Innovative Control of the Cucurbit Powdery Mildew: Combining Nanoencapsulated dsRNA and Low-Dose Fungicides for Sustainable Crop Protection

Nisrine Bakhat,[1,2] Yandira Morales,[1,2] David Fernández-Salvatierra,[1] Leonardo Velasco,[3] Alejandro Pérez-García,[1,2] and Dolores Fernández-Ortuño[1,2,†]

[1] Departamento de Microbiología Facultad de Ciencias, Universidad de Málaga, Málaga 29010, Spain
[2] Instituto de Hortofruticultura Subtropical y Mediterránea "La Mayora", Universidad de Málaga, Consejo Superior de Investigaciones Científicas (IHSM–UMA–CSIC), Málaga 29010, Spain
[3] Instituto Andaluz de Investigación y Formación Agraria, Pesquera, Alimentaria y de la Producción Ecológica (IFAPA), Churriana, Málaga 29140, Spain

Abstract

The rise of fungicide-resistant pathogens and increasingly strict regulatory frameworks have heightened the demand for sustainable alternatives in plant disease control. This study assesses the potential of spray-induced gene silencing (SIGS) targeting *SdhC*, a critical subunit of the mitochondrial succinate dehydrogenase (SDH) complex and determinant of SDHI fungicide resistance, for managing *Podosphaera xanthii*, the causal agent of cucurbit powdery mildew. To evaluate this approach, *PxSdhC* double-stranded RNA (dsRNA) was initially infiltrated into melon cotyledons, resulting in a 65% reduction in fungal biomass and a 60% decrease in *SdhC* gene expression, validating its effectiveness for SIGS applications. A key achievement of this study was the demonstration that strong disease control can be achieved with substantially reduced doses of SDHI fungicides when combined with *PxSdhC*-dsRNA, providing the first direct evidence of the synergistic potential of this strategy. Building on these results, greenhouse trials were conducted in which dsRNA was applied in both naked and carbon dot (CD)-nanoencapsulated forms, alone or in combination with low doses of the SDHI fungicides boscalid and fluopyram. Nanoencapsulation enhanced dsRNA stability and extended gene silencing effects, achieving up to 69 and 53% disease suppression at 14 and 21 days postinoculation, respectively. Moreover, combined treatments with *PxSdhC*-dsRNA-CD and low-dose fungicides exhibited synergistic effects, with disease suppression ranging from 33 to 91%, depending on isolate resistance levels. Sensitive and low-resistance isolates responded effectively, while efficacy declined in highly resistant ones. In vitro specificity assays confirmed that *PxSdhC*-dsRNA selectively inhibited *Botrytis cinerea*, a species with high *SdhC* sequence homology, without affecting unrelated fungi. Altogether, these results highlight the promise of gene-targeted, nanoformulated dsRNA as an effective, precise, and environmentally friendly strategy for integrated disease management, capable of boosting the performance of low-dose fungicide applications and reducing chemical input.

Keywords: carbon dots, cell respiration, *Podosphaera xanthii*, RNA interference, SDHI fungicides, spray-induced gene silencing, succinate dehydrogenase

Powdery mildew diseases, caused by various fungal species, pose a significant threat to global agriculture due to their devastating impact on crop yield (Vela-Corcía et al. 2015). These fungi are obligate biotrophs, requiring living host cells to complete their life cycle. Through haustoria, specialized parasitic structures, they extract nutrients from plant cells and release effector proteins that can manipulate host defense mechanisms (Godfrey et al. 2009; Polonio et al. 2019). Among the fungal species responsible for powdery mildew, *Podosphaera xanthii* is the primary causal agent affecting cucurbit crops worldwide (Álvarez 1993; del Pino et al. 2002; Fernández-Ortuño et al. 2006). Its rapid spread and severe impact on plant development make it a major concern for growers. Disease management strategies typically rely on a combination of cultural practices, resistant crop varieties, biological control, and chemical fungicides with different modes of action (Gilardi et al. 2013; Ons et al. 2020). However, chemical control remains the most widely used approach due to its immediate effectiveness, despite concerns about resistance development (Bellón-Gómez et al. 2012; Vielba-Fernández et al. 2021).

One of the most effective chemical strategies for managing fungal disease is the use of succinate dehydrogenase inhibitors (SDHIs), a widely employed class of fungicides that target the succinate dehydrogenase (SDH) enzyme complex. This complex plays a critical role in the mitochondrial electron transport chain and is essential for ATP production in fungal cells (Ceballos Alcantarilla 2017). Structurally, the SDH complex comprises four main subunits (SdhA, SdhB, SdhC, and SdhD), with SdhB, SdhC, and SdhD being directly involved in fungicide binding (Sierotzki and Scalliet 2013). SDHIs have been commercially available for more than 45 years and represent one of the fastest-growing classes of fungicides in terms of the number of active compounds introduced to the market. To date, 24 SDHI active ingredients, belonging to 12 different chemical classes and offering broad-spectrum antifungal activity, have been registered for plant pathogen control (http://frac.info). Due to their mode of action, SDHIs are classified by the Fungicide Resistance Action Committee (FRAC) as having a medium to high risk of resistance development. This is largely attributed to the specific and limited binding sites within the SDH complex, where point mutations in subunits B, C, and D can significantly reduce fungicide binding and efficacy (FRAC 2019; Vielba-Fernández et al. 2021). In *P. xanthii,* resistance to SDHI fungicides has been primarily associated with point mutations in the *Sdh*C gene. These mutations result in varying degrees of resistance and can also lead to cross-resistance among different SDHI compounds. In Spain, for example, amino acid substitutions such as A86V and G151R in the SdhC subunit have been linked to field resistance to boscalid and fluopyram (Vielba-Fernández et al. 2021). Similarly, in Japan, the A86V mutation has also been identified as a key factor contributing to resistance against a range of SDHI fungicides, including

†Corresponding author: D. Fernández-Ortuño; dfernandez-ortuno@uma.es

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penthiopyrad, isopyrazam, pyraziflumid, and particularly isofetamid (Miyamoto et al. 2020).

The increasing resistance to fungicides has intensified the search for sustainable disease control strategies. RNA interference (RNAi)-mediated gene silencing is a natural mechanism that is part of the post-transcriptional gene silencing process, primarily described in eukaryotic organisms and directed by small interfering RNAs (siRNAs; Ray et al. 2022; Zand Karimi and Innes 2022). However, Argonaute-related proteins, the core component of RNAi, have also been identified in some prokaryotes, suggesting that RNA-guided gene regulation may also occur in these organisms through analogous mechanisms (van der Oost et al. 2014). This process involves the enzymatic processing of double-stranded RNA (dsRNA) into siRNAs (21 to 25 nucleotides in length), which guide RNA-induced silencing complexes to degrade target fungal mRNAs, effectively suppressing their expression (Koch and Wassenegger 2021). This RNAi system has been described in numerous fungal species, allowing for bidirectional RNAi communication between the host and the pathogen (Padilla-Roji et al. 2023). Based on it, the spray-induced gene silencing (SIGS) emerged as a nontransgenic alternative to prevent pathogen infection in plants. This approach relies on spray application of dsRNA onto the host plant, where it can be readily absorbed by both the plant and the pathogen, leading to gene silencing and inhibiting infection (Bakhat et al. 2025; Koch and Wassenegger 2021; McRae et al. 2023). However, the efficiency of dsRNA uptake can be influenced by several factors, including the plant species, tissue type, developmental stage, and the pathogen's lifestyle or structural characteristics (Qiao et al. 2021). Recent studies have demonstrated the effectiveness of SIGS in controlling various fungal diseases (Degnan et al. 2023; Hu et al. 2020; Koch et al. 2016; McLoughlin et al. 2018; Mumbanza et al. 2013) including *P. xanthii* (Bakhat et al. 2025; Ruiz-Jiménez et al. 2021), highlighting the importance of continuing to explore this strategy as a novel tool for disease control in the field.

Despite the promising results of SIGS technology for controlling fungal diseases, several factors limit its widespread adoption in agriculture. One major challenge is the instability of dsRNA molecules in the environment, as they are highly susceptible to degradation by radiation, rain, and especially nucleases present on plant tissue surfaces (Yang et al. 2022). To overcome this limitation, researchers have developed various strategies to protect these dsRNAs, ensuring effective delivery to pathogens and extending their silencing effect under field conditions (Karny et al. 2018). Between them, the use of nanoparticles is one of the most promising solutions (Hoang et al. 2022; Delgado-Martín et al. 2022; Mitter et al. 2017; Ray et al. 2022; Zhang et al. 2020). Different organic and inorganic nanoparticles have been developed for the application of these dsRNAs in plants. Among the latest, carbon dots (CDs; Delgado-Martín et al. 2022) have been used successfully in SIGS assays. For example, recent studies have indicated that the spray application of dsRNA-CD in *Nicotiana benthamiana* and tomato plants resulted in strong silencing of GFP transgenes in both species (Schwartz et al. 2020). In cucurbits, Delgado-Martín et al. (2022) showed that dsRNA encapsulated in CDs extended its activity over time, marking a significant breakthrough in viral disease control. Further research has demonstrated that dsRNAs targeting two *P. xanthii* genes loaded into CDs provide extended protection against *P. xanthii*, significantly reducing fungal biomass and disease symptoms (Bakhat et al. 2025).

Building on existing knowledge, this study investigates the use of RNAi and nanotechnology, in combination with fungicides, for the control of cucurbit powdery mildew (Supplementary Fig. S1). Initially, the efficacy of *PxSdhC*-dsRNA was evaluated through infiltration in melon cotyledons, assessing its impact on fungal growth, target gene expression, and disease development. Subsequently, *PxSdhC*-dsRNA, both naked and nanoencapsulated with CDs, was applied via SIGS under greenhouse conditions to determine its ability to reduce disease severity. Combined treatments with reduced doses of commercial SDHI fungicides (boscalid and fluopyram) were also tested to explore potential synergistic effects. Additionally, the specificity of *PxSdhC*-dsRNA against nontarget fungal species was assessed to ensure targeted action and support its integration into sustainable and precise plant disease management strategies.

