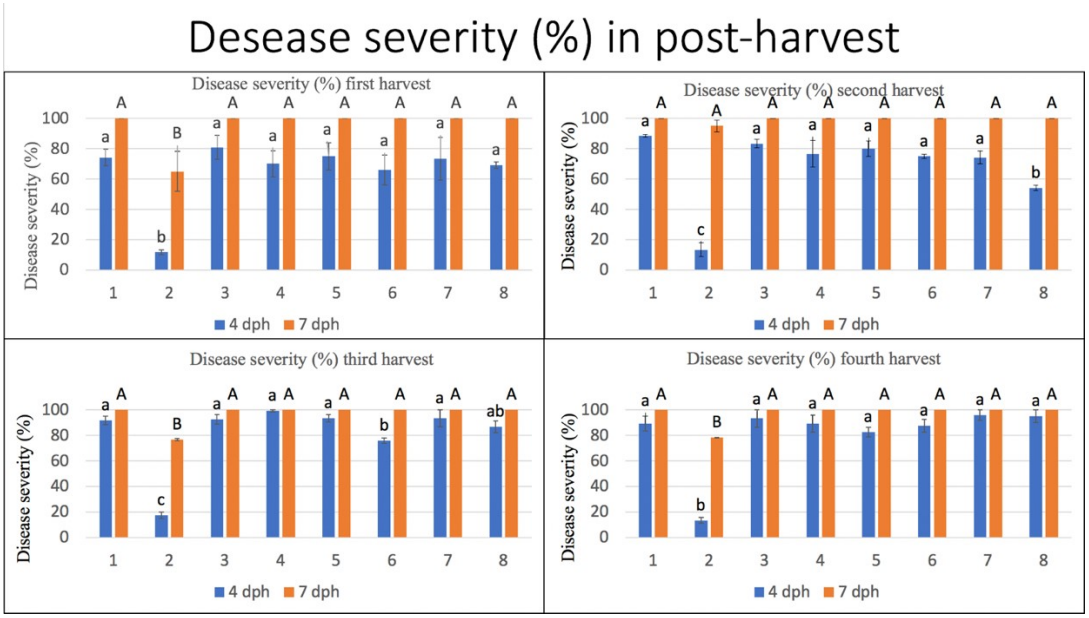

Figure 16: 4 and 7days post-harvest (dph) average of disease severity (%) and disease control value (%) of grey mold on strawberry fruits recorded in four harvests after dsRNAs and fungicide application under greenhouse cultivation system. Means with different letters are significantly different according to the Student- Newman-Keuls test ($p < 0.05$) ± SE. Error bars represent the standard errors of three replications

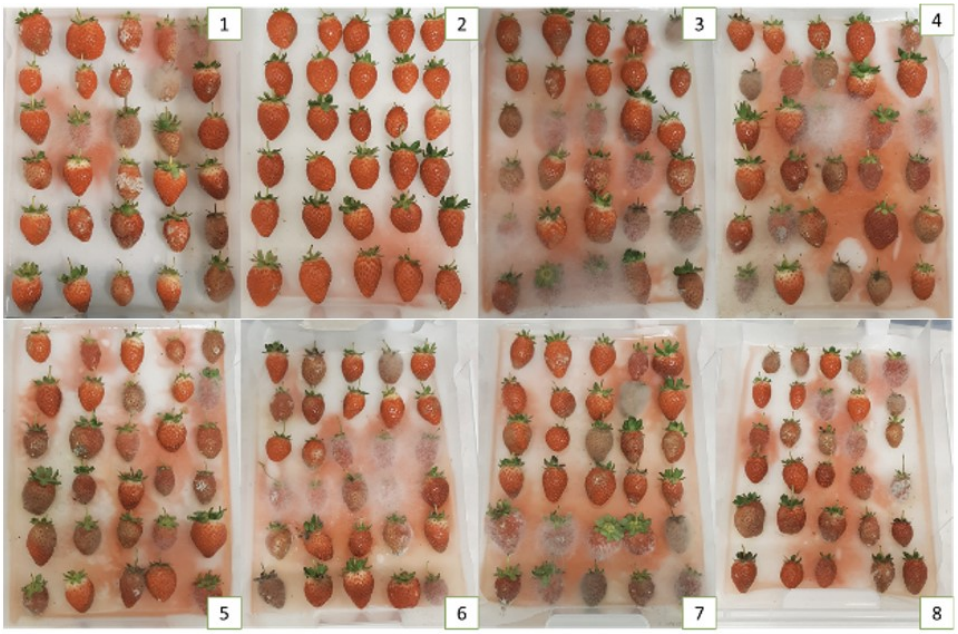

Figure 17: Fruits stored at room temperature 4 days post-harvest of 8 treatments

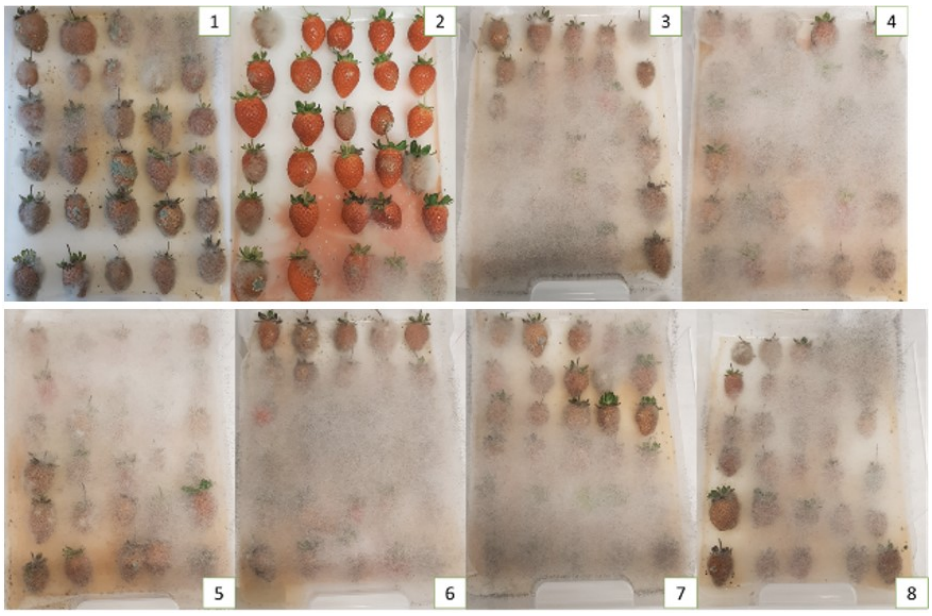

Figure 18: Fruits stored at room temperature 7 days post-harvest of 8 treatments

Final observations:

- The pesticide-applied treatment in greenhouse conditions had a very high effect on fungi protection both at the time of harvesting and post-harvest.
- A moderately positive effect in controlling fruit *Botrytis* infection was observed in almost all the dsRNA formulations applied.
- The most effective treatments are 5 and 6, which are formulated dsRNA A molecules, compared to those of B-formulated and naked dsRNA A.
- All dsRNA formulations had no effect in continuing the protection of fruit infection after 4 and 7 days of storage at ambient temperature.

Sugar and acidity content

The ANOVA analysis performed on the data of the "Vivara" variety fruits treated with 8 different formulations against *B. cinerea* and harvested at 4 different dates, revealed that the treatment did not affect the sensorial quality of the fruits, in terms of Soluble Solids Content (° Brix) and Titratable Acidity (% of Citric Acid) (table 4). The harvest date influenced a significant manner on the content of sugar and acid of fruits, and also the interaction between treatment and harvest date has a significant impact on fruit sensorial quality. These results underline that the harvest date is the main factor affecting the fruit quality (Table 4). All the statistical analyses were made through the STATISTICA 7 software, and the means were compared through the Fisher LSD test, $p < 0.05$.

Factors	Soluble Solids Content	Titratable Acidity
Treatment	n.s.	n.s.
Harvest date	*	*
Treatment - harvest date interaction	*	*

*Table 4: ANOVA analysis for the factors (treatment and harvest date) and the parameters (Soluble Solids Content and Titratable Acidity. n.s. = not significant interaction; * = significant interaction for p<0.05.*

As previously discussed, different treatments did not affect the Soluble Solids Content (Fig. 17) with the average value from 6.5 to 7. The harvest date, and its interaction with the treatment applied, seems to have the highest impact on the fruit quality (table 4)

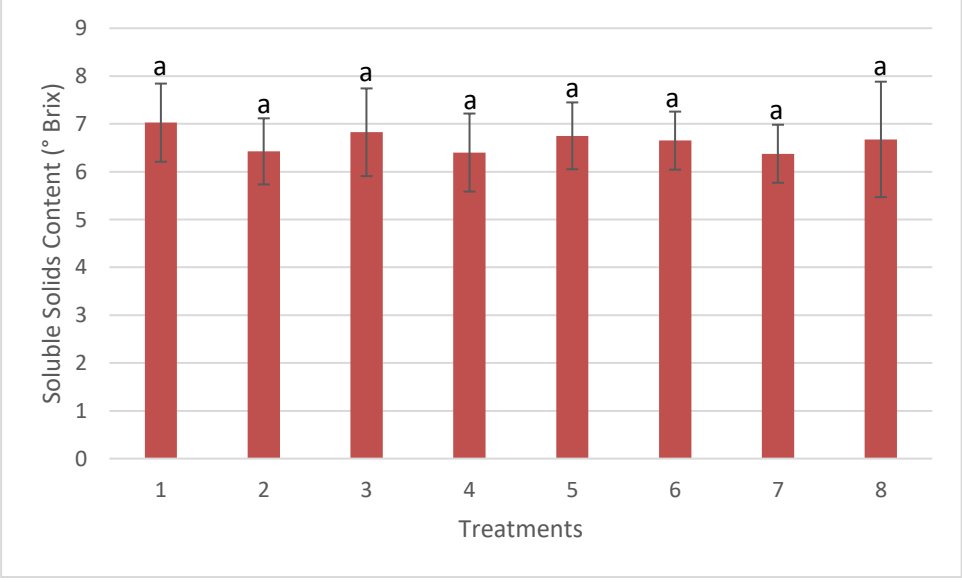

Figure 19: Soluble Solids Content of fruits obtained with different treatments. Different letters indicate a significant difference, Fisher LSD test, p<0.05.

