Control efficacy of a new SIGS-based biofungicide against *Penicillium digitatum* on citrus fruits

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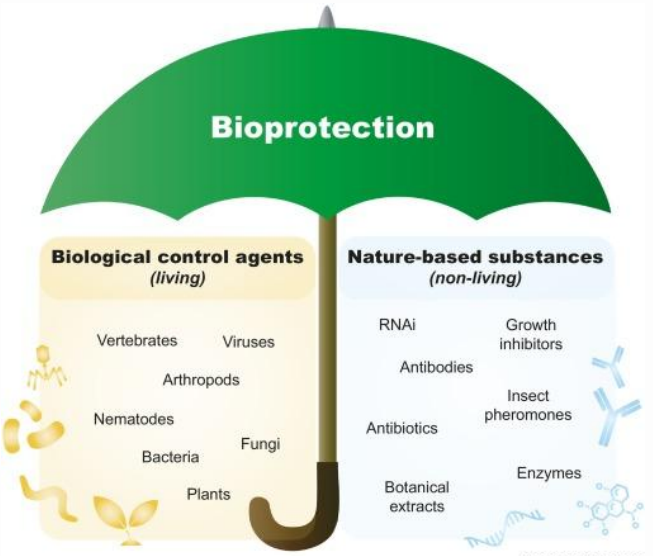
The project titled "Innovative solutions for sustainable and environmentally friendly plant protection of Greece's horticultural crops in the Europe of the future" (TAEDR-0535675) was implemented under the National Recovery and Resilience Plan "Greece 2.0", with funding from the European Union – NextGenerationEU.

Current Challenges in Agriculture

- Going through the new era of the European Green Deal, the need for more sustainable methods to control plant pests and diseases is a necessity.
- A targeted 50% reduction in chemical pesticide use is a key objective under current sustainability policies.
- Developing innovative and sustainable solutions for plant protection

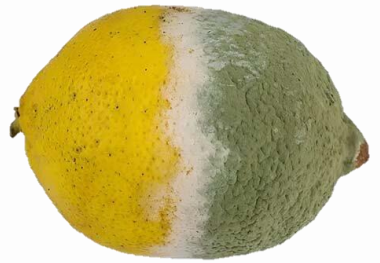
- **Biotechnological tools** → RNAi (RNA interference)
  - HIGS (Host Induced Gene Silencing) → GMO → EC 2001/18 & EC 1829/2003
  - **SIGS** (Spray Induced Gene Silencing) → PP → EC 1107/2009



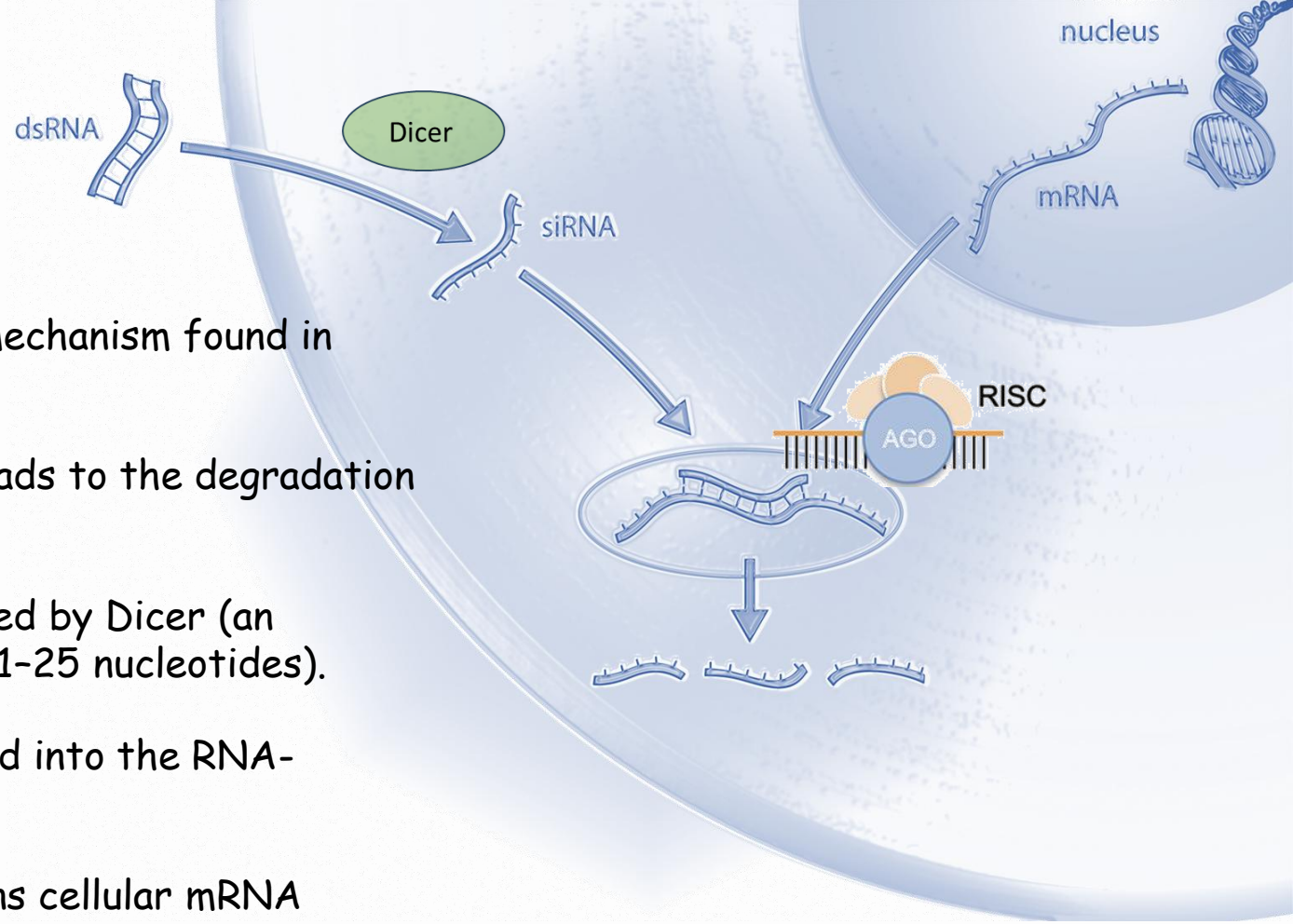
(Stenberg et al., 2023)

Citrus vs. *Penicillium digitatum*

- Green Mold of citrus fruits caused by the fungus *P. digitatum*
- Opportunistic pathogen→ Entry through wounds
- Is considered as the most important postharvest disease of citrus fruits
  - It accounts for approximately 90% of postharvest losses
  - Postharvest application with synthetic fungicides (Imazalil, thiabendazole, pyrimethanil etc.)
- Risk of resistance development and increasing consumer concern about product safety → Leads to the search for alternative control methods