Materials and Methods

Cultivation conditions for fungi, plants, and bacteria

This study used six *P. xanthii* single-spore isolates collected from several countries between 1988 and 2021 (Table 1) which were characterized and kept at −80°C until use as previously described (Braun and Cook 2012; Pérez-García et al. 2006). As cucurbit hosts, melon (*Cucumis melo* L. cv. Rochet; Semillas Fitó) and zucchini (*Curcubita pepo* cv. Negro Belleza; Semillas Fitó) plants were used for gene silencing assays and fungal maintenance, respectively. In both cases, the seeds were germinated on filter paper moistened with distilled water in 8-cm Petri dishes at 25°C in the dark. After 5 days, seedlings were transferred to plastic seedbeds containing peat moss and maintained in a growth chamber at 25°C with a 16-h light/8-h darkness photoperiod and regular irrigation. Once the cotyledons reached the desired stage (4 to 5 days), they were excised and disinfected in 15% bleach solution for 45 s. Afterward, they were rinsed twice in sterile distilled water for 2 min each and dried on sterile filter paper. The disinfected cotyledons were then transferred to 5-cm Petri dishes containing Bertrand medium (Álvarez and Torés 1997) and inoculated with the fungal spores. These were incubated in a growth chamber at 22°C under the same 16-h light/8-h darkness photoperiod for 1 week. For *Aspergillus nidulans*, *Botrytis cinerea*, *Fusarium graminearum*, and *Penicillium digitatum*, cultures were maintained on potato dextrose agar (PDA) at 23°C under the same light/dark conditions for 7 to 10 days. For RNAi silencing vector maintenance and propagation, the *Escherichia coli* strain DH5α was used. The strain was routinely grown at 37°C in lysogeny broth (LB) medium supplemented with ampicillin (100 μg/ml) when required.

Fungicide sensitivity studies for SDHI fungicides: boscalid and fluopyram

To determine the sensitivity of the *P. xanthii* isolates to the SDHI fungicides boscalid and fluopyram, we conducted a leaf-disc bioassay following the method described by Vielba-Fernández et al. (2021). In brief, leaf discs (1 cm in diameter) were obtained from 8- to 10-day-old zucchini cotyledons, cut with a cork borer, placed upside down in Petri dishes, and incubated upside down for 1 h on sterile filter paper that had absorbed 3 ml of sterile distilled water (untreated control) or SDHI solutions at different concentrations (10, 25, 50, and 100 μg/ml). The tested fungicides were boscalid (Cantus, BASF S.L.) and fluopyram (Luna Privilege; Bayer CropScience S.L.), with the highest concentration (100 μg/ml) corresponding to the field dose recommended by both manufacturers. After treatment, the discs were left to dry on Bertrand medium (40 g of sucrose, 30 mg of benzimidazole, 10 g of agar, and 1 liter of distilled water) in 5-cm-diameter Petri dishes, and the *P. xanthii* isolates were inoculated onto their adaxial surface using an eyelash. After 10 days of incubation under the same conditions described earlier, powdery mildew growth was assessed. Based on classification proposed by Vielba-Fernández et al. (2021), the isolates were grouped into five

Table 1. Sensitivity of *Podosphaera xanthii* isolates collected from different years, cucurbit hosts, and countries to SDHI fungicides

			Phenotype[a]	
Isolate (Country)	**Year**	**Host**	**Boscalid**	**Fluopyram**
2086 (Greece)	1997	Melon	S	S
SF9 (Spain)	1988	Zucchini	LR	S
31430 (Spain)	2003	Melon	LR	S
FR1D (France)	2020	Zucchini	LR	S
NL1J (Netherlands)	2021	Cucumber	HR	LR
IT1H (Italy)	2020	Cucumber	HR	HR

[a] Fungicide sensitivity assays were performed as previously described by Vielba-Fernández et al. (2021). S = sensitive, LR = low resistance, and HR = high resistance.

categories: (i) sensitive (S), showing no growth at any test concentration; (ii) low resistance (LR), with growth at 10 and 25 μg/ml but complete inhibition at 50 and 100 μg/ml; (iii) moderate resistance (MR), with growth up to 50 μg/ml and complete inhibition at 100 μg/ml; (iv) resistant (R), with growth at all concentrations but without fully covering the leaf disc; and (v) high resistance (HR), with vigorous growth at all tested concentrations. Twelve discs from three different cotyledons were used for each concentration. The assay was performed three times to ensure reproducibility.

Nucleic acid extraction and complementary DNA (cDNA) synthesis

To isolate nucleic acids and synthesize cDNA from zucchini cotyledons infected with *P. xanthii*, the infected cotyledons were stored at −80°C until processing. Frozen samples were then ground in liquid nitrogen using a mortar. Genomic DNA was isolated with the MasterPure Yeast DNA Purification Kit (Epicentre), while total RNA was extracted using the TRI Reagent System (Sigma-Aldrich), following the manufacturer's instructions. Total RNA was eluted in diethyl pyrocarbonate-treated water and stored at −80°C until further use. RNA concentration was measured using a NanoDrop ND-2000 spectrophotometer (Thermo Fisher Scientific). From the obtained RNA, cDNA synthesis was performed using SuperScript III reverse transcriptase (Thermo Fisher Scientific) and Oligo dT (20) primers (Thermo Fisher Scientific), following the manufacturer's recommendations.

Construction of silencing vectors

From the previously obtained cDNA, a 312-bp region of the *PxSdhC* (LC522548.1) gene was amplified to construct RNAi silencing vectors. Primers were designed using Primer3 software (http://bioinfo.ut.ee/primer3/; Koressaar and Remm 2007), incorporating recognition sites for the *Nco*I or *Bgl*II restriction enzyme (Supplementary Table S1). Amplification was carried out in a 50-μl reaction mixture containing Milli-Q water, 5x HF Phusion buffer, dNTPs, primers, cDNA, and Phusion High-Fidelity DNA polymerase enzyme (Thermo Fisher Scientific). The amplified fragments were then digested and linearized using FastDigest *Nco*I and *Bgl*II enzymes. A digestion mixture was prepared with Milli-Q water, PCR product, FastDigest buffer, *Nco*I, and *Bgl*II, and the L4440 vector was treated similarly. These mixtures were incubated in a thermocycler, followed by enzyme inactivation. Digestion was confirmed via agarose gel electrophoresis. The amplified and digested fragments were then ligated into the L4440 plasmid using T4 DNA Ligase (Invitrogen) and incubated, and the enzymes were inactivated according to the manufacturer's instructions. The recombinant plasmids were introduced into *E. coli* DH5α cells using the heat shock method (Mandel and Higa 1970), with transformed cells plated on LB agar containing ampicillin and incubated overnight. Plasmid purification was performed using the GenElute Plasmid Miniprep Kit (Sigma-Aldrich), involving bacterial culture collection, lysis, neutralization, centrifugation, and plasmid elution with warm sterile water. Finally, purified plasmids were sequenced to verify the accuracy of the inserted gene fragments (Stab Vida).

In vitro synthesis of dsRNA

Before producing dsRNA, the purified plasmids were linearized by digestion with the same restriction enzymes used in their construction, either *Nco*I or *Bgl*II. dsRNA synthesis was then performed using the HiScribe T7 Quick High Yield RNA Synthesis Kit (New England Biolabs) following the manufacturer's instructions. The final 20-μl mixture contained 10 μl of dNTPs, 8 μl of the linearized plasmid, and 2 μl of T7 RNA polymerase, which later was incubated at 37°C for 2 h. The initial yield of crude dsRNA product was up to 20 μg/μl in some cases, depending on the template and reaction efficiency. The dsRNA was subsequently purified by ethanol precipitation and resuspended in RNase-free water, resulting in final concentrations of approximately 2 μg/μl in a 20-μl resuspension volume.

Cotyledon infiltration assays

To evaluate the effects of dsRNA on fungal development and gene expression, infiltration assays were conducted using 8-day-old melon cotyledons, following the protocol described by Martínez-Cruz et al. (2018). Prior to each assay, a solution of dsRNA targeting *PxSdhC* was prepared in infiltration buffer (10 mM MES and 10 mM $MgCl_2$) at a final concentration of 0.1 μg/ml. This solution was infiltrated into the adaxial surface of melon cotyledons using a needleless insulin syringe. Fifteen cotyledons were infiltrated per treatment to allow sampling for different analyses. The infiltrated cotyledons were then incubated in a growth chamber at 25°C under a 16-h light/8-h dark photoperiod for 24 h. After incubation, the cotyledons were sprayed with a suspension of *P. xanthii* conidia (1×10^5 spores/ml) and returned to the same growth conditions. Cotyledons infiltrated with dsRNA corresponding to the nonspecific sequence located between the two T7 promoters in the pL4440 plasmid, lacking predicted targets in both melon and *P. xanthii*, were used as a negative control. In contrast, *PxTUB2*-dsRNA was employed as a validated positive control, owing to its previously demonstrated strong inhibitory effects on fungal growth and its essential role in *P. xanthii* biology (Bakhat et al. 2025; Ruiz-Jiménez et al. 2021). Subsequent analyses included bright-field microscopy, qPCR, and RT-qPCR as described below. The entire experiment was repeated three times to ensure reproducibility of all analyses.