Although treatments applications did not affect the acidity of the fruits, there was a variation highlighting that the fungicide-treated fruits were statistically less acid rather than control fruits. The acidity value of treatments 4 to 8 was in between treatment 1 and 2, the highest

values and the lowest value, respectively (Fig. 18).

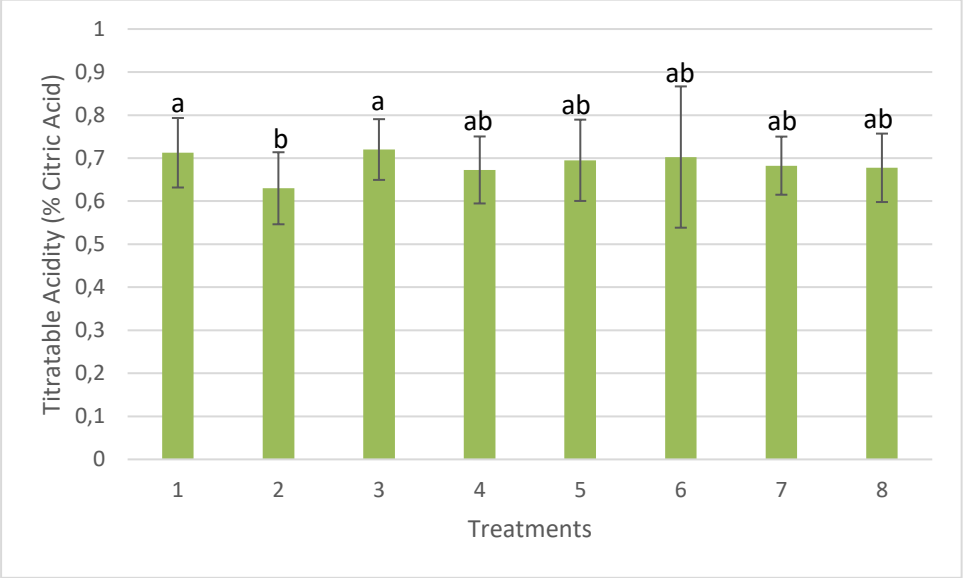

Figure 20: Fruit Titratable Acidity obtained with different treatments. Different letters indicate a significant difference, Fisher LSD test, p<0.05.

As shown in graph (Fig. 19), the solid soluble content of the third harvest ranked the top value contrary to the last harvest corresponding to the lowest value. The sugar content of fruits harvested at the first and second harvest achieved intermediate values.

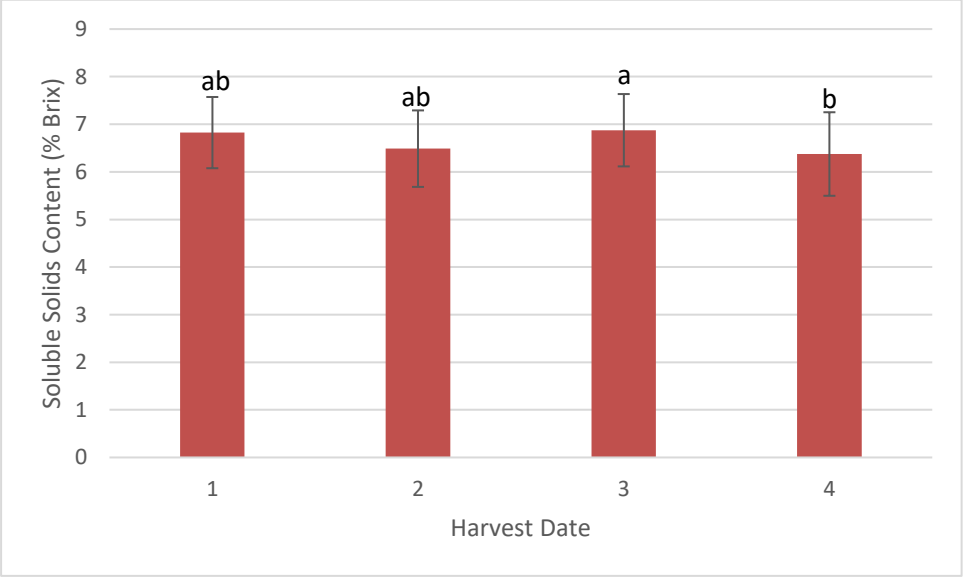

Figure 21: Soluble Solids Content of fruits obtained at different harvest dates. Different letters indicate a significant difference, Fisher LSD test, p<0.05

The analysis of the effect of harvest date on fruit Titratable Acidity reported in Figure 20 revealed that the fruits harvested on the first date are significantly more acid than the fruits harvested at the second and fourth date, while fruits harvested at the third date presented intermediate values.

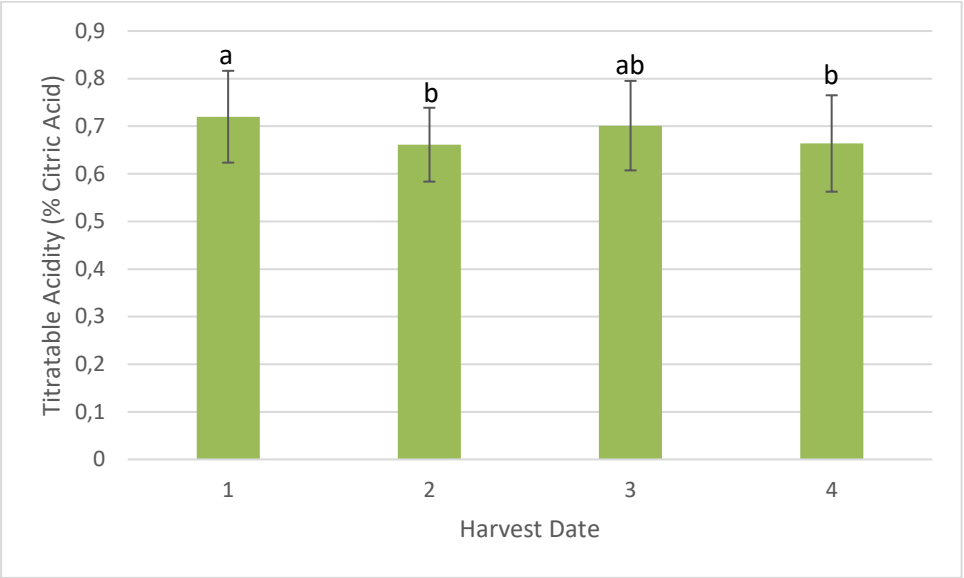

Figure 22: Fruit Titratable Acidity obtained at different harvest dates. Different letters indicate a significant difference, Fisher LSD test, $p<0.05$.

4.2 Open field production system

Strawberries have been grown under normal commercial production conditions in the open field. During the trials, daily temperatures were higher than 25° C; relative humidity was higher than 40%; and the rainfall was scarce, accumulating approximately 39.4 mm of rain in second decades of April-May, and 16.5 mm in June, managed with adequate fertilization and irrigation regime to produce good healthy strawberry plants during the period from mid-May to mid-June. Strawberries were harvested two days a week, considering the optimal condition of the photoperiod and term-period during the spring season in central Italy.

Strawberry Productive parameters

The average fruit weight is statistically and practically similar, with above 20 grams per fruit. The total harvested fruit production ranges from 135-165 g per plant, of which about 100-130 g were attributed to marketable ones. The extent to which the commercial production was protected against fungal diseases per fungicide-treated plant was greater when compared to those from the non-treated experimental units. Considering the weight of commercial production, none of the dsRNA treatments were effective as the chemical pesticides; all plots were better or comparable to untreated formulate, while plots no.10 and 12 had the lowest yields and were statistically different compared to the rests. Among three control treatments, chemical fungicide-treated plants (2) had the highest commercial products compared to untreated plants (1) and Serenade-based regime (3) in which treatment no.1 and 3 had the intermediate value even though there was no statistical difference between them. In contrast, the Serenade-based regime had the highest total production values to the rest 11 treatments,

was the second-highest in terms of commercial yield following chemical fungicide-treated plants. In terms of dsRNA treatments, the highest marketable yield has been reached in plot no.7, followed by no.8, proportionate to the lowest control plot (1) (Fig. 23).