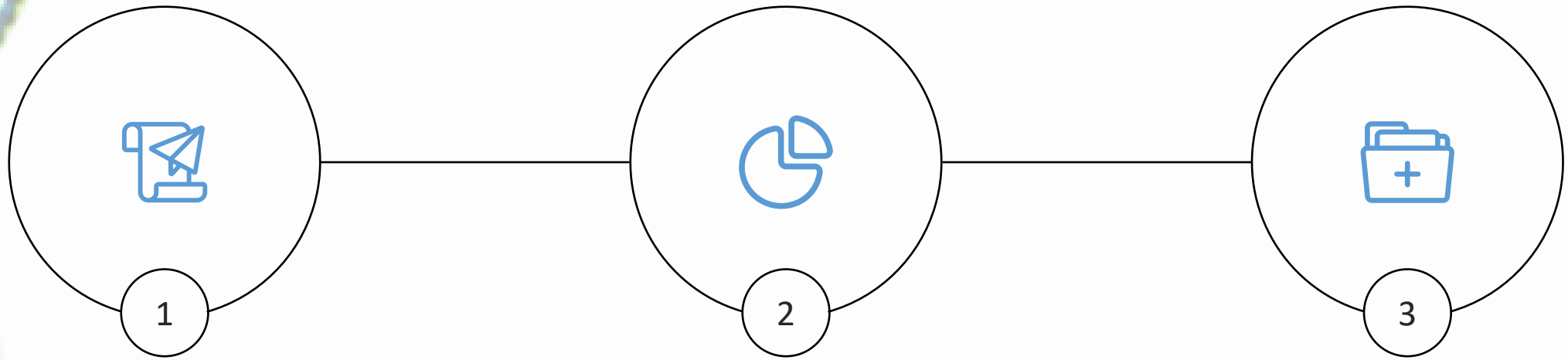


RNAi



- RNA interference (RNAi) is a natural gene-silencing mechanism found in eukaryotes
- It is triggered by double-stranded RNA (dsRNA) →leads to the degradation of specific messenger RNAs (mRNAs)
  - **Dicer processing**: dsRNA is recognized and cleaved by Dicer (an endonuclease) into short fragments (siRNAs) (~21–25 nucleotides).
  - **RISC assembly**: siRNA (antisense) is incorporated into the RNA-induced silencing complex (RISC).
  - **Target recognition**: The siRNA-loaded RISC scans cellular mRNA molecules for sequences complementary to the guide strand.
  - **Argonaute**: After binding to the target mRNA→ cleaves the mRNA, leading to its degradation and gene silencing.

Aims of the study



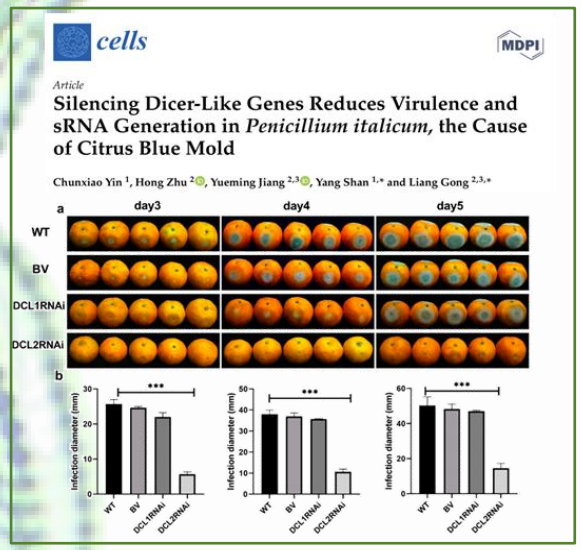
Selection of target genes & *in vitro* synthesis of dsRNA

Evaluate the effectiveness of dsRNA application against *P. digitatum* on citrus fruits

Investigate the control efficacy of siRNAs against P. *digitatum*

Selection of target genes and *in vitro* synthesis of dsRNA

Existing literature


cells

MDPI

Article

Silencing Dicer-Like Genes Reduces Virulence and sRNA Generation in *Penicillium italicum*, the Cause of Citrus Blue Mold

Chunxiao Yin [1], Hong Zhu [2], Yueming Jiang [2,3], Yang Shan [1,*] and Liang Gong [2,3,*]

The Idea

Develop a SIGS-based biofungicide

Selection of target genes essential for the RNAi machinery

AGO1 & AGO2

DCL1 & DCL2

Control efficacy of dsRNA

In vitro synthesis of dsRNA (MEGAscript™RNAi Kit)

In vivo efficacy assessment on citrus fruits:

- Oranges (cv. Salustiana)
- Mandarins (cv. Satsuma)
- Lemons (cv. Maglini)

In vivo efficacy assessment on citrus fruits

Plant Material

cv. Salustiana

Experimental Design

Sample Setup

- Total fruits: 16
- Replicates: 4
- Fruits per replicate: 4

Incubation Conditions

Temperature: 25°C

Incubation dur. :
5 days
10 days

Disease Assessment

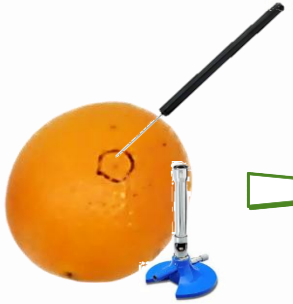
5 & 10 dpi

Disease Severity (cm)

Disease Pressure Index

$$DSI = \frac{DS \times DI}{100}$$

Surface sterilization (NaClO 1% - 3min)



Artificial wound (3mm)

Application of dsRNA (800ng)

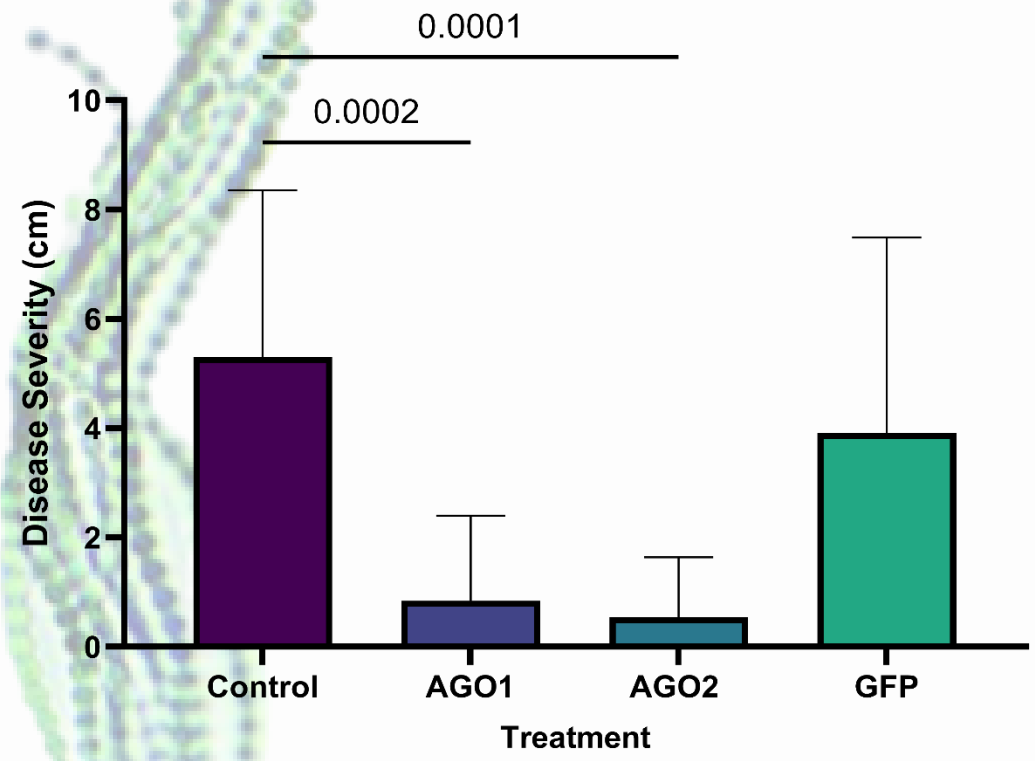
Incubation (2 hours – 25°C)

Artificial inoculation (10^5 conidia ml^{-1})