Haustorium count and fungal development visualization

The progression of *P. xanthii* infection was evaluated by histochemical analysis using the lactophenol blue staining method described by Romero et al. (2008). For this, 8-mm-diameter cotyledon discs were collected at 24, 48, and 72 h postinoculation (hpi) with *P. xanthii* and subjected to a decolorization step by boiling in ethanol for 15 min. Discs were prepared from three cotyledons per time point, with each cotyledon typically providing 4 to 5 discs. Afterward, they were immersed in a lactophenol blue/ethanol solution (1:10 ratio) and placed in a water bath at 87°C for 90 s to fix the stain. Samples were kept in darkness at room temperature overnight. The next day, discs were washed with acetic acid/ethanol solution (1:3 ratio) and examined using light microscopy with an Eclipse E800 microscope (Nikon). Haustoria appeared as dark blue spots beneath the *P. xanthii* hyphae, inside the epidermal cells. Images were captured with a Leica DFC450 camera attached to the microscope and processed using LAS-AF LCS Lite software (Leica Microsystems).

Estimation of fungal biomass and gene expression analysis

To quantify *P. xanthii* biomass in dsRNA-infiltrated melon cotyledons, qPCR analysis was performed targeting the *P. xanthii* β-tubulin gene *PxTUB2* (KC333362) and the *C. melo actin-7* gene *CmACT7* (XM_008462689.2) using specific primers (Supplementary Table S1), following the method of Vela-Corcía et al. (2014). Total DNA was extracted from infected cotyledons 96 hpi, and qPCR was carried out using the CFX Connect Real-Time PCR Detection System and the SsoFast EvaGreen reagent (Bio-Rad). Each biological replicate was run in triplicate. Reactions included template DNA, primers, reagents, and Milli-Q water, with thermal conditions set to 95°C for 5 s and 65°C for 5 s. The ratio of *P. xanthii* DNA to melon genomic DNA was calculated to estimate fungal biomass. Additionally, RT-qPCR was used to assess the effect of dsRNA silencing on the expression of the *P. xanthii PxSdhC* gene, using cDNA from dsRNA-infiltrated cotyledons infected at 72 hpi. The *P. xanthii* elongation factor 1-alpha gene *PxEF1* (MK249653) served as the reference for normalization. RT-qPCR reactions were performed using the same system and reagents, under the same conditions: enzyme activation at 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 65°C for 5 s. Each RT-qPCR included three technical replicates per biological replicate. Data were analyzed using the CFX Manager Software.

Determination of *PxSdhC*-dsRNA concentration and CDs-dsRNA ratio for SIGS assays

To evaluate the effectiveness of *PxSdhC*-dsRNA in controlling cucurbit powdery mildew via SIGS, a preliminary assay was

conducted. Various concentrations (10, 20, 30, 40, 60, and 80 μg/ml) were sprayed onto melon plants at the second leaf stage. This experimental design follows the dose-response strategy previously described by Ruiz-Jiménez et al. (2021) who reported significant disease suppression. In our study, the concentration range was expanded to include higher doses to explore potential improvements in efficacy. As controls, sterile distilled water and nonspecific dsRNA sequence and *PxTUB2*-dsRNA at 40 μg/ml were used as negative and positive treatments, respectively, consistent with the same study (Ruiz-Jiménez et al. 2021). For each treatment, six plants were used to ensure statistical reliability, with only one leaf sprayed per plant (six biological replicates). After 24 h, leaves were inoculated with a conidial suspension of *P. xanthii* (1×10^4 spores/ml) and kept in a greenhouse for 2 weeks. Disease severity was quantified by analyzing the percentage of leaf surface coverage by mildew using Fiji software (Rueden et al. 2017). This experiment was repeated three times for reproducibility. After identifying the most effective dsRNA concentration and considering the potential of nanoparticle encapsulation to enhance dsRNA stability and cellular uptake, *PxSdhC*-dsRNA was encapsulated in CD nanoparticles. Preliminary assays were conducted using different dsRNA-to-nanoparticle ratios to determine the most effective formulation for SIGS applications.

The CDs were obtained by hydrothermal synthesis from glucose and polyethylenimine (2 KDa) following the methodology described elsewhere (Delgado-Martín et al. 2022). These CDs exhibited an average particle size of 4 to 5 nm as determined by TEM analysis, and according to the determination made with dynamic light scattering (DLS), they exhibited a positive surface charge (zeta potential of +22 mV), which facilitated electrostatic interaction with the negatively charged dsRNA. Fourier-transform infrared spectroscopy confirmed the presence of amine, hydroxyl, and carboxyl functional groups on the CD surface, which enabled both ionic and hydrogen bonding interactions with RNA molecules. The stabilization of dsRNA was primarily achieved through electrostatic binding and physical shielding, which protected the molecule from nuclease degradation and environmental exposure. This complexation also enhanced cellular uptake by promoting adhesion to the plant cuticle and pathogen surfaces, thereby improving gene silencing efficiency in SIGS applications (Delgado-Martín et al. 2022).

Determination of SDHI fungicide concentrations to combine with SIGS assays

To explore a combined approach using RNAi, nanotechnology, and SDHI fungicides for cucurbit powdery mildew control, different concentrations of boscalid (5, 7, 10, 20, and 100 μg/ml) and fluopyram (2, 5, 7, 10, 20, and 100 μg/ml) were tested with 100 μg/ml being the dose recommended in the field by the manufacturer. The goal was to determine optimal doses that, when combined with SIGS, might produce a synergic effect. After fungicide application, plants were inoculated with *P. xanthii* spores under the same conditions described earlier and incubated in a greenhouse for 2 weeks. Plants treated with sterile distilled water served as a negative control. For each treatment, six leaves from six different plants were sprayed. This experiment was repeated three times to confirm the reproducibility of results and to evaluate the consistency of treatment effectiveness.

Combined assays of dsRNAs, CDs, and SDHI fungicides

A combined assay was conducted to evaluate the effectiveness of naked and CD-nanoencapsulated dsRNA in conjunction with the low doses of SDHI fungicides determined previously. For this purpose, the following treatments were tested: naked *PxSdhC*-dsRNA (40 μg/ml), encapsulated *PxSdhC*-dsRNA (40 μg/ml)-CD, naked *PxSdhC*-dsRNA combined with fungicides (boscalid at 10 μg/ml or fluopyram at 2 μg/ml), and *PxSdhC*-dsRNA-CD combined with the same fungicides and concentrations. Positive controls included *PxTUB2*-dsRNA (40 μg/ml), *PxTUB2*-dsRNA-CD, boscalid, and fluopyram at the recommended field dose of 100 μg/ml. Sterile distilled water, nonspecific dsRNA sequence (40 μg/ml), and empty CDs served as negative controls. For each treatment, six melon plants at the second leaf stage were sprayed with dsRNA (naked or nanoencapsulated), followed by fungicides after 4 h, and inoculated with *P. xanthii* spores (1×10^4 spores/ml) after 24 h. Six plants per condition were grown for 21 days, with samples collected at 14 and 21 days postinoculation. Disease symptoms were analyzed using the Fiji software, and sterile water-treated plants were considered as 100% infection controls. This experiment was repeated three times for reproducibility.

Repeatability of the combined assay testing *P. xanthii* isolates collected from different countries

To assess the effect of the combined treatment (*PxSdhC*-dsRNA-CD with SDHI fungicide), six *P. xanthii* isolates representing different SDHI resistance phenotypes, commonly found in natural populations, were tested (Table 1). Treatments included the application of *PxSdhC*-dsRNA (40 μg/ml)-CD in combination with fungicides (boscalid at 10 μg/ml or fluopyram at 2 μg/ml); and positive and negative controls (sterile distilled water and SDHI fungicides at the recommended field dose) were also included. Six plants per condition were treated at the second leaf stage, followed by fungicide application after 4 h. The next day, plants were inoculated with *P. xanthii* spores (1×10^4 spores/ml) and maintained in greenhouse conditions for 14 days. Disease severity was evaluated through image analysis using Fiji software, using the sterile water-treated plants as the reference for 100% infection. This experiment was repeated three times.

In vitro evaluation of the specificity of *PxSdhC*-dsRNA across other fungal species

To assess the specificity of the *PxSdhC*-dsRNA treatment across different fungal species, an in vitro assay was performed using *A. nidulans, B. cinerea*, *F. graminearum*, and *P. digitatum*. PDA plates were prepared, and 100 μl of *PxSdhC*-dsRNA at a concentration of 500 μg/ml was applied to each. This concentration was selected based on preliminary dose-response experiments (data not shown) that indicated it was sufficient to induce observable gene silencing effects in vitro. Then, a 1-mm PDA disc, taken from a 5-day-old PDA plate preinoculated with the respective fungus, was placed at the center of each dsRNA-treated plate. Three biological replicates were performed per fungal species, along with three control plates treated with sterile distilled water. Additional controls included plates treated with nonspecific dsRNA obtained from the pL4440 plasmid and *BcDCL2*-dsRNA at 500 μg/ml (López-Laguna et al., unpublished). Fungal growth was measured 48 h after treatment to determine any potential off-target effects of *PxSdhC*-dsRNA. This experiment was repeated three times.

Statistical analysis

Experimental data were analyzed using Tukey's test to determine whether the fungal growth inhibition observed under each treatment was statistically significant. A P value of <0.05 was considered the threshold for significance.

Results

Boscalid and fluopyram sensitivity studies

The four previously described discriminatory concentrations of boscalid and fluopyram were tested to assess the sensibility of *P. xanthii* isolates. Based on their ability to grow on discs treated with these doses, isolates were classified into three categories: sensitive (S), showing no growth at any tested concentration; low resistance (LR), with isolates able to grow at 25 μg/ml but showing complete inhibition at 50 and 100 μg/ml; and high resistance (HR), with isolates capable of vigorous growth at all concentrations tested (Table 1).

The study included *P. xanthii* isolates collected from different host plants, countries, and years, encompassing a wide range of SDHI sensitivity phenotypes. These isolates were selected to represent the diversity of resistance profiles that may be encountered in natural field populations. Although some were collected decades ago, they

remain valuable for comparative and validation purposes, potentially reflecting pathogen populations from environments with limited fungicide pressure, such as organic cropping systems. Isolate 2086 (Greece 1997, melon) was classified as S to both boscalid and fluopyram. In contrast, isolates SF9 (Spain 1988, zucchini), 31430 (Spain 2003, melon), and FR1D (France 2020, zucchini) exhibited LR to boscalid but remained S to fluopyram. Isolate NL1J (Netherlands 2021, cucumber) showed HR to boscalid and LR to fluopyram, while IT1H (Italy 2020, cucumber) displayed HR to both fungicides (Table 1).