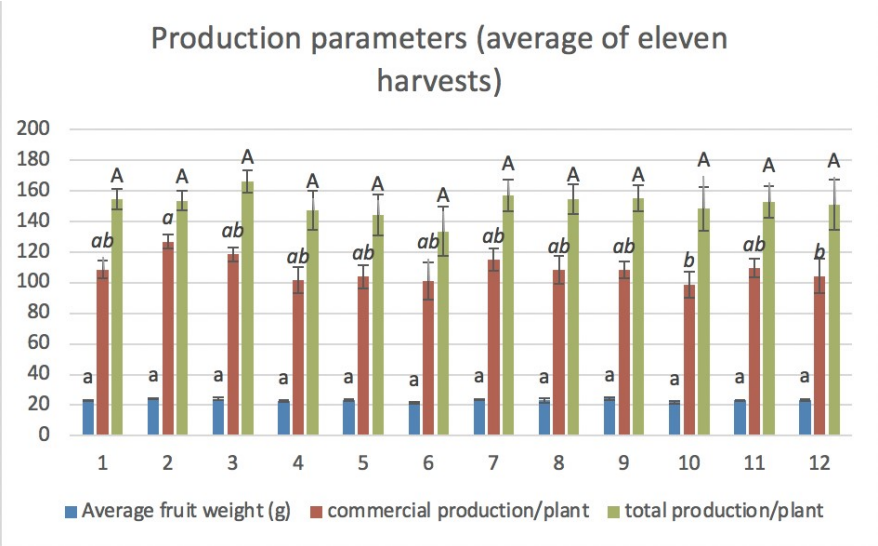

Figure 23: Average fruit weight (g), commercial production/plant (g), and total production/plant (g) in eleven harvests. Means with different letters are significantly different according to the Duncan test ($p < 0.05$) ± SE. Error bars represent the standard errors of three replications for each treatment.

The commercial production during the harvesting period followed a bell-shaped distribution, beginning from lower than 200g/experimental unit in the initial two harvests. From the third to the fifth harvest, the commercial production was dramatically expanding, arriving at its pinnacle higher than 2500 g/plot. From the 6th gather to the 9th, the marketable fruit mass showed a declining pattern, resulting in a few ripe products at the last reap. The chart featured high commercial production of 3597 g/plot came to at the fifth reap in fungicide-treated plots, producing 191 strawberries. The 5th, 6th, and 7th were the most efficient harvesting times with higher than 1500 g/plot (Fig. 24).

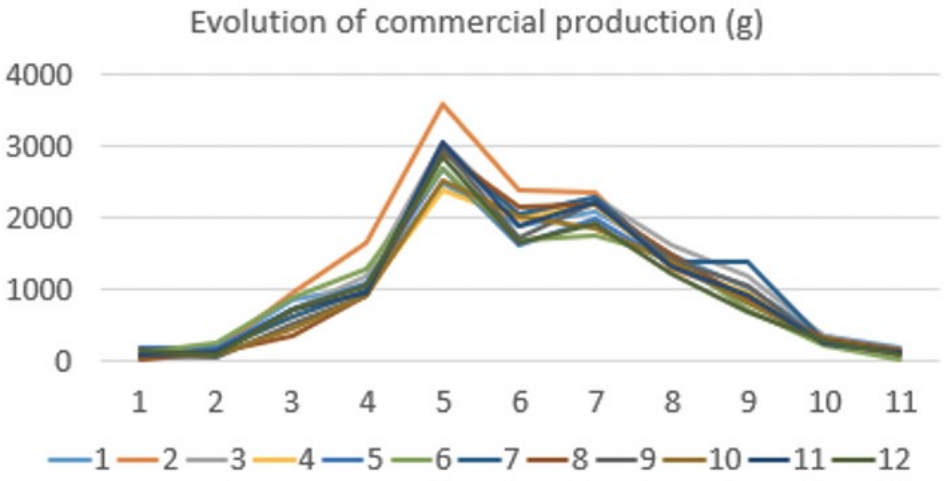

Figure 24: Evolution of commercial production in different treatments at each harvest time under open-field cultivation system.

At each harvest, waste products were collected and classified to allocate fruits affected by pathogen or fungi in minor incidence, notably Botrytis fungus as the main pathogenic agent. Among the eleven harvests, the production of infected/wasted fruit on plants treated with chemical fungicide and Serenade (biological fungicide) was 23.54 g and 45.65 g, respectively (Fig. 25). Overall, plots 5, 6, and 7 had the intermediate value of waste products and the best dsRNA regime minimizing mostly the damaged fruits caused by Botrytis was treatment no.6 following by 5 and 7.

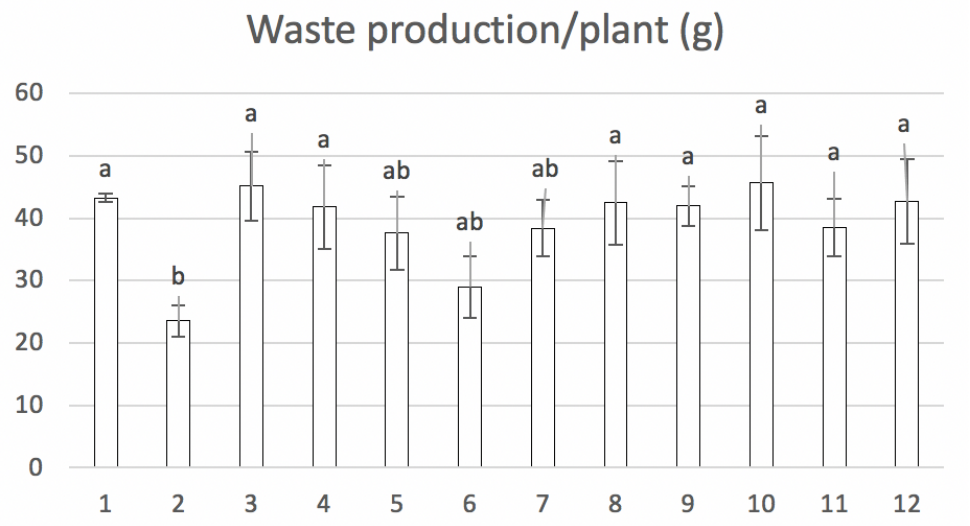

Figure 25: Waste production/plant (g) in eleven harvests. Means with different letters are significantly different according to the Duncan test ($p < 0.05$) ± SE. Error bars represent the standard errors of three replications for each treatment.

As shown in the graph, the climatic and humidity conditions were becoming less favorable for *Botrytis* infection, especially in June, corresponding to a reduction in waste/infected

production; nonetheless, this trend increased at the 9th harvest due to rain. For 10 out of 11 parcels considered, the fifth harvest reached the highest values of fruit waste correlating with the highest peak of fruit production, followed by a significant decrease. The lowest waste production was achieved during the first two and in the last harvests as the plants were near the end of the fruit production period (Fig. 26).

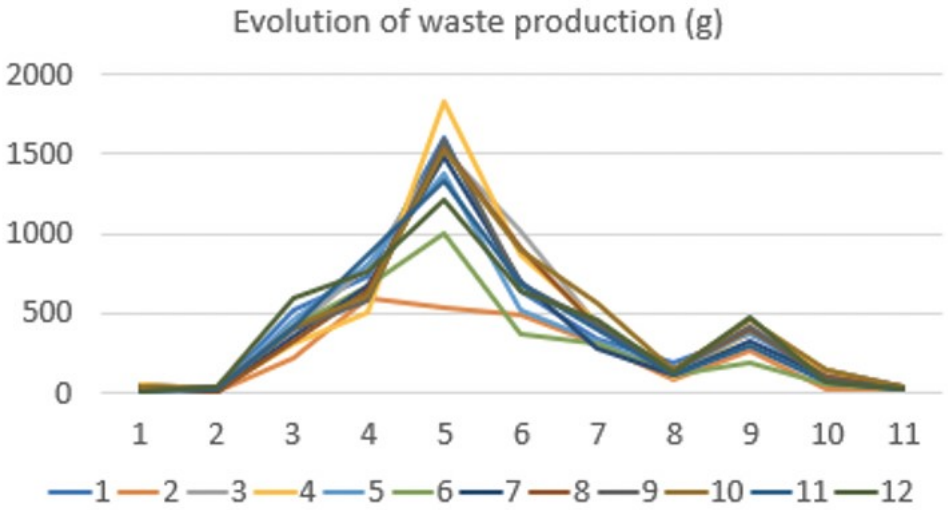

Figure 26: Evolution of waste production/experimental unit (g) of 11 harvests under classical open-field cultivation system

Disease severity at the harvest

Disease severity at the 1st and 2nd harvest

The low yield led to a high standard deviation among treatments; therefore, no statistical differences were detected. In general, it was observed that plants of blocks 1 and 3, in the lower side of the row, were earlier-matured, reaching average values of commercial productions of 221,5 and 138,75 g respectively than the plants grown in blocks 2 (91,46 g) and 4 (61.21 g) located in the upper side of the row, attributed to higher time exposure to the sun.