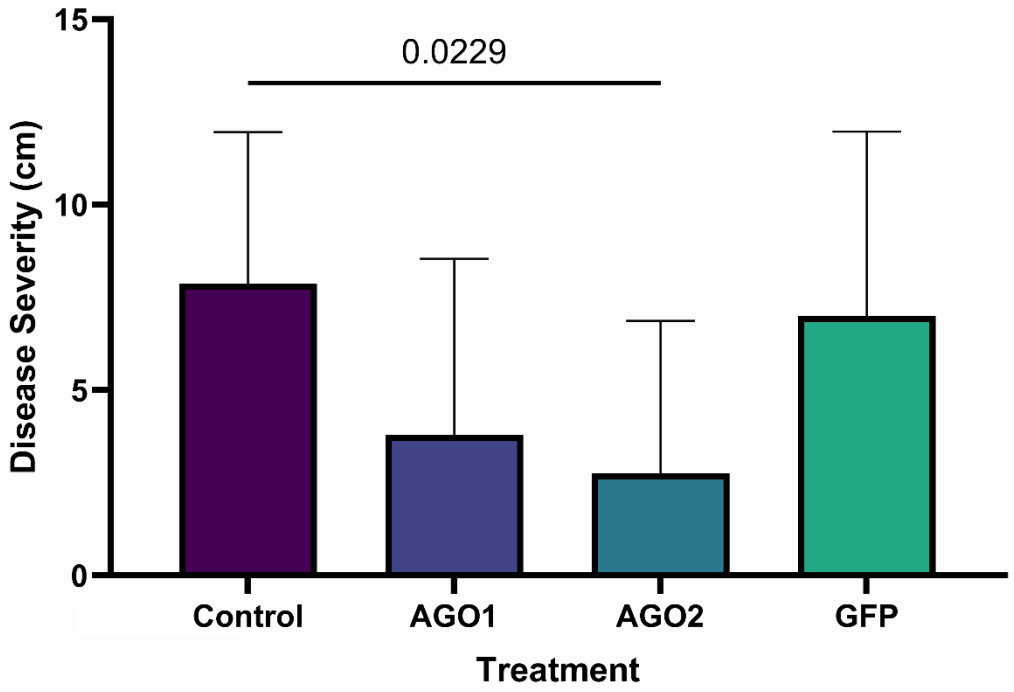
Incubation (5 & 10 days)

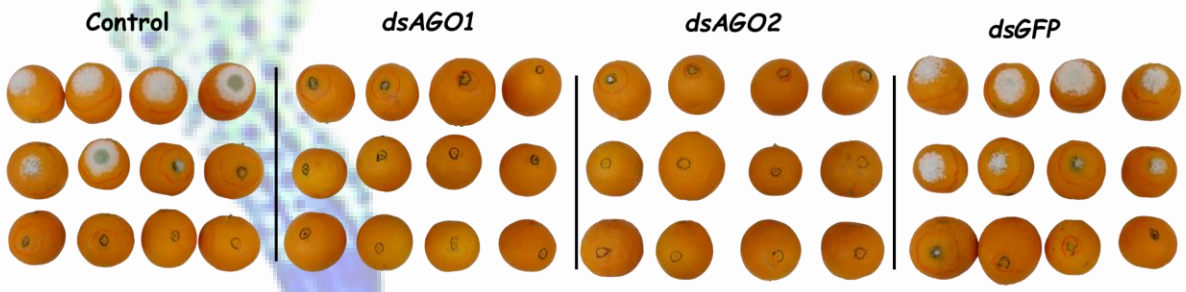
In vivo efficacy assessment on orange fruit | AGO1 & AGO2