From this point onward, reference *P. xanthii* isolate 2086 (Polonio et al. 2021a), S to both SDHI fungicides, was used to optimize all assays described below, while the remaining isolates were employed to evaluate the effect of the combined assay (*PxSdhC*-dsRNA-CD-SDHI fungicide).

dsRNA gene silencing of the *SdhC* subunit in *P. xanthii*

As a preliminary step, infiltration experiments were conducted on melon cotyledons. This stepwise strategy was used to validate the silencing potential of *PxSdhC*-dsRNA under controlled conditions before progressing to more complex whole-plant SIGS trials. Specifically, histochemical, quantitative PCR, and gene expression assays were carried out, following the methodology described by Martínez-Cruz et al. (2018), to evaluate the effects of *PxSdhC*-dsRNA on fungal colonization, biomass accumulation, and target gene transcript levels.

To monitor the progression of *P. xanthii* infection, histochemical analysis was conducted using the lactophenol blue staining method on infected melon cotyledon discs collected at 24, 48, and 72 hpi. As shown in Figures 1A and B, gene silencing of *PxTUB2* and *PxSdhC* using dsRNAs significantly reduced fungal growth compared with the negative control (nonspecific dsRNA sequence obtained from the pL4440 plasmid). At 24 hpi, all treatments showed low haustorium counts; however, fungal growth markedly increased in the negative control at 48 and 72 hpi, while treatments with *PxTUB2*-dsRNA and *PxSdhC*-dsRNA maintained significantly lower and more consistent growth levels. These results suggest that silencing both genes effectively hinders *P. xanthii* development under controlled conditions. Furthermore, fungal biomass was quantified using qPCR at 96 hpi, based on the ratio of *P. xanthii* genomic DNA to *C. melo* genomic DNA (Px/Cm gDNA). As shown in Figure 1C, fungal biomass was significantly reduced by approximately 65% (95% CI: 30.4–108.0%; $P = 0.003$) in samples infiltrated with *PxSdhC*-dsRNA compared with the negative control. No significant differences were observed between the effects of *PxTUB2*-dsRNA and *PxSdhC*-dsRNA, indicating that both have a similar impact on reducing *P. xanthii* biomass. Next, RT-qPCR was used to assess the impact of dsRNA silencing on *PxSdhC* gene expression. For this, cDNA obtained from dsRNA-infiltrated melon cotyledons infected with *P. xanthii* at 72 hpi was used to quantify the relative expression of the *PxSdhC* gene. *PxEF1* served as a reference gene for normalization, following the methodology described by Polonio et al. (2019). The expression of the *PxSdhC* gene was significantly reduced in cotyledons infiltrated with *PxSdhC*-dsRNA compared with the negative control, showing results comparable with the *PxTUB2*-dsRNA's gene silencing (Fig. 1D). This 60% reduction confirms that dsRNA silencing has a strong impact on fungal development, as the marked decrease in expression of an essential gene results in a significant reduction in fungal growth.

Selection of dsRNA and fungicide doses for the integrated control of the disease

To optimize the combined use of dsRNA and commercial fungicides within integrated disease management programs, preliminary assays were conducted to determine the optimal concentration of each treatment individually. The goal was to achieve approximately 40 to 65% disease inhibition and to assess any potential synergistic effects when combining them. For *PxSdhC*-dsRNA, after testing a range of dsRNA concentrations, a positive correlation was observed between increasing dsRNA doses and greater inhibition of fungal development. This suggests that higher dsRNA concentrations enhance gene silencing, thereby increasing disease control efficacy (Fig. 2A). Based on these results, a concentration of 40 μg/ml was selected for subsequent experiments, as it produced around 64% with no significant differences compared with the positive control, the *PxTUB2* gene.

To determine the optimal dsRNA-to-nanoparticle ratio for effective nanoencapsulation, various ratios of *PxSdhC*-dsRNA and CDs (1:1, 1:2, 1:5, and 1:10) were tested using agarose gel electrophoresis (Fig. 2B). The gels revealed a clear shift in dsRNA migration patterns depending on the degree of encapsulation. At a 1:1 ratio, only partial encapsulation was observed, indicated by slower migration compared with free dsRNA. Complete encapsulation was achieved at a 1:2 ratio, where the dsRNA showed strong interaction with CDs and remained near the wells indicating significantly reduced migration. At higher ratios (1:5 and 1:10), dsRNA was largely retained in the wells, likely due to over-encapsulation or aggregation of the dsRNA-nanoparticle complexes. Based on these findings, the 1:2 ratio was selected as the most effective for subsequent SIGS assays, offering a balance between full encapsulation and complex stability.

When different concentrations of the SDHI fungicides boscalid and fluopyram were tested, the *P. xanthii* isolate 2086 used in these assays exhibits differential sensitivity to the SDHI (Fig. 3). In the case of boscalid, disease inhibition increased progressively with higher concentrations, reaching 100% inhibition at the recommended field dose (100 μg/ml), indicating a clear dose-dependent effect (Fig. 3A), and confirming its sensitive phenotype to this compound, as demonstrated in the in vitro study described above. Fluopyram showed a similar response, having high efficacy even at low concentrations with inhibition levels of approximately 60% starting from 2 μg/ml, and no significant differences were observed among the higher doses (Fig. 3B). For further experiments, concentrations of 10 μg/ml and 2 μg/ml were selected, as they achieved approximately 45 and 60% disease inhibition for boscalid and fluopyram, respectively. These concentrations represent a 10-fold (boscalid) and 50-fold (fluopyram) reduction compared with their respective field application rates (100 μg/ml), highlighting the potential of this approach to significantly reduce chemical input.

Evaluation of dsRNAs naked or nanoencapsulated in CDs in combination with SDHI fungicides

Once the optimal concentrations for the different treatments were selected, a greenhouse assay was conducted to evaluate the efficacy of combining dsRNAs targeting the *PxSdhC* gene, either naked or nanoencapsulated in CD nanoparticles, with the SDHI fungicides at low doses. Melon plants were sprayed with different treatments under previously described experimental conditions. Photographs were taken at 14 and 21 dpi to assess the persistence of the treatment effects over time.

At 14 dpi, leaves treated with *PxSdhC*-dsRNA showed a significant reduction in *P. xanthii* development, with approximately 57% disease inhibition comparable with the positive control *PxTUB2*-dsRNA (58%; Fig. 4A). Nanoencapsulation of *PxSdhC*-dsRNA further improved the stability and persistence of gene silencing. *PxSdhC*-dsRNA-CD treatment achieved 69% inhibition, which was statistically similar to *PxTUB2*-dsRNA-CD and to the 57% observed with naked *PxSdhC*-dsRNA ($P > 0.05$). Regarding the combination of the *PxSdhC*-dsRNA and the fungicides at low doses, a synergistic effect was observed, resulting in greater disease inhibition compared with the individual application for each component. Specifically, 85% disease reduction was achieved for *PxSdhC*-dsRNA-CD-boscalid treatment and 87% for *PxSdhC*-dsRNA-CD-fluopyram (Fig. 4A). Representative images of treated leaves at this time point are shown in Figure 4, visually illustrating the differences in disease severity among treatments.

The benefits of nanoencapsulation were even more evident at 21 dpi, when all treatments showed some decrease in efficacy, but those using CD-encapsulated dsRNA maintained superior inhibition (Fig. 4B). At this time point, naked *PxSdhC*-dsRNA and *PxTUB2*-dsRNA treatments showed 35 and 36% inhibition, respectively,

whereas nanoencapsulated *PxSdhC*-dsRNA-CD and *PxTUB2*-dsRNA-CD achieved 53 and 48%, respectively. Importantly, the highest levels of disease inhibition were obtained again under conditions where dsRNA-CD was combined with low-dose fungicide treatments.

Efficacy and limitations of the *PxSdhC*-dsRNA-CD-SDHI fungicide combination across *P. xanthii* isolates

The combined treatment of *PxSdhC*-dsRNA nanoencapsulated with CDs (*PxSdhC*-dsRNA-CD) and SDHI fungicides at low doses demonstrated variable levels of disease suppression across *P. xanthii*