Nevertheless, the 9th treatment resulted in the least effective control of grey mold, with the highest disease severity, although was not significantly different from the untreated plants, reaching the minor value of disease control value (Fig. 27). Treatments 2, 3, 5, 6 seemed to provide a good level of protection against grey mold at this early time of harvest. In the first treatment, the protection for strawberries provided by chemical fungicide was as equal as the biological fungicide; however, on the consecutive harvest, treatment 3 had a higher protective effect against *Botrytis.*

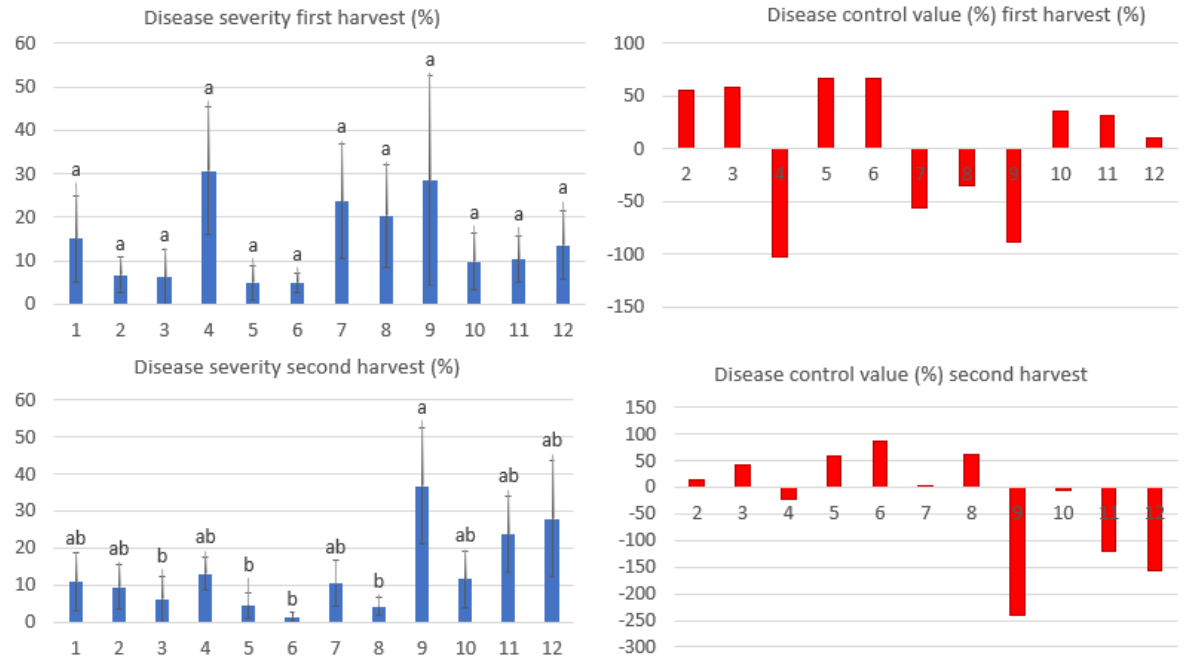

Figure 27: Disease severity (%) and disease control value (%) of grey mold on strawberry plants (at the 1st and 2nd harvests). Means with different letters are significantly different according to the Duncan test ($p < 0.05$) ± SE. Error bars represent the standard errors of three replications.

Disease severity at the 3rd harvest

At the third harvest, each plot produced at least 380 g of marketable production. The high variability among the various treatments in four blocks did not permit the identification of the best treatment to control grey mold, even though the amount of disease severity in fungicide-treated plants is half of that in the control treatment.

The graph indicated that the highest disease severity, in absolute values, was obtained on the control treatment; consequently, every fungicide/dsRNA-based treatment had an influence in reducing the disease severity with mild to high effect. All dsRNA treatments had a better control effect of the diseases compared to what was achieved from plants treated only with Serenade ASO (3) which was as infected as non-treated plants. (Fig. 28).

Among dsRNA-based treatments, no.11 and no.6 showed the highest level of protection, conferring control values higher than 40%, close to those obtained after the application of fungicides (52.50%).

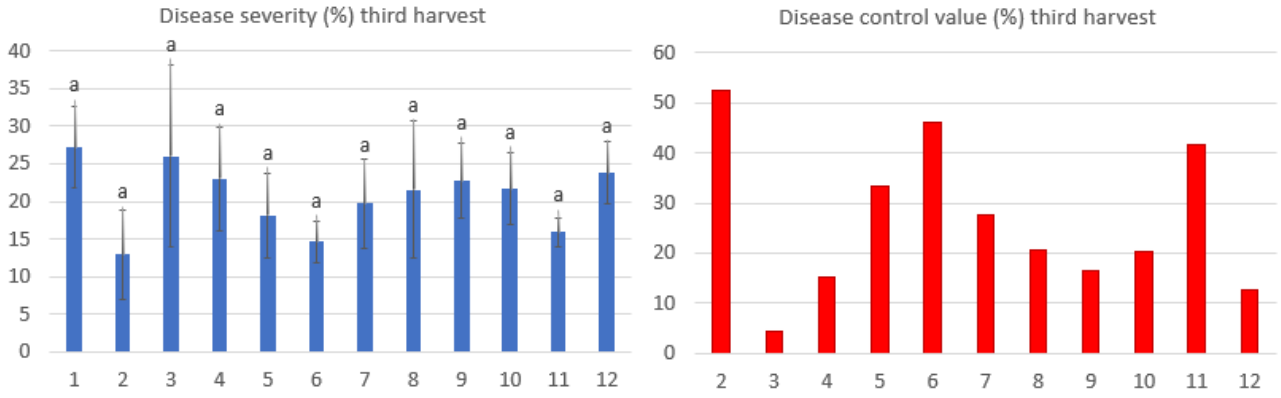

Figure 28: Disease severity (%) and disease control value (%) of grey mold on strawberry plants (at the 3rd harvests). Means with different letters are significantly different according to the Duncan test ($p < 0.05$) ± SE. Error bars represent the standard errors of three replications.

Disease severity at the 4th harvest

At this harvesting time, the lowest disease severity values were obtained in the second treatment and the sixth without a noticeable difference, followed by treatment no.4; general protection against *Botrytis* was observed in all treatments dealt with dsRNA. In contrast, treatment no.7 had disease severity as highest as a control treatment, even though no statistically differences have been detected from those that were treated with fungicide (Fig. 29).

Among the C formulations (4 to 7), the best performance was achieved by treatment 6, which seems to exert greater control than the Serenade ASO (treatment 3) and D group dsRNAs (8 to 12), to an extent comparable to what was observed in pesticides treatment. Even though based on the same formulation but in greater concentration, treatment seven performed with high disease severity, quite comparable to the untreated control, there may be a saturation effect due to high concentrations employed. Regarding the D formulation, the most efficient was the treatment at the highest concentration, able to reduce the disease severity by 22% (Fig. 29).

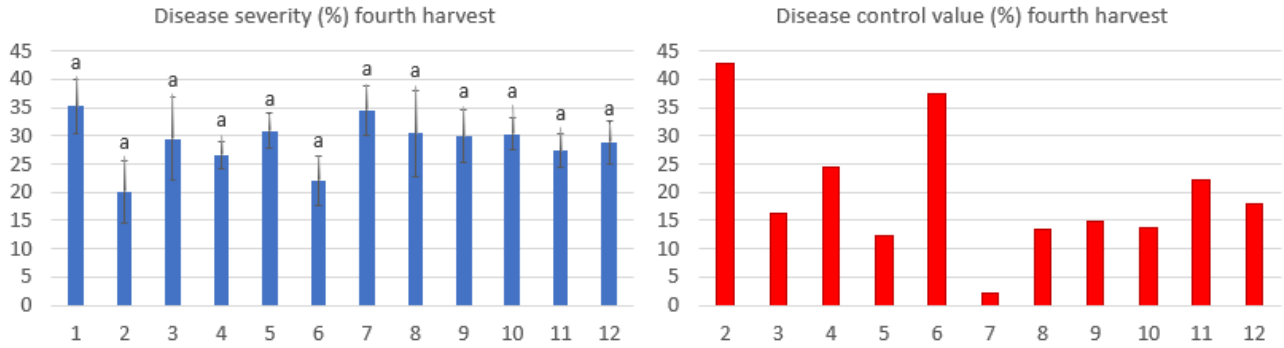

Figure 29: Disease severity (%) and disease control value (%) of grey mold on strawberry plants (at the 4th harvests). Means with different letters are significantly different according to the Duncan test ($p < 0.05$) ± SE. Error bars represent the standard errors of three replications.

Disease severity at the 5th harvest

At the first impression, except for treatment no.6, 11, and 12, all dsRNA-treated and organic treatment plants exerted the higher or comparable disease severity to untreated plants, ranging from 35 to 40 %. The commercial fungicide exhibited the best efficiency correlating with more than 60% of protection (Fig. 30).

This harvest was the most productive in terms of marketable yield, total, and waste production, in which the lowest values of disease severity were recorded in treatment 2, statistically different from the rest (Fig. 30).

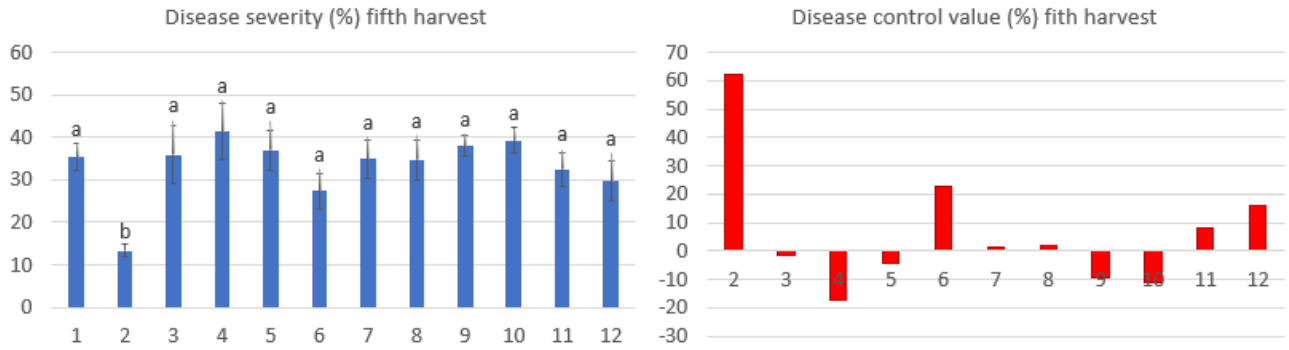

Figure 30: Disease severity (%) and disease control value (%) of grey mold on strawberry plants (at the 5th harvests). Means with different letters are significantly different according to the Duncan test ($p < 0.05$) ± SE. Error bars represent the standard errors of three replications

Disease severity at the 6th harvest

The level of disease severity at the sixth harvesting decreased overall by 10% in respect to the previous harvest, probably due to drier and windier seasonal conditions. As with all other harvestings, treatment no.2 still performed the best resistance to grey mold and it was statistically different from all others. The disease severity values of control plants and the treatment 4, 5, 8, 9, 11 were similar in statistical points, ranging from 15 to 23% (Fig. 31).