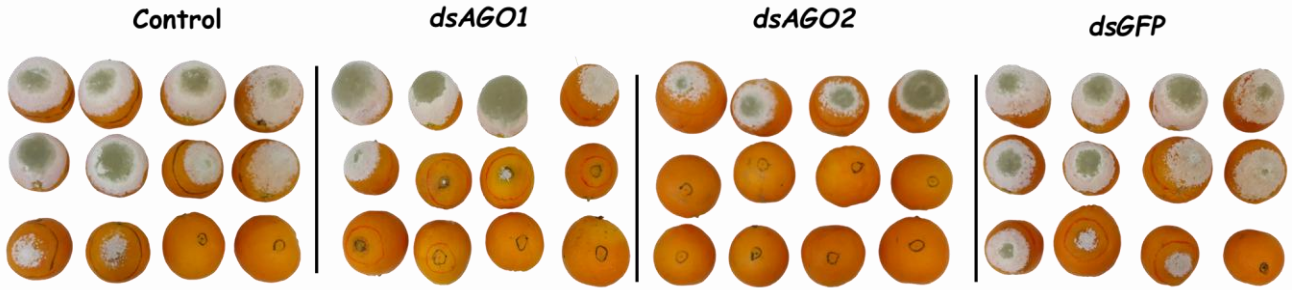
5 dpi



10 dpi

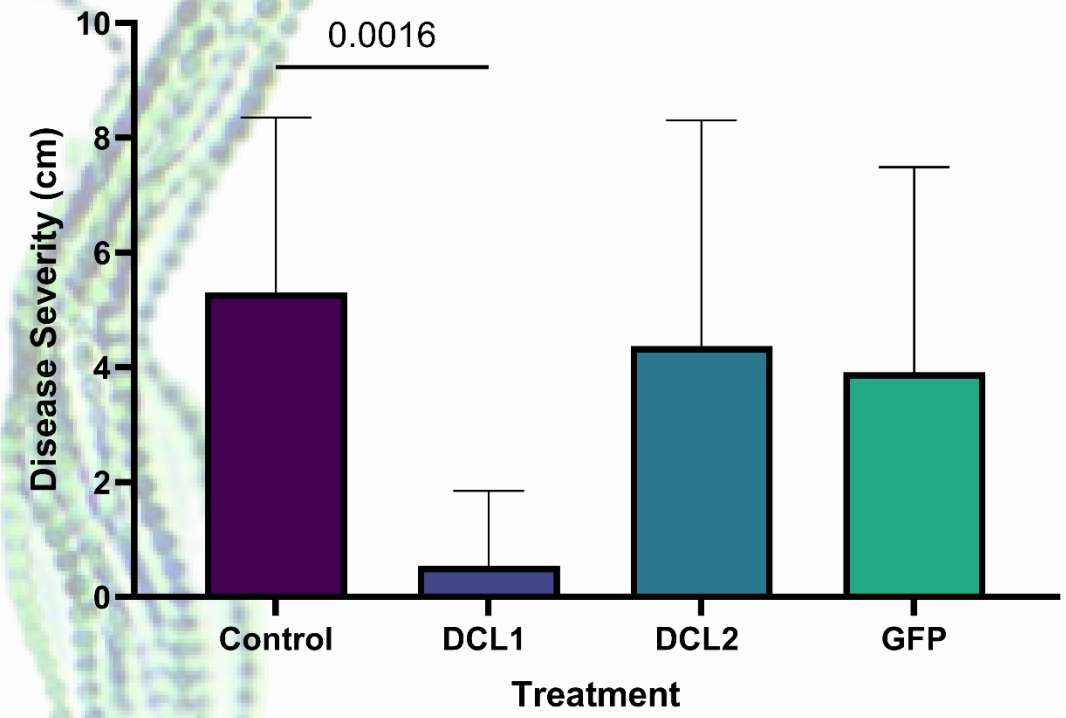




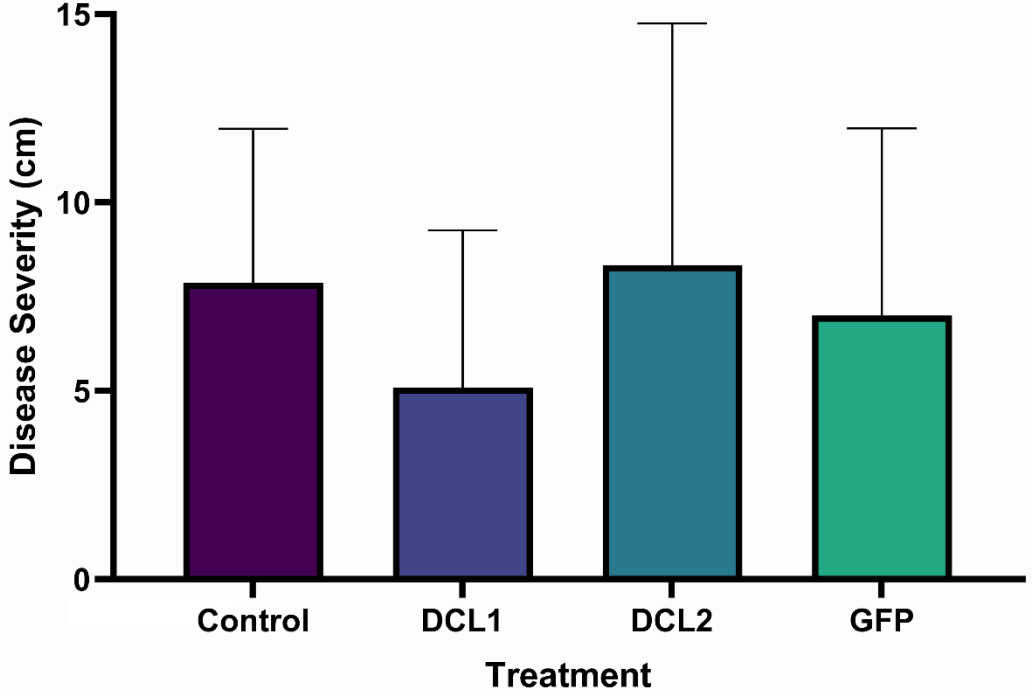


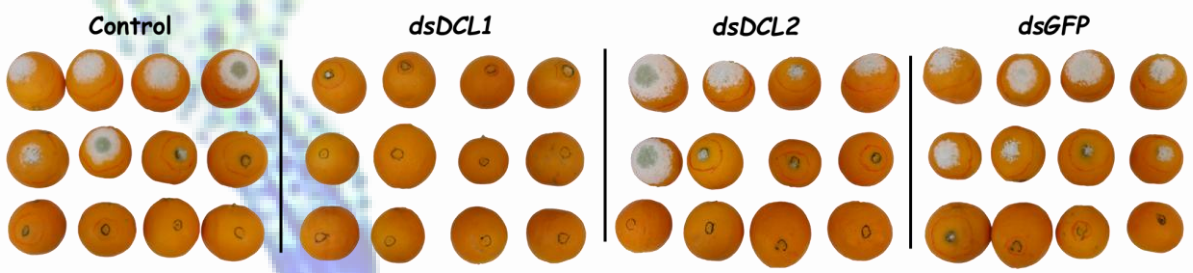
In vivo efficacy assessment on orange fruit | DCL1 & DCL2

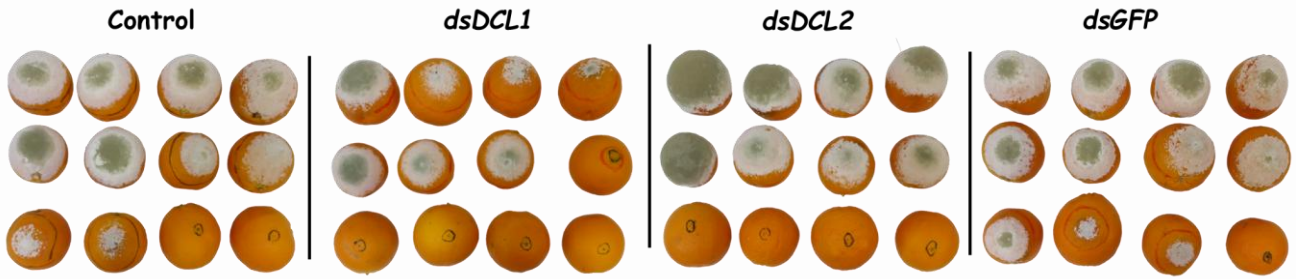
5 dpi



10 dpi





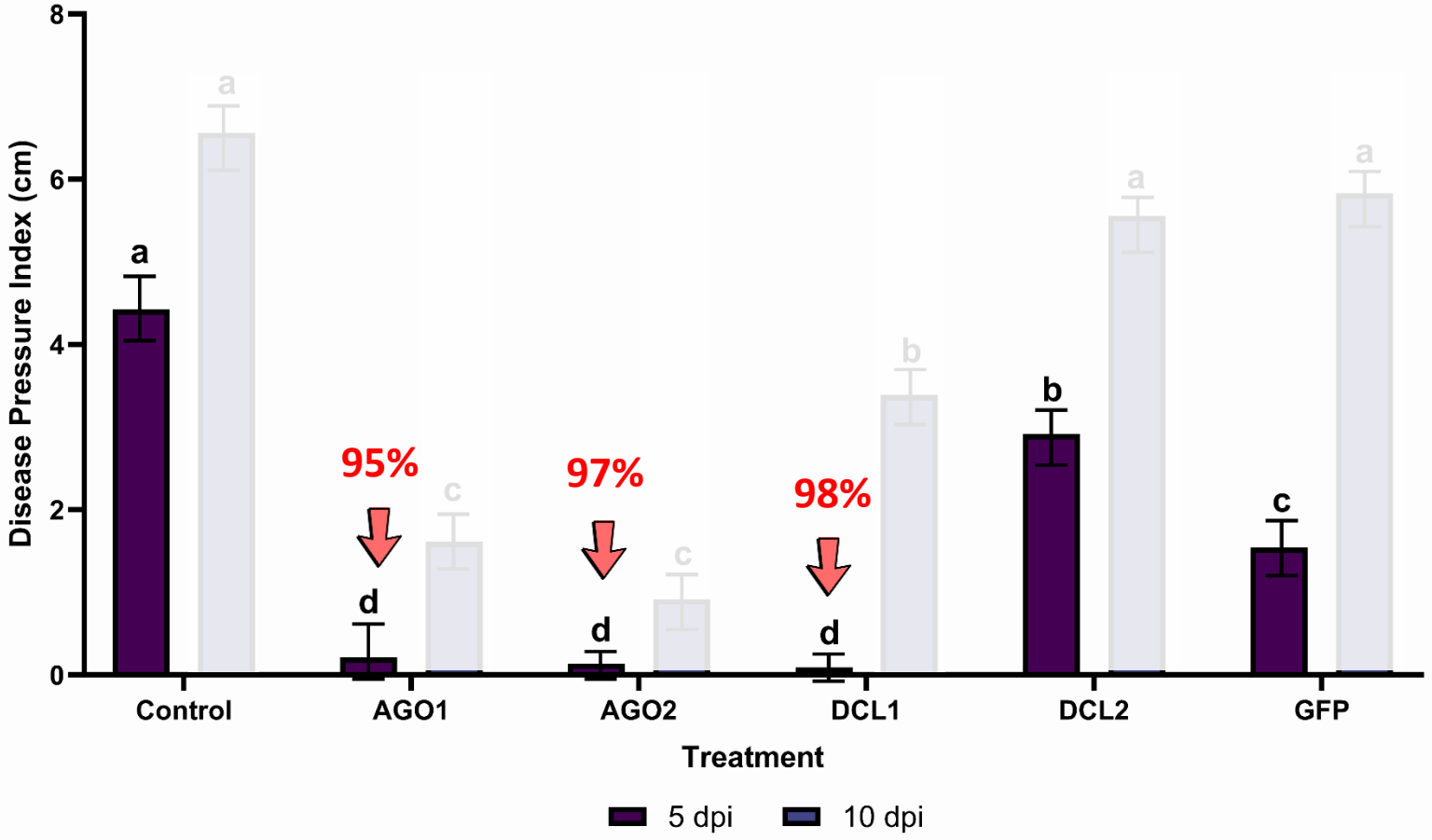


In vivo efficacy assessment on orange fruit

5 dpi

- dsRNA targeting the AGO1, AGO2, and DCL1 genes exhibited sig. lower DPI values compared to the control.
- Application of dsAGO1, dsAGO2, and dsDCL1 inhibited disease development by 95%, 97%, and 98%, respectively.