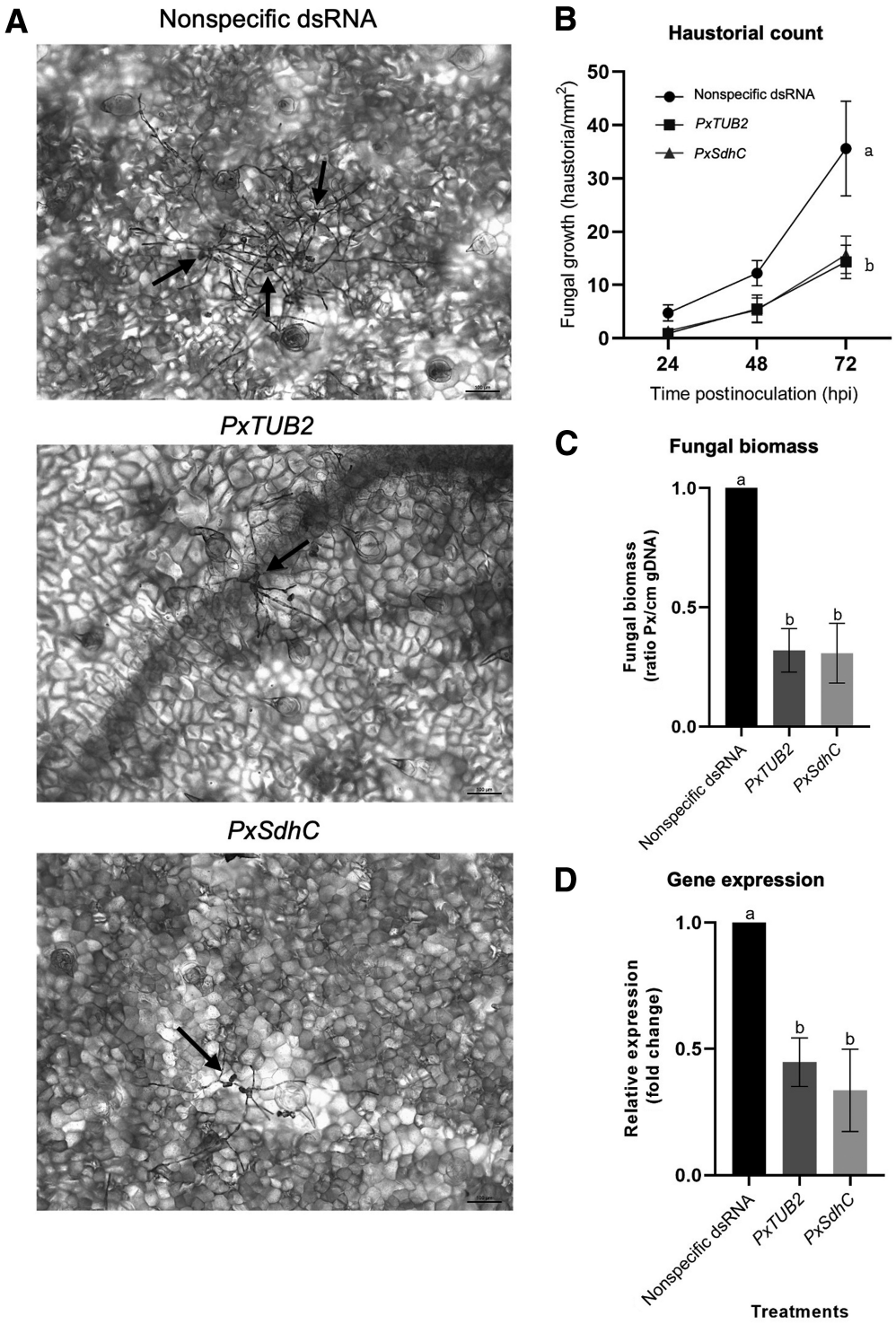
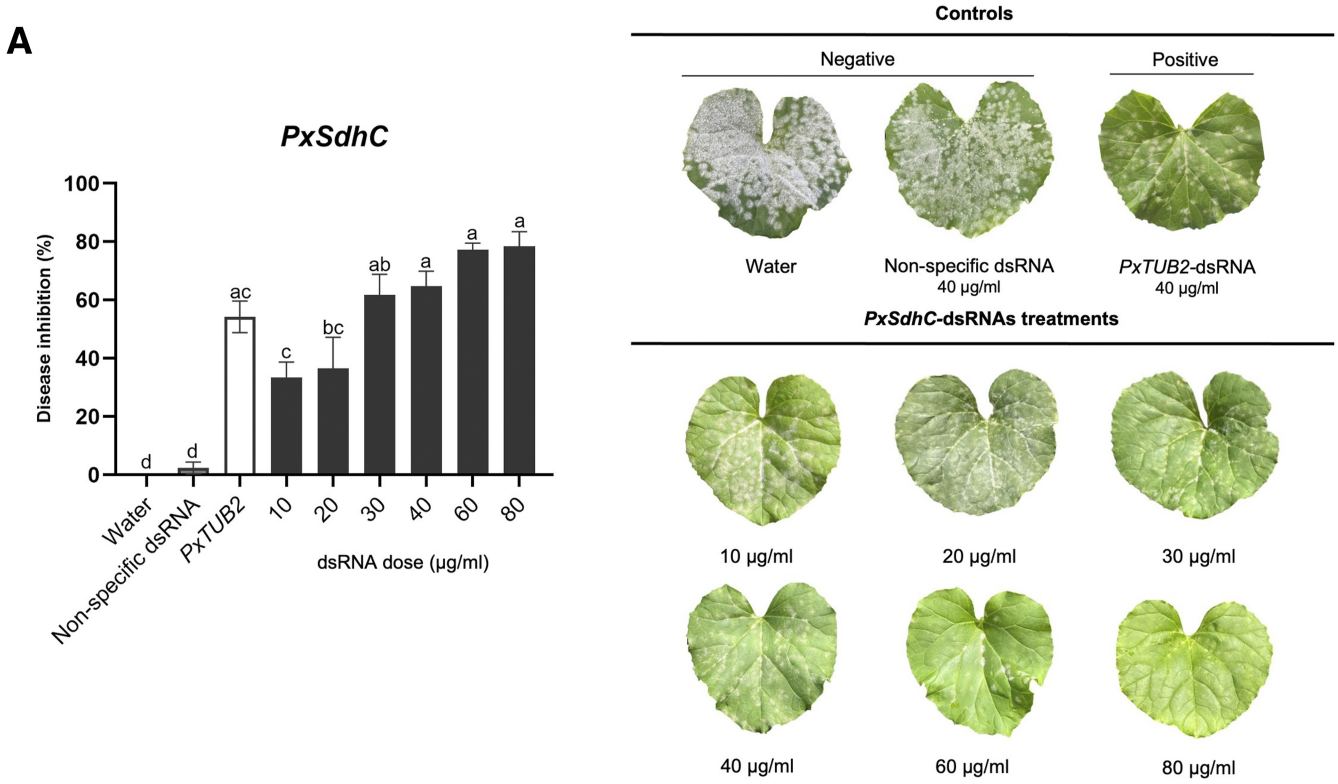


Fig. 1. Effect of *PxSdhC* silencing *Podosphaera xanthii* growth in melon plants. **A,** Visualization of fungal penetration points was performed using in situ lactophenol staining and bright-field microscopy. Images were captured 48 h postinoculation with *P. xanthii.* Arrowheads indicate *P. xanthii* haustoria. Bars = 100 μm. **B,** Estimation of fungal growth was based on haustorium counts. Fungal colonization was quantified by counting the number of penetration points (haustoria) per unit area (mm^2) on melon cotyledons at different times postinoculation (24, 48, and 72 h). Values represent the mean of 15 samples from three independent experiments. **C,** Fungal biomass was quantified by qPCR and expressed as the ratio of *P. xanthii*-specific genomic DNA to *Cucumis melo* genomic DNA (*Px/Cm* gDNA). **D,** Relative gene expression was measured by RT-qPCR and expressed as fold change compared with the negative control (nonspecific dsRNA obtained from the pL4440 plasmid). The *P. xanthii* translation elongation factor 1-alpha gene (*PxEF1*) was used as a reference gene for normalization. Treatments with *PxTUB2*-dsRNA and *PxSdhC*-dsRNA significantly downregulated the *PxSdhC* gene compared with the negative control. Bars represent the mean ± standard error (SE) of three independent experiments. Different letters above the bars indicate statistically significant differences. Data points with the same letter do not differ significantly at $P = 0.05$ according to Tukey's test.

isolates with differing SDHI resistance phenotypes (Fig. 5). In general, coapplication of *PxSdhC*-dsRNA-CD with either fluopyram (2 μg/ml) or boscalid (10 μg/ml) significantly reduced powdery mildew severity compared with the untreated control (100% disease severity). However, treatment efficacy was strongly influenced by each isolate's specific SDHI resistance profile.

Boscalid applied at the recommended field rate was highly effective in the SDHI-sensitive Greek isolate 2086, completely suppressing powdery mildew (100%). In isolates with LR, such as SF9 (Spain), 31430 (Spain), and FR1D (France), the fungicide achieved moderate control of 74, 69, and 81% disease suppression, respectively. In HR isolates, efficacy dropped substantially: NL1J (Netherlands) showed 51% suppression, while IT1H (Italy) showed only 39%. In the combined treatment (*PxSdhC*-dsRNA-CD-boscalid at 10-fold reduction field dose), the combination achieved 85% suppression in the sensitive isolate 2086, confirming the potential of RNAi to maintain high efficacy while reducing fungicide input. For LR isolates, suppression was modest but still notable: 58% (SF9), 66% (31430), and 63% (FR1D). In HR isolates, the combined treatment led to minimal improvements: 55% in NL1J and 40% in IT1H, showing limited benefits in highly resistant populations (Fig. 5).

Fluopyram alone completely suppressed disease in the sensitive isolates (2086, SF9, 31430, and FR1D). In the Dutch isolate NL1J,