Similar to the fifth reap, treatment 6 confirmed the greater protection as the disease control value was the second-highest with 35%, preceded by fungicide-protected plants with nearly 50% (Fig 31). Noticeably, the biological pesticide-treated plants had the lowest disease control value in correlation with the highest disease severity and were statistically different from all other treatments.

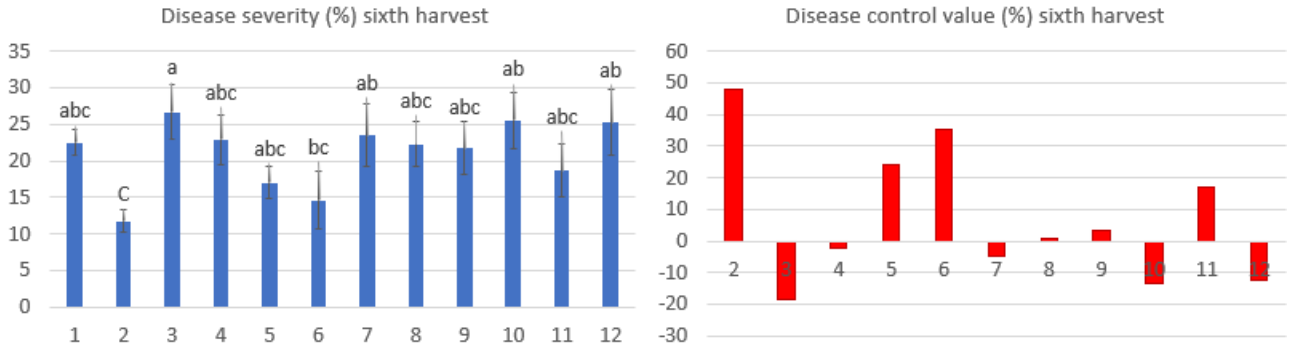

Figure 31: Disease severity (%) and disease control value (%) of grey mold on strawberry plants (at the 6th harvest). Means with different letters are significantly different according to the Duncan test ($p < 0.05$) ± SE. Error bars represent the standard errors of three replications.

Disease severity at the 7th harvest

A general reduction of the disease severity was experienced in almost all fungicide and dsRNA-based treatments, starting from the fifth to the sixth harvest. As in previous harvesting times, treatment 2 stood out as the most effective treatment, followed by treatment 6 (Fig. 32). Although all treatments were statistically resembling, the C group treatments (from 4 to 8), notably those with higher concentration, showed greater resistance to grey mold compared to the D group (8 to 12) counterpart (Fig. 32).

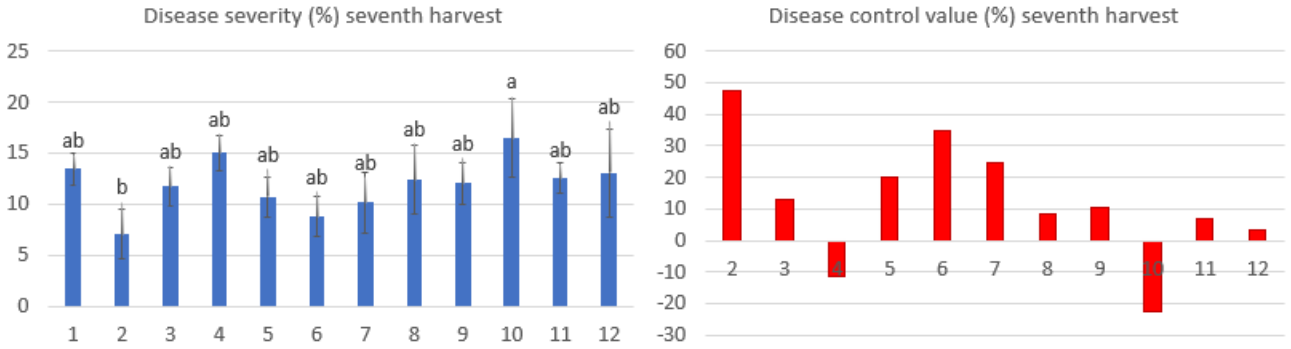

Figure 32: Disease severity (%) and disease control value (%) of grey mold on strawberry plants (at the 7th harvests). Means with different letters are significantly different according to the Duncan test ($p < 0.05$) ± SE. Error bars represent the standard errors of three replications.

Disease severity at the 8th and 9th harvest

As in all treatments, in the 8th treatment, the lowest disease severity values were nearly always observed in the favourable treatment no.2, and those of highest values were obtained in the negative control (treatment 1). Regarding dsRNA-treated groups, treatment 6 acquired the lowest disease control value for the first time, approximately to the non-treated plants (Fig. 33). In contrast, probably due to the rain of some days before the 9th harvesting, *B. cinerea* had the

ideal environment to attack the fruits. As shown in the graphs (Fig. 33), the disease severity values were almost double compared to the previous harvest time. Unlike the 8th harvest, the six treatments at this time increased the protective effect against grey mold but not significantly. Regime 3 also exhibited a noticeable resistance compared to itself in previous harvest times.

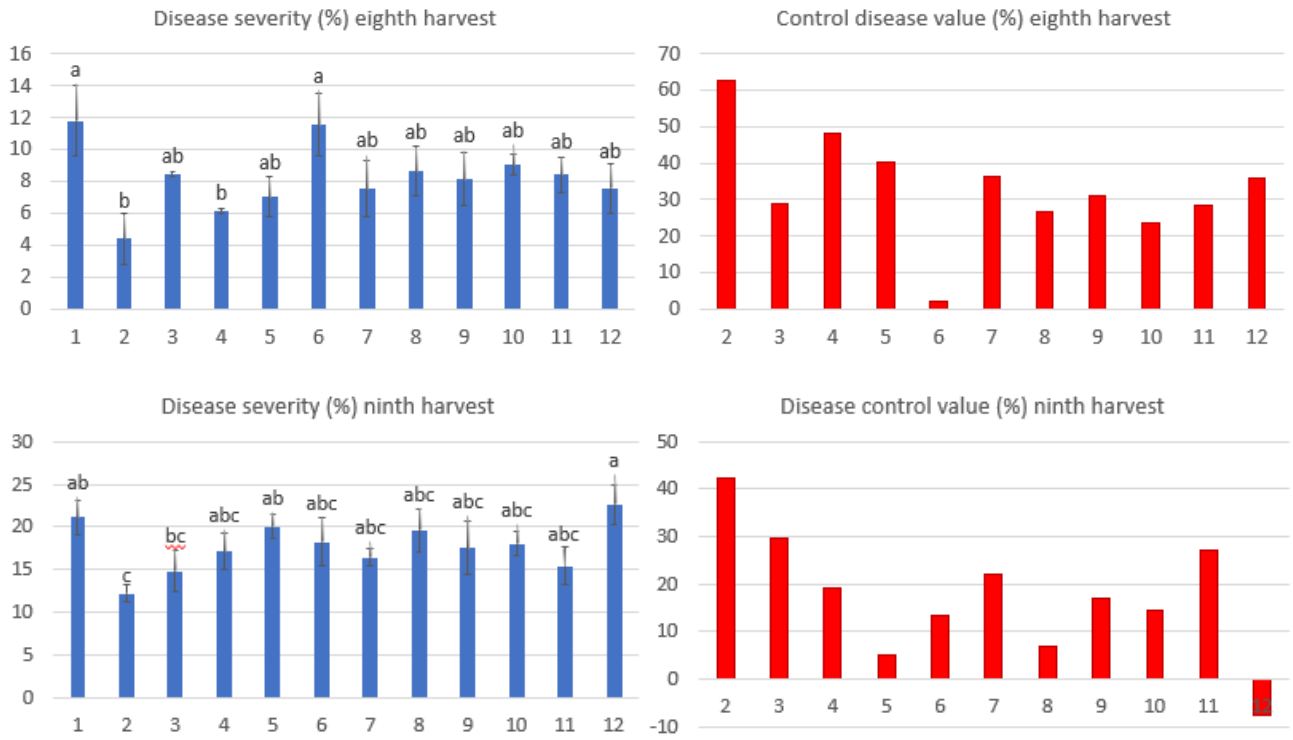

Figure 33: Disease severity (%) and disease control value (%) of grey mold on strawberry plants (at the 8th and 9th harvests). Means with different letters are significantly different according to the Duncan test ($p < 0.05$) ± SE. Error bars represent the standard errors of three replications.

Disease severity at the 10th and 11th harvest

At the 10th harvest, a generally higher infection was detected in all regimes from 10 to 15%. A higher level of resistance was detected at the increasing concentration of C formulations. Treatment 9 of the D group was the most effective among all others, reducing the disease severity by 50%, comparable to those exerted by treatment 2 (Fig. 34).