Disease Inhibition (%)



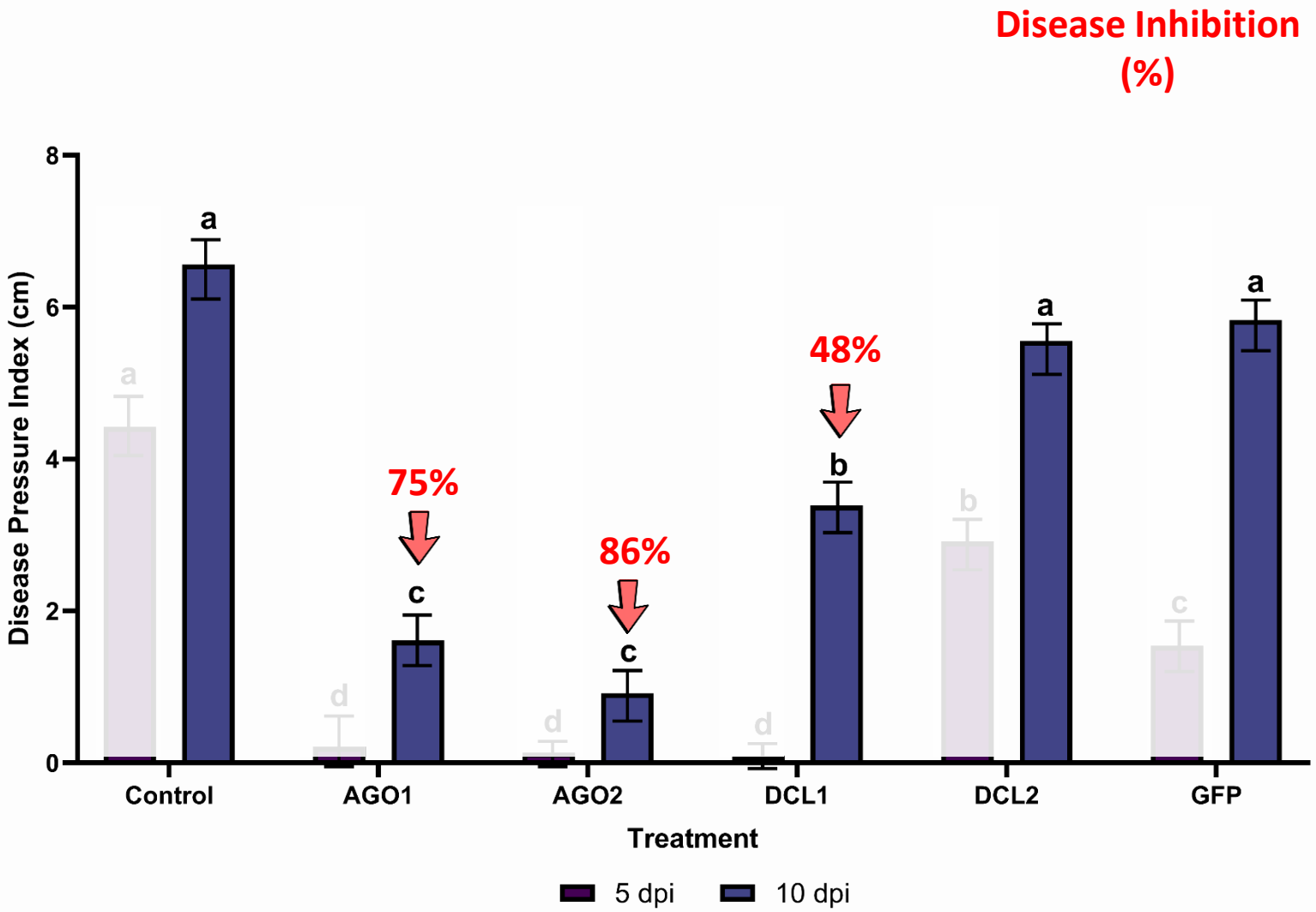
In vivo efficacy assessment on orange fruit

5 dpi

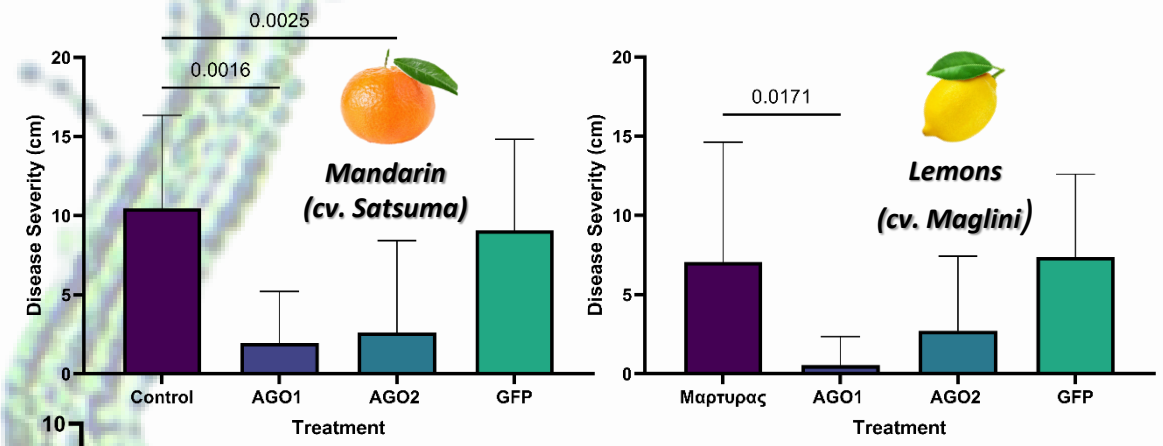
- dsRNA targeting the **AGO1, AGO2**, and **DCL1** genes exhibited sig. lower DPI values compared to the control.
- Application of dsAGO1, dsAGO2, and dsDCL1 inhibited disease development by **95%, 97%,** and **98%,** respectively.