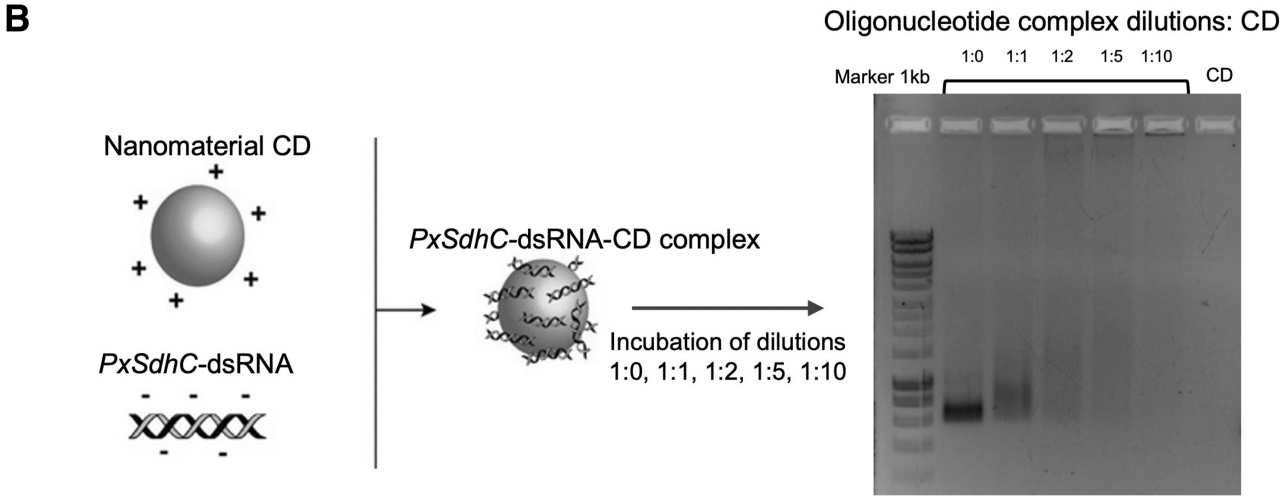


Fig. 2. Effects of *PxSdhC*-dsRNA on *Podosphaera xanthii* development and dsRNA-CD complex migration. **A,** Effect of different concentrations (10, 20, 30, 40, 60, and 80 μg/ml) of *PxSdhC*-dsRNA on *P. xanthii* development in melon leaves. The negative controls consisted of sterile distilled water and nonspecific dsRNA (40 μg/ml), while the positive control consisted of *PxTUB2*-dsRNA applied at 40 μg/ml to silence the *PxTUB2* gene. Bars represent the mean ± SE from three assays, each with six biological replicates. Bars sharing the same letter are not significantly different according to Tukey's test (one-way ANOVA, $P = 0.05$). Images of treated melon plants were taken at 14 days postinoculation. **B,** Delay of dsRNA migration in 1% agarose gel electrophoresis when coated with increasing amounts of carbon dot (CD). Lanes: (1) 1-kb DNA ladder (Meridian Bioscience); (2) *PxSdhC*-dsRNA; (3) *PxSdhC*-dsRNA-CD [1:1]; (4) *PxSdhC*-dsRNA-CD [1:2]; (5) *PxSdhC*-dsRNA-CD [1:5]; (6) *PxSdhC*-dsRNA-CD [1:10]; (7) CDs only.

which exhibited low resistance to fluopyram, suppression was also high (93%). However, in the HR Italian isolate IT1H, fluopyram achieved only 39% disease reduction. Combined with *PxSdhC*-dsRNA-CD (low dose, 50-fold reduction), the combination treatment maintained high levels of control in most isolates, even at significantly reduced fungicide doses. Notable suppression levels were observed in sensitive (2086, SF9, 31430, and FR1D) and low-resistant (NL1J) isolates, ranging from 80 to 90% disease reductions, slightly below the fungicide alone but still highly effective. Conversely, the HR isolate IT1H showed reduced efficacy, with disease suppression dropping to 33% (Fig. 5).

Specificity of *PxSdhC*-dsRNA against different fungal species

To evaluate the specificity of *PxSdhC*-dsRNA across different fungal species, an in vitro assay was conducted using *A. nidulans, B. cinerea, F. graminearum,* and *P. digitatum*. PDA plates were treated with 100 µl of *PxSdhC*-dsRNA (500 µg/ml), and fungal growth was assessed by measuring colony diameters at 48 hpi. Among the tested fungi, only *B. cinerea* showed significant growth inhibition in response to *PxSdhC*-dsRNA treatment (Fig. 6A and B). The average colony diameter of *B. cinerea* in *PxSdhC*-dsRNA-treated plates was 3.9 cm, compared with 5.2 cm in the control treatment with sterile distilled water (Fig. 6B). In contrast, *A. nidulans, F. graminearum*, and *P. digitatum* did not show significant differences in growth between treated and control plates, with colony diameters remaining consistent (Fig. 6A and B). To confirm that the inhibition observed in *B. cinerea* was due to *PxSdhC*-dsRNA and not a nonspecific dsRNA effect, additional controls were included. Plates treated with nonspecific dsRNA obtained from the pL4440 plasmid showed no significant difference compared with the water control, ruling out any effect from the dsRNA backbone. However, treatment with *BcDCL2*-dsRNA (targeting the *Dicer2* gene of *B. cinerea*) also resulted in reduced colony size (average 4 cm), demonstrating that targeted RNAi can effectively inhibit *B. cinerea* (Fig. 6C). A phylogenetic analysis based on the *PxSdhC*-dsRNA sequence revealed that *B. cinerea* clusters separately from the other tested fungi but is closely related to *P. xanthii* (Fig. 6D). Sequence alignment using UniProt showed that the *SdhC* gene of *B. cinerea* (XM_001547025.2) shares 64.81% identity with the dsRNA targeting *SdhC* of *P. xanthii* (Fig. 6D). In contrast, the *SdhC* sequences from *A. nidulans* (*XM_676970.2*), *F. graminearum* (*XM_011330488.1*), and *P. digitatum* (*XM_014678844.1*) showed lower sequence identity. This relatively high sequence similarity between *B. cinerea* and *P. xanthii* likely explains why *PxSdhC*-dsRNA was able to inhibit *B. cinerea*, while the other fungi, with lower *SdhC* homology, remained unaffected.

Discussion

Global food production is increasingly threatened by pests and diseases, with powdery mildews among the most damaging fungal pathogens. These fungi severely impact important crops with *P. xanthii* representing the main causal agent of powdery mildew in cucurbits across southern Europe and the Mediterranean

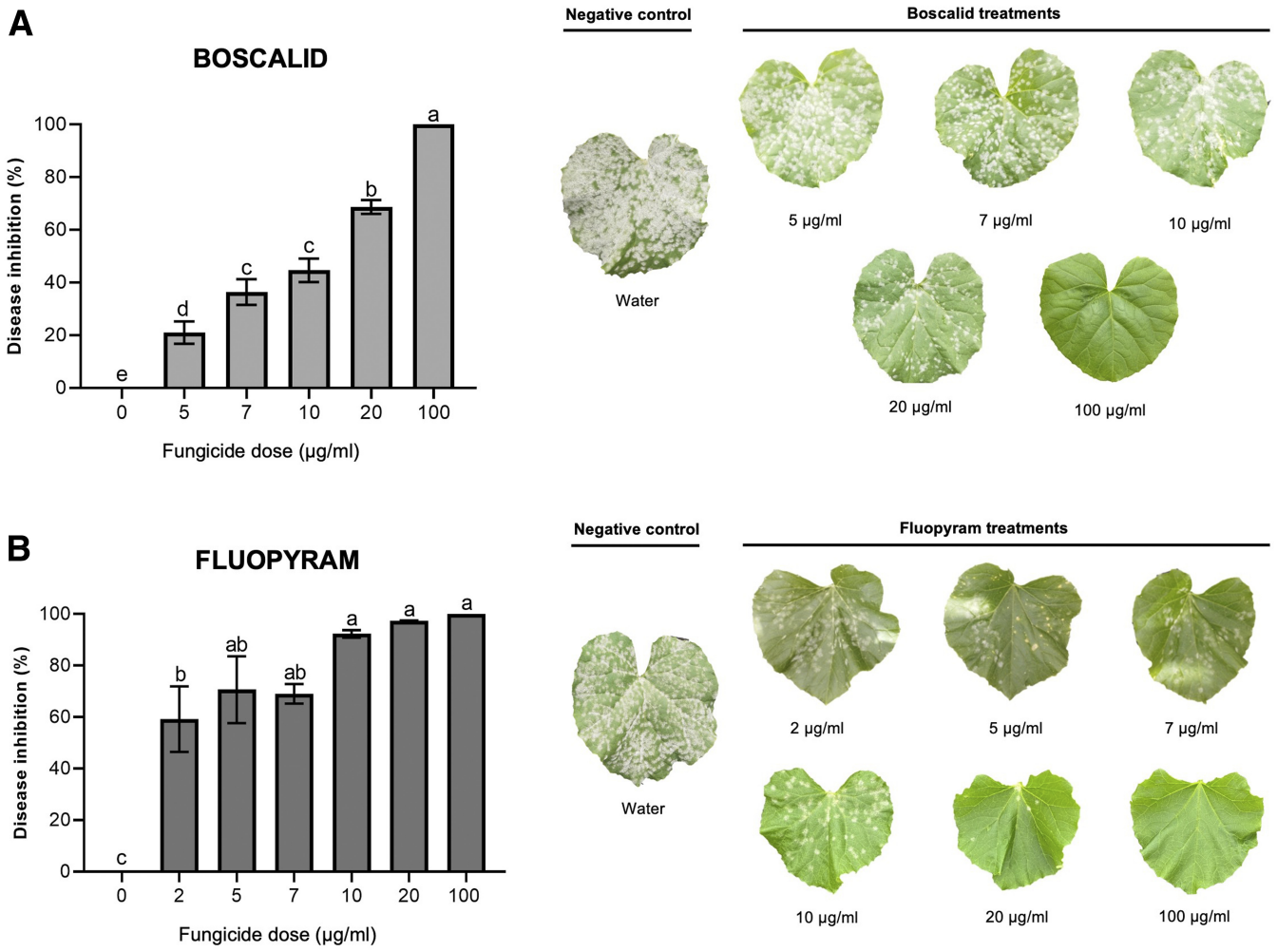


Fig. 3. Development of *P. xanthii* on melon leaves following treatment with different concentrations of the fungicides boscalid (5 to 100 µg/ml) (**A**) and fluopyram (2 to 100 µg/ml) (**B**). Negative controls consisted of sterile distilled water, while positive controls were treated with the recommended field dose (100 µg/ml for boscalid and fluopyram). Bars represent mean values ± SE from three assays with six biological replicates each. Bars sharing the same letter indicate no significant differences according to Tukey's test (one-way ANOVA, $P = 0.05$). Images of treated melon plants were taken at 14 days postinoculation.