This was the ending of the harvesting period; disease symptoms started generally declining from 10-15%; all treatments effectively performed the *Botrytis*-control function. In the 11th harvest, fungicides and all dsRNA-based treatments had an excellent capacity to control the infection of the disease, on average about 40% in comparison with the non-treated control. Regarding the C group, the lower concentration of dsRNA gave higher protection than those of greater dsRNA content. In contrast to the C group, only the highest concentration in D regimes allowed considerably reducing grey mold on fruits (Fig. 34).

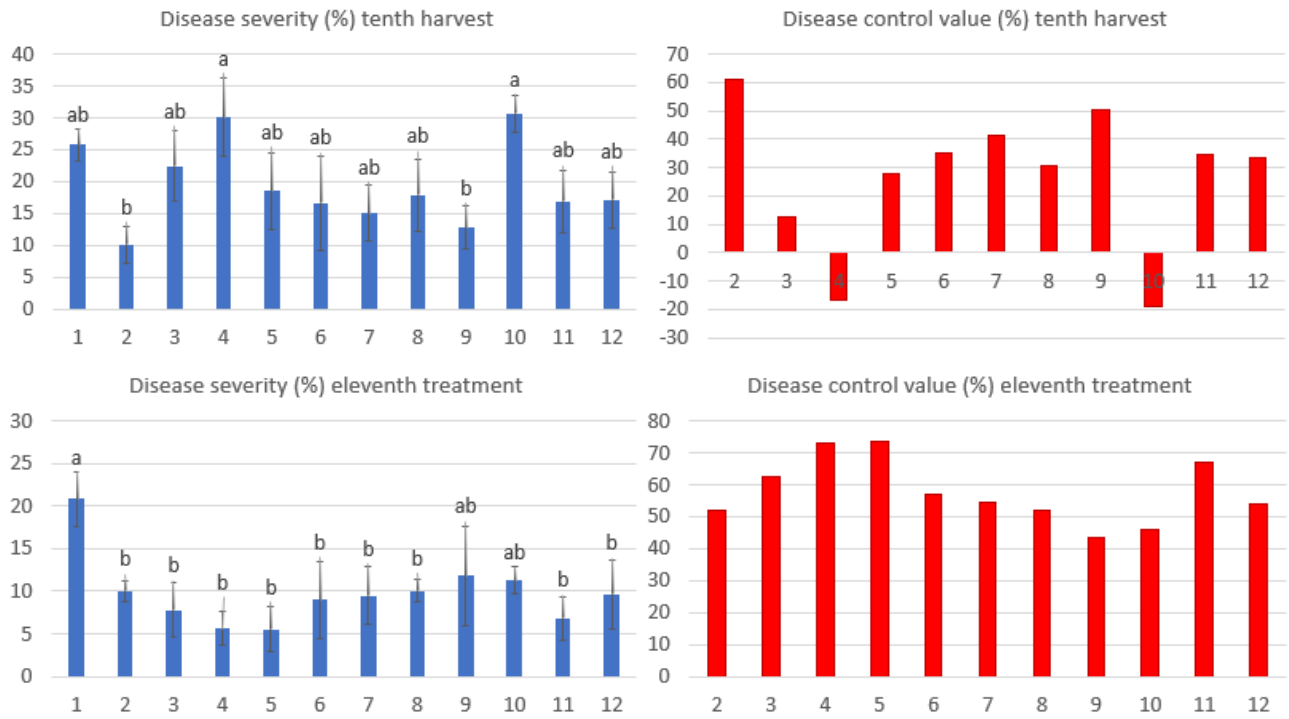

Figure 34: Disease severity (%) and disease control value (%) of grey mold on strawberry plants (at the 10th and 11th harvests). Means with different letters are significantly different according to the Duncan test ($p < 0.05$) ± SE. Error bars represent the standard errors of three replications.

Disease severity (%) and Disease control (%) value general considerations during the period of 11 harvests

Resuming what has been observed during the eleven harvests, general evaluations are reported below, discerning the different classes of treatments (Fig. 35):

-The fungicide treatment was the best performant to counteract grey mold with 50% efficacy.

-Serenade (organic fungicide) allowed controlling the grey mold by 20%.

-Concerning the treatments with C dsRNA, the most effective formulations were 5 (25 g/ha) and 6 (50 g/ha), offering quantifiable protection between 35 and 40% compared to the untreated control. Although belonging to the same category, treatment four was found to be almost ineffective, and treatment 7 was as effective as treatment 3 and treatment 8 of the D group. This is probably because the concentration of treatment 6 is the critical point of C-dsRNA, above which the performance starts decreasing.

-D formulations reached a good level of protection, only treatment 8 and 11 comparable to treatment 3, while the other seems to have a slight or nil effect, especially treatment 12 for the reason that it was applied once a week. The C formulations (4 to 7) offered higher protection against *Botrytis* compared to the D group (8 to 12) that has a different silencing target.

→In a nutshell, ds-RNA treatments provided an effect in reducing the pathogenicity of grey mold on strawberries, with an extent dependent on the concentration, formula, environmental

conditions.

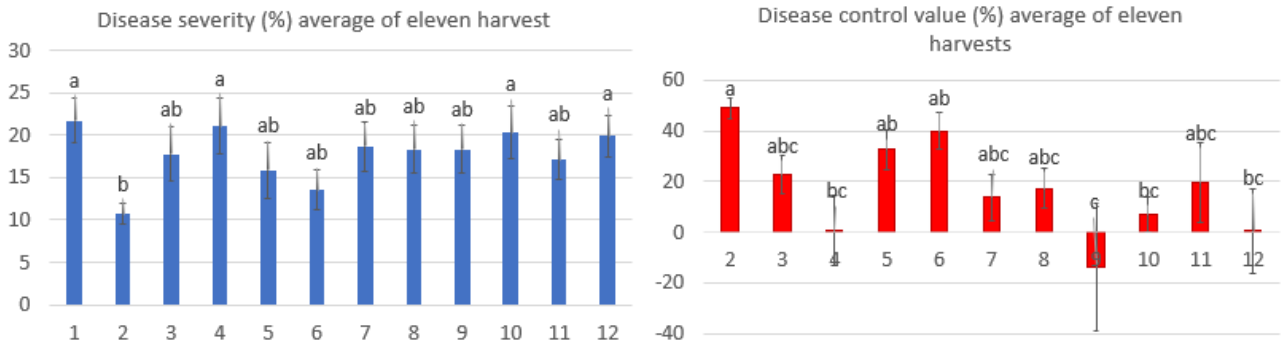

Figure 35: Average of disease severity (%) and disease control value (%) of grey mold on strawberry fruits recorded in eleven harvests. Means with different letters are significantly different according to the Duncan test ($p < 0.05$) ± SE. Error bars represent the standard errors of three replications.

Disease severity during post-harvest

After counting and weighing all harvested fruit, the best 30 fruits were used to evaluate the disease progression in post-harvest conditions. These fruits were placed above a filter paper soaked with distilled water to ensure a high humidity content in transparent plastic boxes. During the trial, the boxes were maintained at room temperature in dark conditions. At three- and seven days of post-harvest conditions, fruits were examined regarding the disease severity. Data have been recorded only for fruit from the first 4 weeks. For the last harvest (5th week), data were not reported due to the higher temperatures which facilitated the reduction of the strawberry shelf-life period.

At first glance, non-treated fruits were strongly attacked by *Botrytis* in the first 3 days of post-harvesting and were statistically different from all other treatments, whereas the treatment 6 belonging to the C group had the lowest value of disease severity nearly homologous to the best option, CHEMICAL PESTICIDES - treatment 2, correlating with the highest disease control value of this two treatment (Fig. 36). The extent to which dsRNA-treated strawberries infected by grey mold were in an intermediate intensity of disease between the negative and positive controls, even though they were not statistically different (Fig. 36).

In terms of 7 dph, half of all treatments were highly infected, almost higher than 95%, resulting in loss of fluid and leakage of color; treatments 5, 6, 7 were infected around 85%, and this value in Teldor-Signum-Switch treatment was around 70% (Fig. 36). The protection effect of all regimes could not last up to 7 days after harvesting leading to a significantly lower effect compared to 3dph. Consequently, 23% of disease control value was observed after the application of positive fungicide treatments, 10% was the percentage of this value regarding the treatments 3, 4, 5, 6.

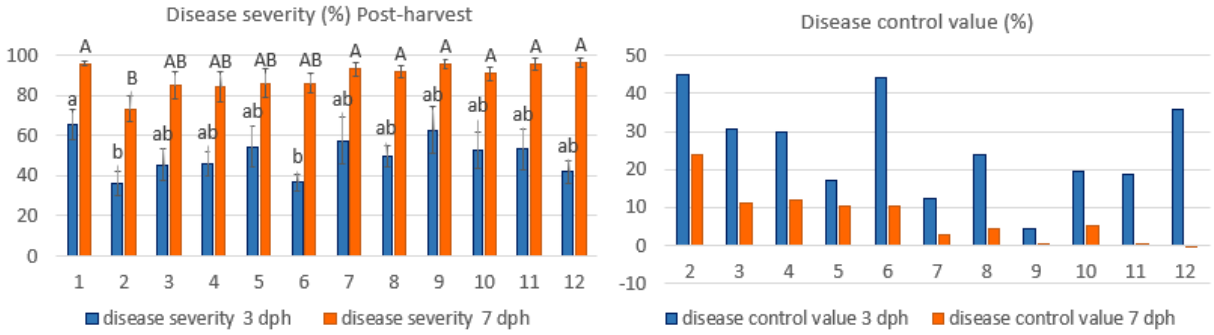

Figure 36: Average of disease severity (%) and disease control value (%) at 3 and 7days post-harvest.