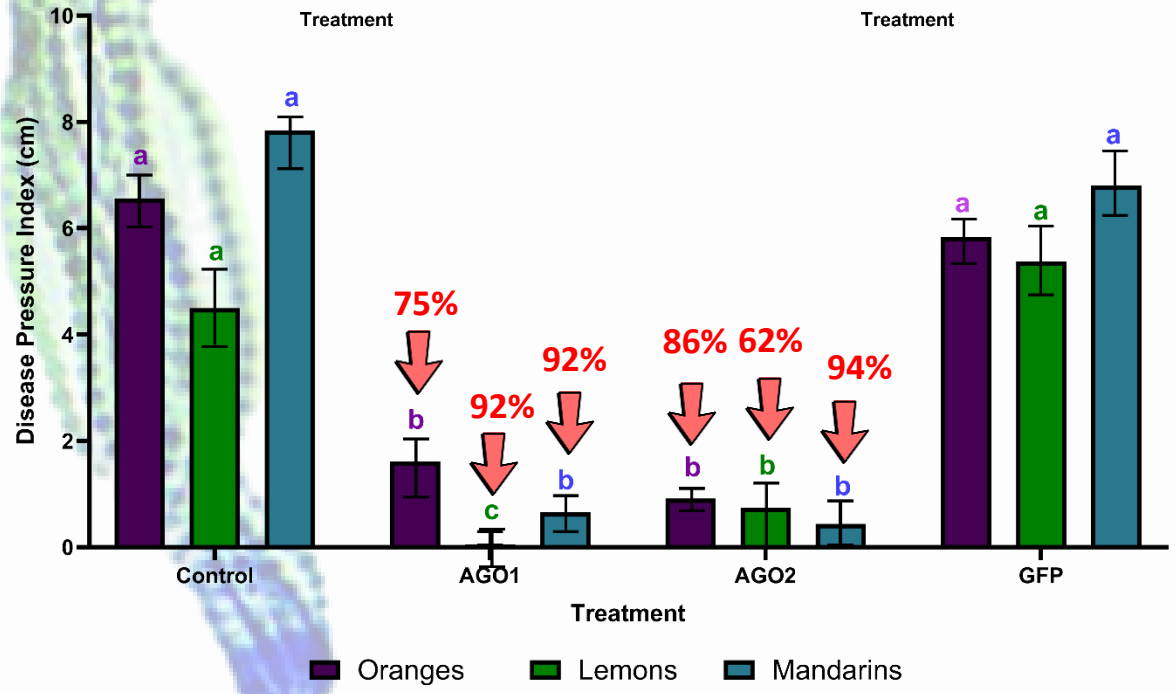
10 dpi

- dsAGO1 and dsAGO2 maintained low disease levels at 10 dpi, indicating sustained silencing.
- **dsDCL1** efficacy **declined** over time, suggesting reduced silencing at 10 dpi.
- Application of dsAGO1, dsAGO2, and dsDCL1 inhibited disease development by 75%, 86%, and 48%, respectively.

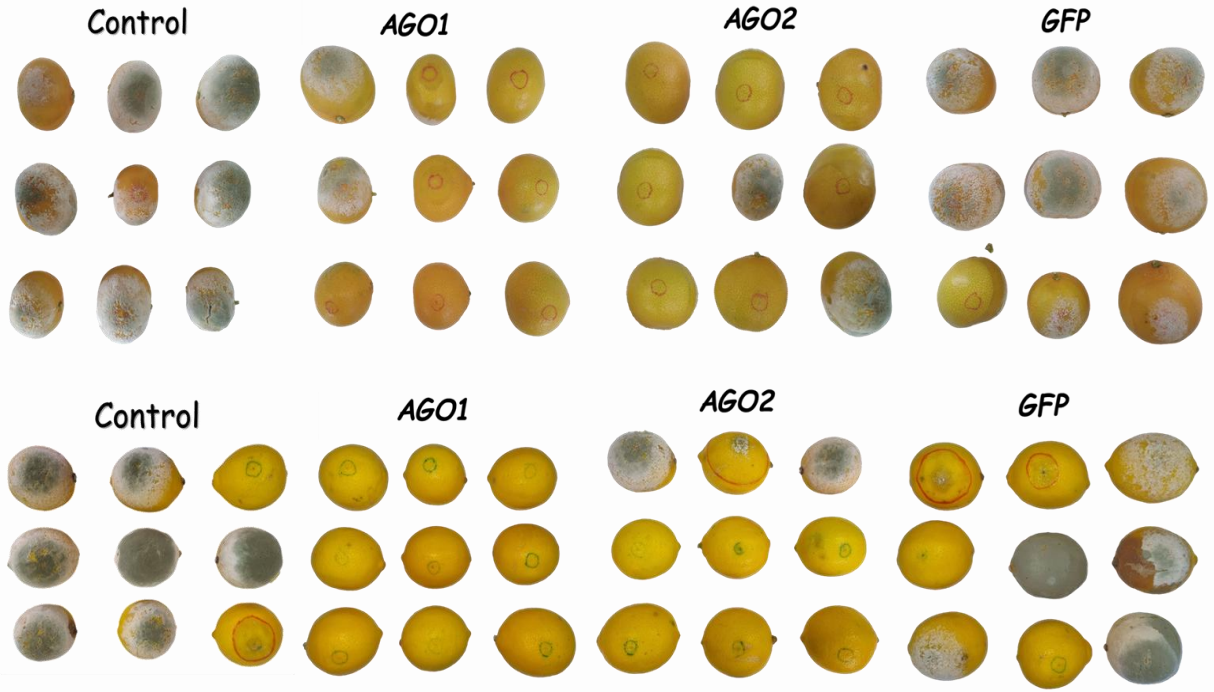


In vivo efficacy assessment on citrus fruits





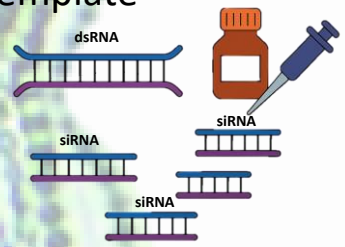
- **Similar** results **obtained** from other **citrus fruits**
- The effectiveness of ***dsAGO1*** was higher compared to ***dsAGO2***
- The **disease inhibition** for all tested citrus ranged from **75-94%**



In vivo efficacy assessment of siRNAs on orange fruit

In vitro production of siRNAs

- siRNA produced using ShortCut® RNase III kit
- ***dsAGO1*** & ***dsAGO2*** used as template



Experimental Design

Sample Setup

- Total fruits: 16
- Replicates: 4
- Fruits per replicate: 4

Incubation Conditions

Temperature: 25°C

Incubation dur. : 5 days 10 days

Disease Assessment

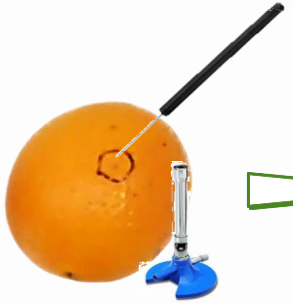
5 & 10 dpi

Disease Severity (cm)

Disease Pressure Index

$$DSI = \frac{DS \times DI}{100}$$

Surface sterilization (NaClO 1% - 3min)



Artificial wound (3mm)

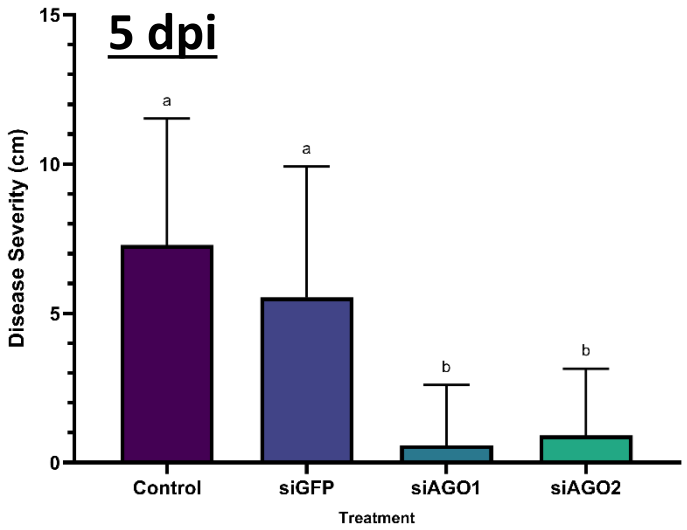
Application of siRNA (800ng)