(Pérez-García et al. 2009). Although fungicides remain the primary control strategy, their effectiveness can be reduced by the rapid emergence of resistant populations (Fernández-Ortuño et al. 2006, 2007; López-Ruiz et al. 2010; Bellón-Gómez et al. 2015; Fisher et al. 2018; Miyamoto et al. 2020; Vielba-Fernández et al. 2018, 2019, 2021). Moreover, this growing resistance problem is further exacerbated by the consumer demand for pesticide-free food, and stricter European regulations underscore the urgent need for sustainable alternatives (Fletcher et al. 2020; European Commission 2022). In this context, RNAi has emerged as a promising approach. While early methods like host-induced gene silencing (HIGS) faced regulatory limitations due to genetically modified organism (GMO; Nowara et al. 2010; Nunes and Dean 2012) concerns, SIGS offers a nontransgenic alternative, allowing exogenous application of dsRNA to silence essential fungal genes (McLoughlin et al. 2018; Qiao et al. 2021; Wang and Jin 2017). Recent studies have demonstrated the efficacy of SIGS to control fungal diseases in important crops (McLoughlin et al. 2018; Mosa and Youssef 2021; Chen et al. 2023; Duanis-Assaf et al. 2022). Regarding cucurbit powdery mildew, Ruiz-Jiménez and colleagues (2021) demonstrated the potential of SIGS for managing this disease using *P. xanthii*-specific dsRNA involved in essential functions such as respiration, glycosylation, and efflux transport. However, to maximize the effectiveness of this strategy, it is essential to continue exploring and validating key fungal genes that can serve as sustainable targets for plant protection tools. Building on this approach, Bakhat et al. (2025) demonstrated that dsRNAs designed against *P. xanthii* effectors involved in chitin deacetylases (CDAs) and their degradation (EWCAs), when nanoencapsulated in CDs, not only improved gene silencing efficiency but also extended the duration of disease control under greenhouse conditions.

The succinate dehydrogenase complex is a key component of the eukaryotic mitochondrial respiratory chain and the primary target of SDHI fungicides. Given the central role of the *SdhC* subunit in mediating resistance to multiple SDHI fungicides, this gene was selected in the present study as the target for preliminary evaluations of RNAi-based approaches to control *P. xanthii*. In addition, this target was considered particularly relevant because several SDHI

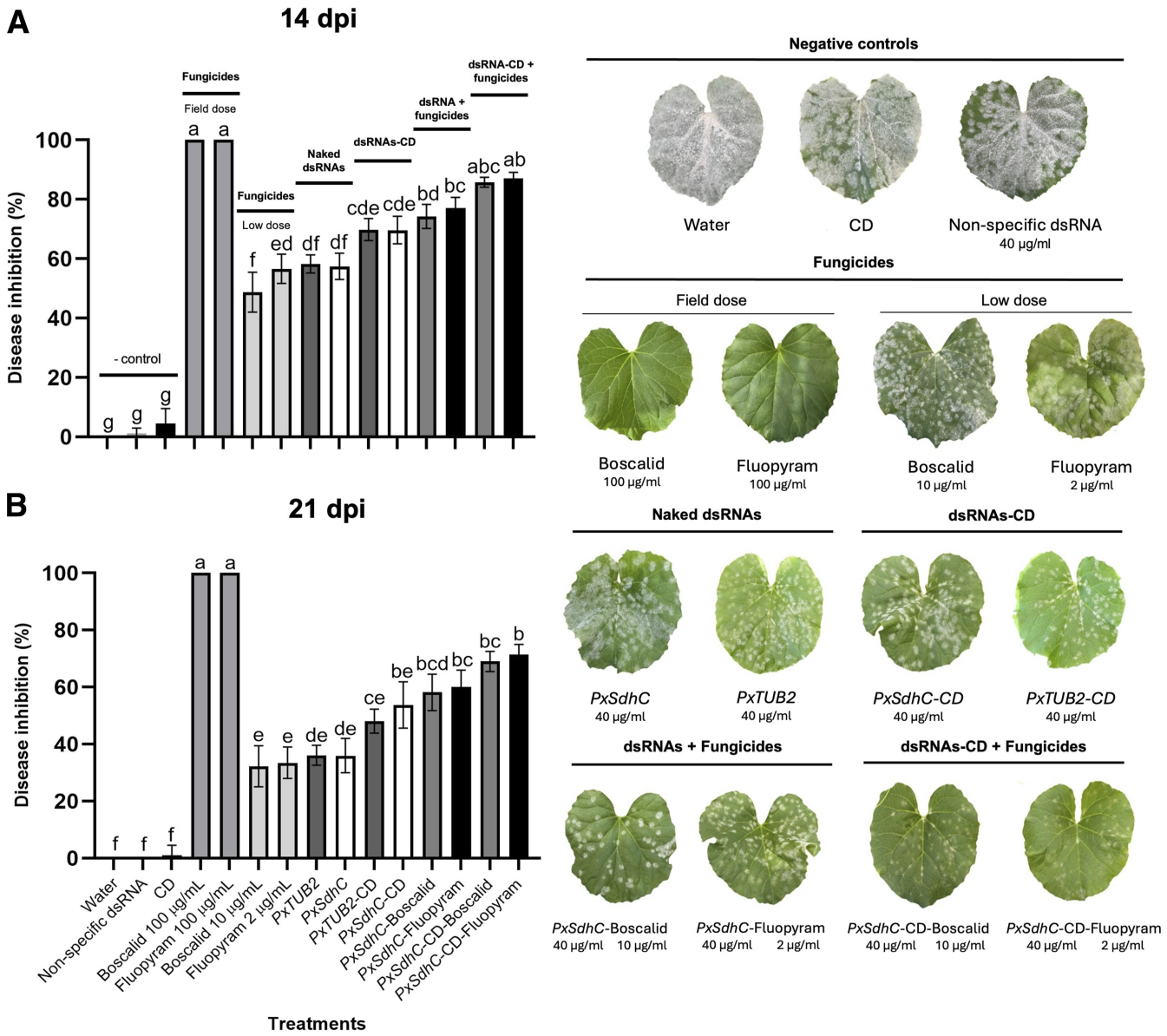


Fig. 4. Disease inhibition (%) of cucurbit powdery mildew on melon leaves at 14 (**A**) and 21 (**B**) days postinoculation (dpi) following application of dsRNA-based treatments targeting *PxSdhC*. Treatments included naked and carbon dot (CD)-encapsulated dsRNAs (*PxSdhC* and *PxTUB2*), applied alone or in combination with low doses of the SDHI fungicides boscalid and fluopyram. Controls included water, CD alone, and nonspecific dsRNA, as well as fungicides applied at both full and reduced field doses. Bars represent mean inhibition values ± SE from three independent experiments, each with six biological replicates. Statistical significance was assessed using one-way ANOVA followed by Tukey's test ($P = 0.05$); bars sharing the same letter are not significantly different. Representative leaf images taken at 14 dpi are shown to illustrate visual symptoms under each treatment.

fungicides, currently registered for agricultural use, act on the same metabolic pathway. Thus, evaluating the combined application of fungicides and RNAi, both acting on succinate dehydrogenase, could offer insights into potential synergistic effects and more sustainable disease control strategies. In cotyledon infiltration assays, silencing of *PxSdhC* resulted in a significant reduction in fungal growth and gene expression. These results were consistent with those observed in previous studies where *Agrobacterium tumefaciens*-mediated (ATM) HIGS and SIGS, targeting other genes in *P. xanthii*, led to strong gene silencing effects (J. M. Martínez-Cruz et al. 2021; J. Martínez-Cruz et al. 2021; Polonio et al. 2021b; Ruiz-Jiménez et al. 2021; Bakhat et al. 2025), further confirming the importance of dsRNA-mediated gene silencing in fungal development. However, targeting the same metabolic pathway with both SDHI fungicides and RNAi-based strategies may raise concerns about potential cross-resistance. Mutations in the *SdhC* gene that reduce the binding efficiency of SDHIs could also hinder the effectiveness of RNAi if they alter gene expression levels or dsRNA recognition pathways. Conversely, selective pressure from RNAi-based silencing could favor alleles with reduced susceptibility to both RNAi and SDHIs, potentially accelerating resistance development. Therefore, while combining SDH-targeting fungicides and RNAi may enhance short-term control, it is essential to monitor resistance dynamics closely. This highlights the need for integrated resistance management strategies when deploying SIGS in combination with chemical control.

To further evaluate the synergistic potential of combining RNAi-based strategies with conventional control measures, the application of *PxSdhC*-dsRNA, both in its naked and nanoencapsulated forms (dsRNA-CDs) using the SIGS approach, was tested. Initial dose-response experiments confirmed that increasing concentrations of *PxSdhC*-dsRNA led to greater inhibition of fungal growth, reaching maximum efficacy at 60 μg/ml. This inhibition pattern aligned with previous studies indicating that dsRNA concentration is a key factor determining SIGS efficacy (Bakhat et al. 2025; Ruiz-Jiménez et al. 2021). Furthermore, encapsulating *PxSdhC*-dsRNA in CDs significantly enhanced its ability to reduce *P. xanthii* development in melon plants at both 14 and 21 dpi. The highest inhibition levels were observed with dsRNA-CD formulations, where fungal growth was substantially reduced compared with naked dsRNA with just one application, suggesting improved stability against enzymatic degradation and environmental exposure (Delgado-Martín et al. 2022;