Figure 37: Fruits stored at room temperature 3 days post-harvest of 12 treatments.

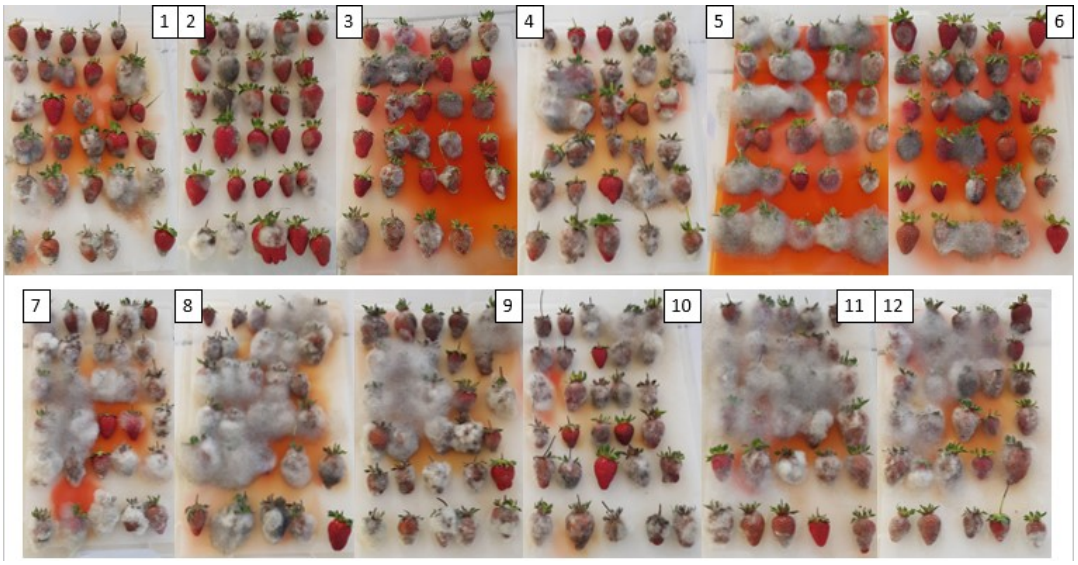

Figure 38: Fruits stored at room temperature 7 days post-harvest of 12 treatments.

Final observation:

The data collected during post-harvest experiments confirmed what was observed in the field

at the time of harvest:

- Chemical fungicides are the best option to handle the number and diffusion of necrotic lesions caused by *Botrytis cinerea* during post-harvest conditions.
- Overall, at almost the same concentration, the C-group regimes granted better insurance to withstand grey mold, near to the extent triggered by chemical fungicide and in greater quantity compared to organic fungicide.
- Regarding the C group (4 to 7), the medium concentration of dsRNA (plots 5 and 6) gave higher protection than those of greater dsRNA concentrations. In contrast to the C group, only the highest concentration-treatment 11, in D (8 to 12) regimes allowed a considerable reduction of grey mold on fruits
- Biological treatment can be considered as comparable to the highest concentrate of C dsRNA (7), also to the lowest and highest concentrations of D group dsRNA.

Sugar and acidity content

The ANOVA analysis performed on the data of the "Tea" variety fruits treated with 12 different formulations against *Botrytis cinerea* and harvested at 5 different dates revealed that the treatment did not affect the sensorial quality of the fruits, in terms of Soluble Solids Content (° Brix) and Titratable Acidity (% of Citric Acid) (Table 5).

Factors	Soluble Solids Content	Titratable Acidity
Treatment	n.s.	n.s.
Harvest date	*	*
Treatment - harvest date interaction	*	*

*Table 5: ANOVA analysis for the factors (treatment and harvest date) and the parameters (Soluble Solids Content and Titratable Acidity) N.s. = not. Significant interaction; * = significant interaction for $p<0.05$.*

In terms of soluble solids content, the highest value was witnessed in plot 5 followed by biological and treatment no. 12 with 8,5 and 8,4 respectively. Treatment 7 had an average value of 7,6 º Brix ranking the lowest position. Overall, treatments did not affect the soluble solid content of strawberries, albeit there was a fluctuation in the sugar content among 12 formulate, ranging from 7,6 to 8,6 º Brix, significantly higher than greenhouse-cultivated fruits in which the values averaged around 6.3 to 7 º Brix (Fig. 37)

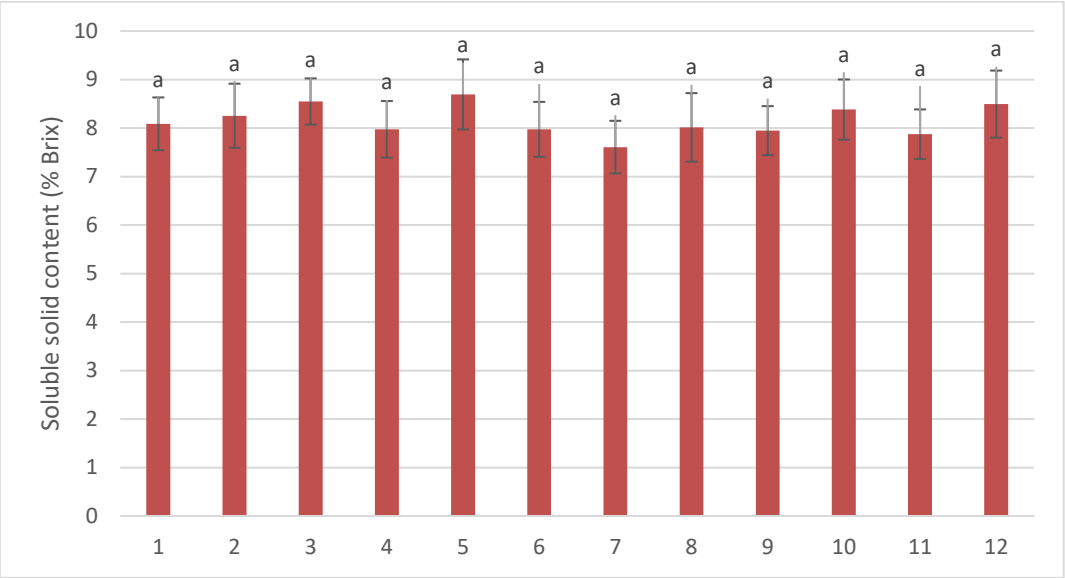

Figure 39: Soluble Solids Content of fruits obtained at different treatments. Means with different letters are significantly different according to the Duncan test ($p < 0.05$) ± SE. Error bars represent the standard errors of three replications for each treatment.

The highest value of acidity was experienced in plots 1 and 9, followed by treatment 3 with hardly noticeable differences. On the contrary, treatment 5 had the lowest value of titratable acidity content. Based on the comparison of the two trials conducted on the greenhouse and the open field, field-based fruits had higher acid content with 0,77 on average in contrast to 0.71 on the tunnel system. Reasonably speaking, the treatments did not affect this sensorial parameter (Fig. 38)

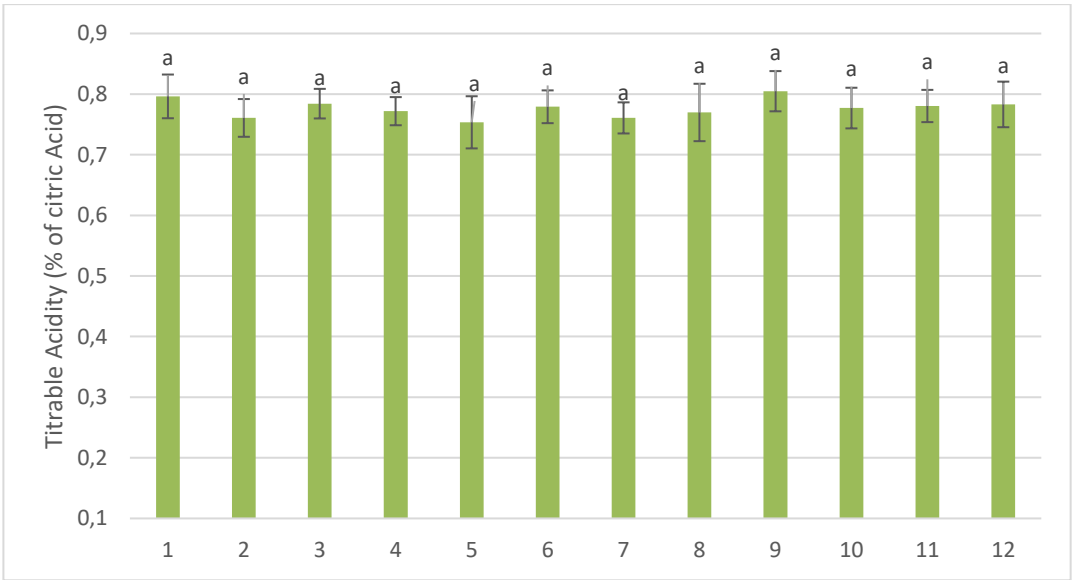

Figure 40: Fruit Titratable Acidity obtained with different treatments. Means with different letters are significantly different according to the Duncan test ($p < 0.05$) ± SE. Error bars represent the standard errors of three replications for each treatment

These results revealed that sensorial quality, notably sugar content and acidity, are affected by

fruit variety and environment for plantation, but not influenced by treatments whether biological, fungicide or dsRNA formulates.