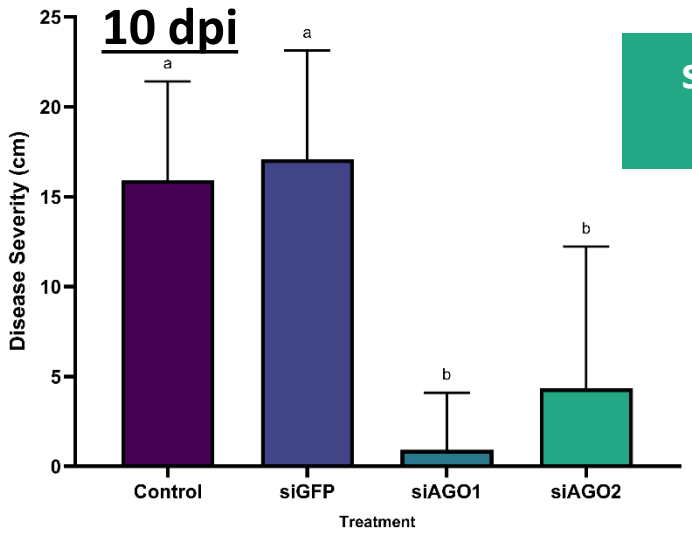
Incubation (2 hours – 25°C)

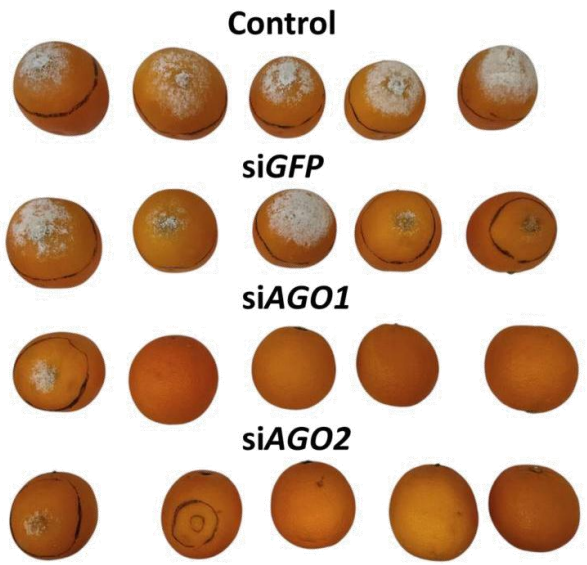
Artificial inoculation (10^5 conidia ml^{-1})

Incubation (5 & 10 days)

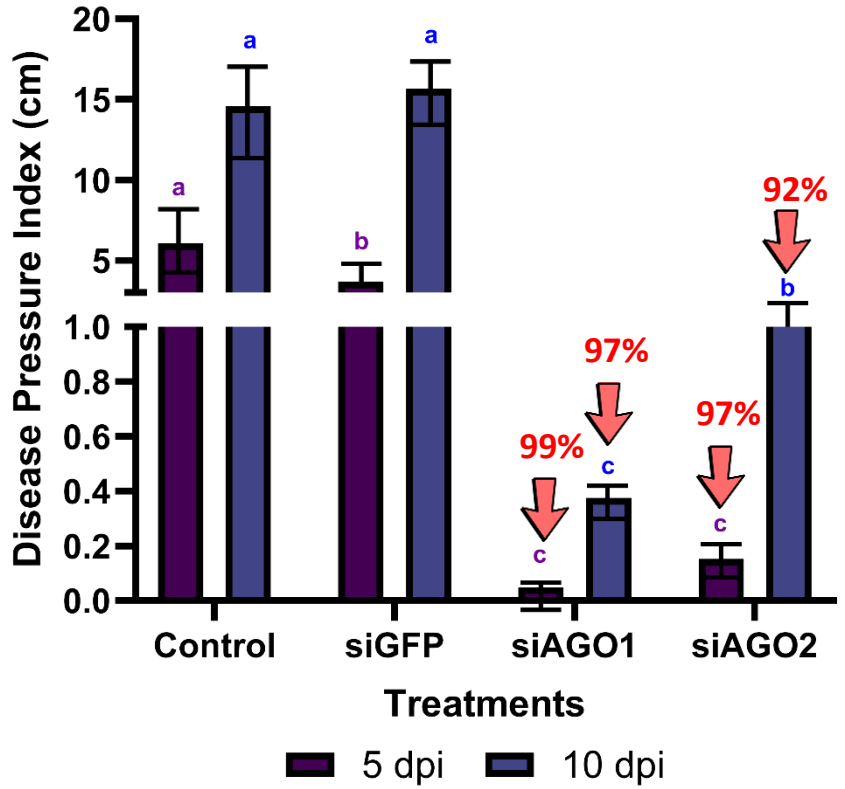
In vivo efficacy assessment of siRNAs on orange fruit







siRNA treatments achieved high disease control efficacy, ranging from 92% to 99%



Disease Inhibition (%)

Small RNA Sequencing

Aim

- Investigate which siRNAs are the most effective (size, sequence)?

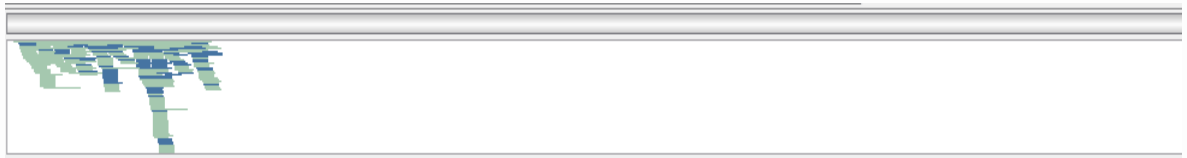
Experimental Design

- Perform small-RNA sequencing on the siRNAs generated from dsAGO1
- Bioinformatic analysis

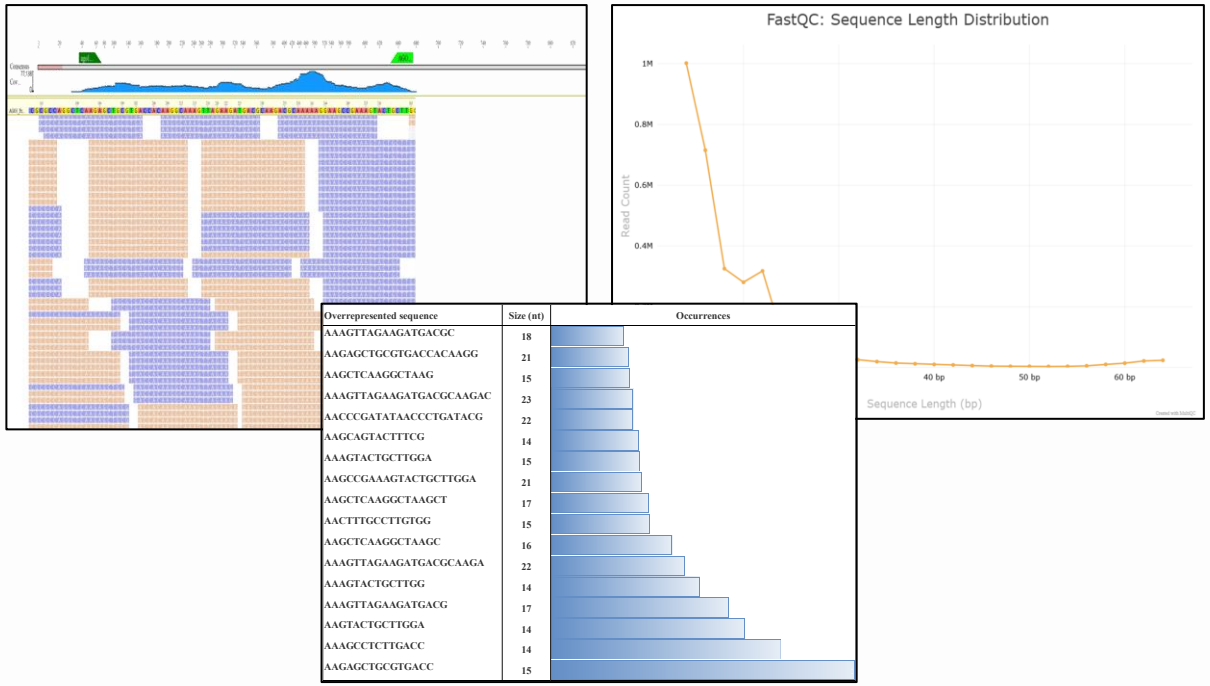
Small RNA Sequencing

Illumina NextSeq
10M depth
GenXpro (Germany)

siRNA sequencing assembly on gene of interest (*pd_AGO1*)

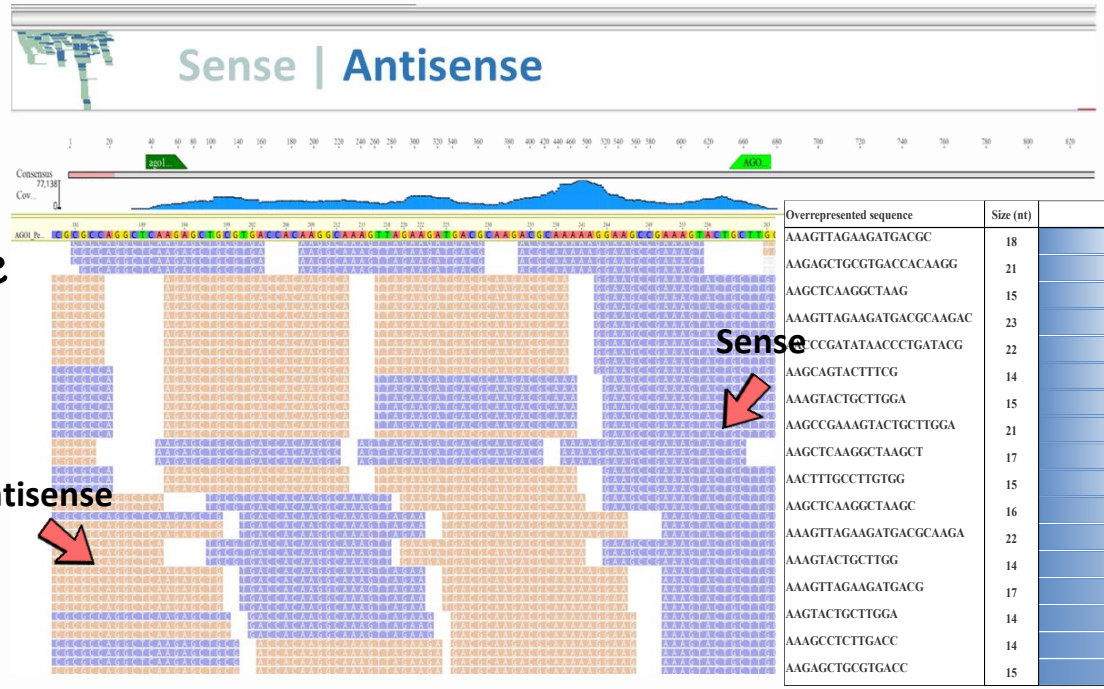


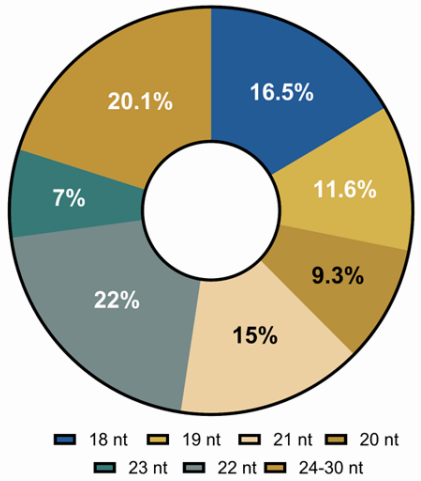
Bioinformatic Analysis

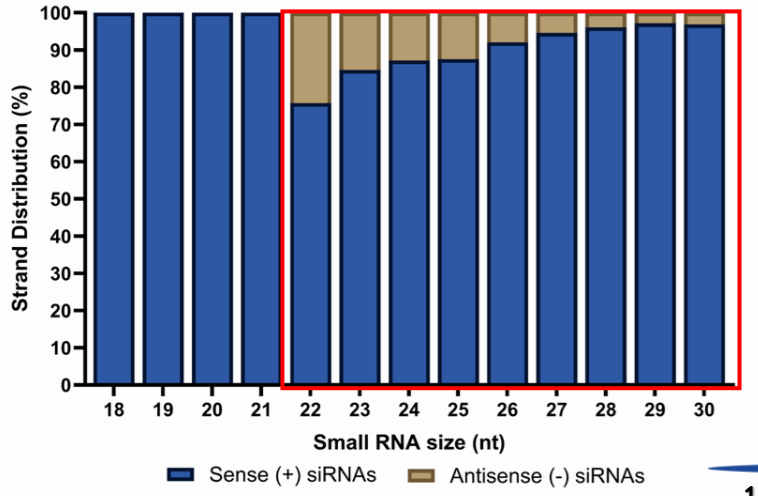


Small RNA Sequencing

- 19,482,770 reads were sequenced
- The majority of small RNA sequences were mapped on the gene of interest
- **Most of the siRNA** sequences identified with a size of **22nt**
- siRNAs with a size of **18-21nt** were characterized as **(+) strand siRNA** (sense)
- **The (-) strand RNA** (antisense) were mostly noticed on siRNA with the size of **22-30nt**
- The **majority** of **antisense siRNAs** were identified in the siRNA class with **22nt** **(essential for gene silencing)**







Conclusions

- Among all target genes the highest control efficacy was noticed in **dsAGO1 & dsAGO2**

- The results obtained for the dsRNA targeting **DCL1 & DCL2** revealed **low efficacy** against Green Mold disease

- The application of **dsAGO1** and **dsAGO2** demonstrated **high efficacy** against *P. digitatum* across various citrus fruits and incubation periods.

Conclusions

- siRNA applications exhibited greater control efficacy compared to the dsRNAs targeting the genes *AGO1* & *AGO2*

- The si*AGO1* and si*AGO2* resulted in **high disease inhibition** rates (>92%) after **10 days** of incubation

- Small RNAseq revealed the prevalence of **22nt antisense siRNAs** targeting ***AGO1*** gene based on the reference sequence

Dr. Athanasios Dalakouras
(ELGO-Demeter)

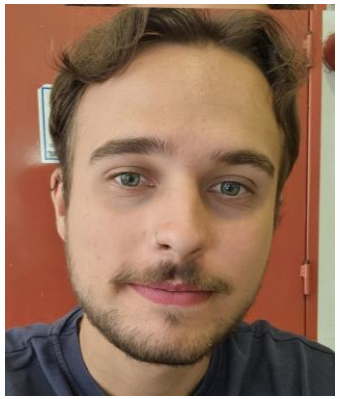
Prof. Kalliope K. Papadopoulou
(UTH)

Dr. Venetia Koidou
(ELGO-Demeter)

Thank you!!!

Prof. George Karaoglanidis
(AUTH)

Vasilis Tsagaris
(AUTH)



Christos Raidis
(UTH)

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Next Steps in Progress

De novo synthesis of the most abundant antisense siRNA identified through small RNA sequencing

In vivo assessment of the synthesized siRNA on citrus fruits

Monitoring the localization and movement of siRNA vs. dsRNA on citrus fruit using Cy3-labeled siRNAs/dsRNAs and confocal microscopy