The harvesting period had a major influence on this trait (Fig. 39). The first three harvests were completely statistically different from one to another; however, the intermediate values were witnessed in the first and the third harvests. Regarding the evolution of intrinsic sugar concentrate throughout 5 harvestings, times of harvesting greatly influenced this parameter, with the highest values that have been detected on the two-last harvest; whereas the least value was obtained at the 2nd time, probably because of the increasing environmental temperature of the last two harvests, corresponding to the end of strawberry season and a consequent lower production load, positively affected the sugar content.

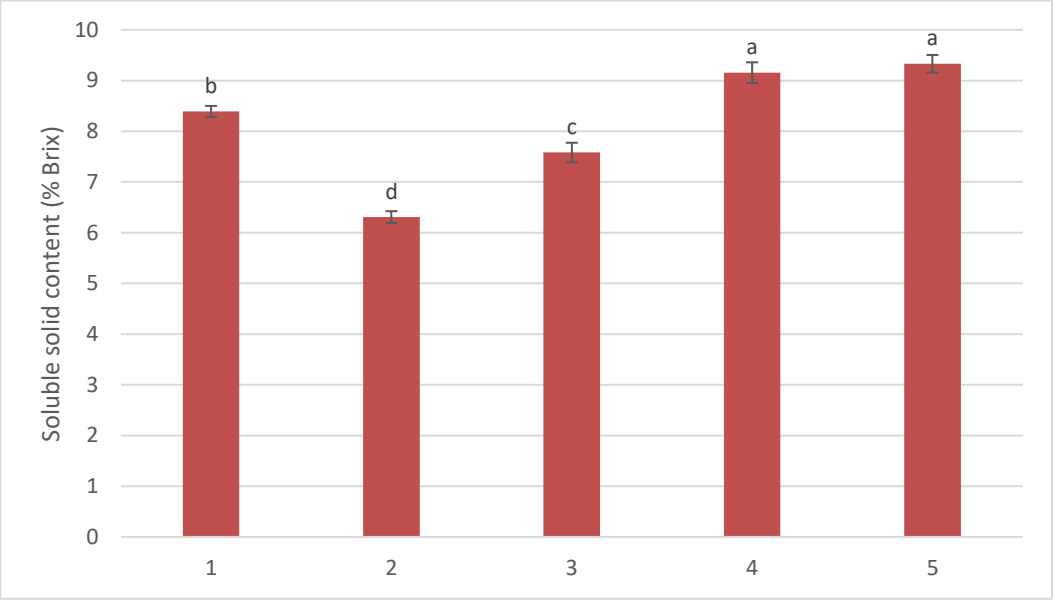

Figure 41: Soluble Solids Content of fruits obtained at different harvest dates. Means with different letters are significantly different according to the Duncan test ($p < 0.05$) ± SE. Error bars represent the standard errors of three replications for each treatment.

The analysis of the effect of harvest date on fruit Titratable Acidity (Figure 40) revealed that the fruits harvested in the first two dates were significantly less acid than the fruits harvested at the third, fourth and fifth date. The fruits harvested at the third date presented intermediate values of acidity, between the fourth and the fifth harvest date (in which the fruits were generally more acid). A hypothetical theory may be withdrawn from these outcomes, higher temperature at the end of the strawberry season may affect this sensorial parameter (Fig. 40).

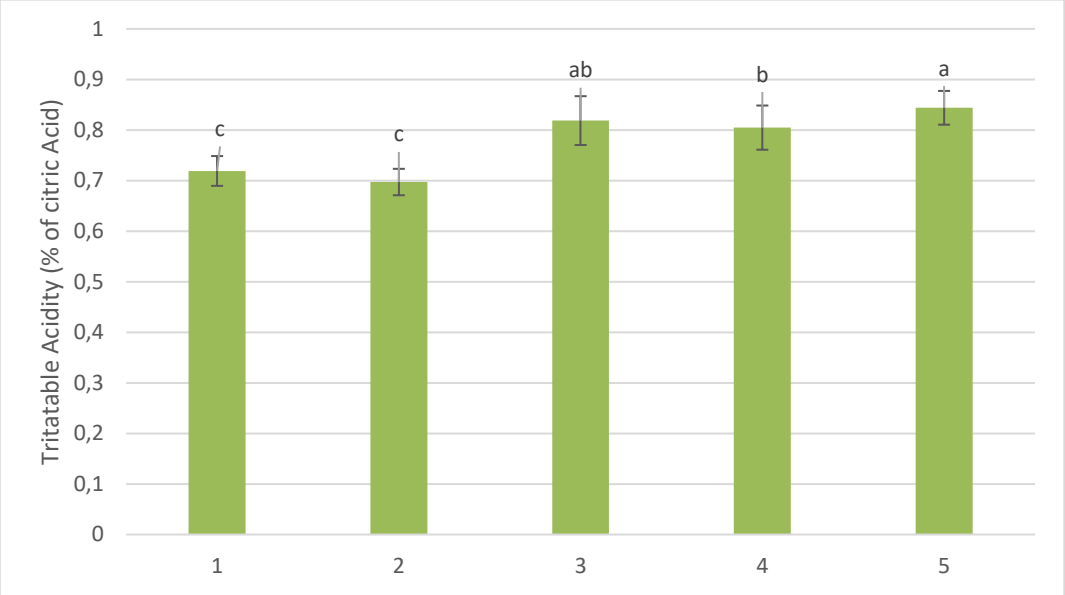

Figure 42: Fruit Titratable Acidity obtained at different harvest dates. Means with different letters are significantly different according to the Duncan test ($p < 0.05$) ± SE. Error bars represent the standard errors of three replications for each treatment.

5. CONCLUSION

The purpose of this study was to determine the impact of Spray-Induced Gene Silencing based on double-stranded RNA toward strawberry against *B. cinerea*, as a basis for the development of new alternative products to chemical pesticides to control strawberry diseases, minimizing food waste. Collected data from two types of environmental and cultivation conditions (greenhouse-soil-less, open field) has proven the positive effects of this approach.

Regarding the greenhouse trial, the fungicide application performed with the best results. Comparing the two trial scales, fungicide treatment in the greenhouse has a higher protective effect in post-harvest than open field A similar event happened during post-harvest. Probably since open field deposited higher infectious conidia in fruits; however, strawberries still get damaged after 7dph in high humidity conditions. The dsRNA in both A and B formulations positively affected *Botrytis* infection with a variation among four harvests, all registering higher than 20% of the grey mold control value; consequently, the difficulty of identifying the best performance had been encountered. All A, B formulations had limited effect in maintaining the protection of fruit after 4 and 7 days of storage at ambient temperature, excluding treatment 6 and 8 over the third harvest at 4 dph. The period from the last treatment to the last harvest was around 11-18 days, during which at fourth harvest the protection was still witnessed; however, at the last harvest the protection effect did not perform effectively; hence, a hypothesis that dsRNA-based products seemed to have 14 days-period of protection could be proposed.

In the open field, *Botrytis*-appealing surroundings, the combination of different fungicides (Teldor, Signum, and Switch) were applied, leading to highly advantageous outcomes; however, similar to greenhouse experiment, strawberries still get damaged after 7dph in high humidity conditions, during post-harvest. In terms of biological fungicide - Serenade ASO, based on Bacillus subtilis strain, exclusively applied in the experimental open field cultivation system, this product had a limited effect in controlling grey mold progression on the fruit surface, around 20% compared to fungicide-combined treatment (50%), at the time of harvesting. Notwithstanding the low protective effect during harvest, Serenade ASO had an acceptable response in post-harvest, almost exclusively at 3dph (30%). Regarding the C regimes, notably with dsRNA-increasing content treatments (5 and 6) offered much more significant control issues, 35 and 40% respectively, near the ratio triggered by the positive fungicide control. Treatment 7 corresponding to the highest concentration, belonging to the same formulation C worked less efficiently compared to treatment 6. This event can be justified by coming up with a theory that the content at treatment 6 is the critical point of this

formulation, above which the efficacy declines. In D dsRNA-based formulations, 19% of fruit protection from *Botrytis* mold was attained by applying treatment 11 with the highest concentrations of dsRNA. Together with treatments 7 and 8, these three concentrations were comparable to the Bacillus subtilis treatment. Among all the dsRNA-based treatments the most efficient were formulated A dsRNA molecules in the greenhouse/soil-less cultivation conditions and C dsRNA in the standard strawberry open field production system. With the same gene target, the production of formulations compared to the naked molecules of dsRNA allows guaranteeing greater effectiveness of resistance, probably due to a prolonged persistence on the surfaces of the fruits of the molecular actors of gene silencing. All C, D formulations had sufficient effect in reducing the fruit stored during post-harvest infection, especially at 3 dph.

Concerning the sensorial quality, especially sugar and acid content, the greenhouse-based fruit was influenced greatly by harvest day and its interaction with different treatments; however, the treatments did not impact the Soluble Solids Content nor Titratable Acidity. A similar trend was detected in the open strawberry field where the sensorial parameters were not affected by different treatments, but the harvest time was the source of the variation to great extent. The high environmental temperature at the end of the harvesting season probably altered these sensorial elements with the higher concentrate at the last two harvests in terms of sugar, the last three harvest regarding acidity.

Limitations and Recommendations from the study

The nutritional analysis of strawberries grown in the open field is primarily important to be conducted for the purpose of comparing nutrients between two cultivars used in the two experiments (open field and greenhouse ones), both highly susceptible to *Botrytis,* and to assess the impact of group C and D treatments toward intrinsic nutrition. Due to the COVID-19 pandemic, this evaluation has not been operated. As a result, this study can not conclude any affirmation about the nutritional value of strawberries in the open field. Therefore, these analyses need to be examined by other colleagues in the foreseeable future.

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