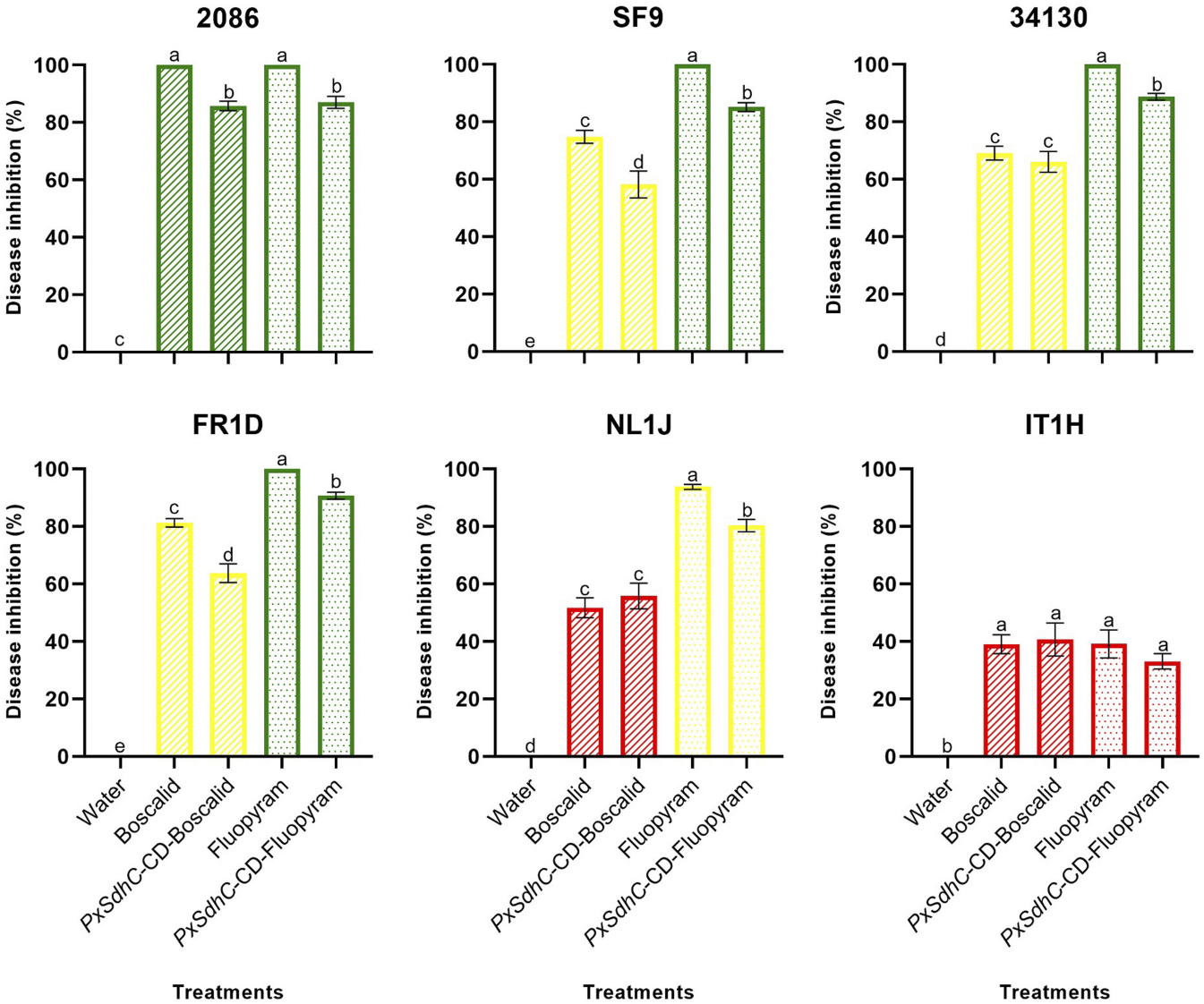


Fig. 5. The effectiveness of the RNAi-CD-fungicide combination for powdery mildew control on different *Podosphaera xanthii* isolates. Disease inhibition on melon leaves was measured at 14 dpi following treatment application. Treatments included control treatments (water and the succinate dehydrogenase inhibitor [SDHI] fungicides boscalid and fluopyram at the field dose rates) and *PxSdhC*-dsRNA-CD in combination with boscalid and fluopyram in low doses. Bars represent mean values ± SE from three assays with six biological replicates each. Bars sharing the same letter indicate no significant differences according to Tukey's test ($P = 0.05$, one-way ANOVA). Bar colors indicate the SDHI resistance profiles of the *P. xanthii* isolates: green = sensitive, yellow = low resistance, and red = high resistance. Treatment types are represented by patterns with inclined lines and dots for boscalid and fluopyram, respectively.

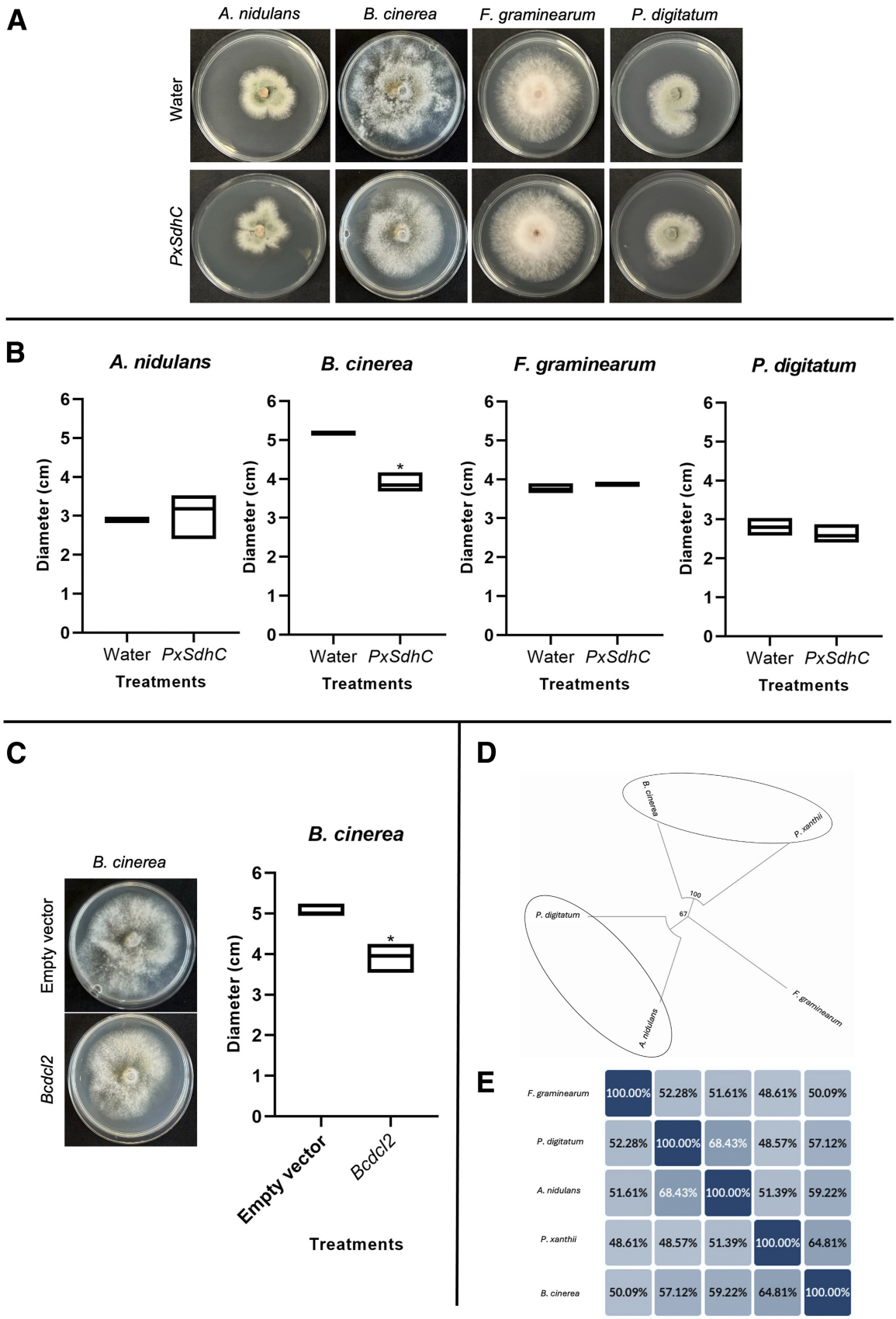


Fig. 6. Effect of applied dsRNA in vitro. **A,** Fungal development on PDA plates in response to *PxSdhC*-dsRNA or water treatment at 48 h. **B,** Growth of *Botrytis cinerea*, *Fusarium graminearum*, *Penicillium digitatum*, and *Aspergillus nidulans* measured by colony diameter (cm), following application of *PxSdhC-dsRNA* and water. Statistical differences were determined using Tukey's test. **C,** In vitro dsRNA assays targeting BcDCL2 in *B. cinerea*, showing significantly reduced colony size in treated samples compared with nonspecific dsRNA obtained from the pL4440 plasmid which showed no effect. **D,** Phylogenetic analysis of plant-pathogenic ascomycetes based on the nucleotide fragment of the *SdhC* gene that was used to design the *PxSdhC*-dsRNA. A radial tree was constructed using neighbor-joining analysis. The nucleotide sequence for *Podosphaera xanthii* was obtained from LC522548.1. Sequences for *B. cinerea*, *F. graminearum*, *P. digitatum*, and *A. nidulans* were obtained from XM_001547025.2, XM_011330488.1, XM_014678844.1, and XM_676970.2, respectively. Bootstrap values from 100 replications are shown on branches. Branch lengths are scaled to reflect evolutionary distances. **E,** Percentage identity of *PxSdhC* compared with other tested fungal species based on UniProt analysis.

Wang et al. 2023). This improvement is consistent with the known properties of these nanoparticles, which enhance dsRNA protection and uptake. Their nanoscale size and surface charge likely facilitated dsRNA stabilization and delivery to fungal and plant cells. CD complexes shield dsRNA molecules during delivery and facilitate cellular internalization, as previously demonstrated in other systems (Iroha et al. 2023; Schwartz et al. 2020; Verma and Modgil 2024), therefore contributing to the extended duration and efficacy of the silencing effect observed. Subsequently, *PxSdhC*-dsRNA-CD in combination with low doses of SDHI fungicides was applied to evaluate their combined efficacy against *P. xanthii* isolates with different SDHI phenotypes. The combined treatments significantly improved disease control in sensitive and low-resistant *P. xanthii* isolates, allowing for major reductions in chemical input while maintaining high efficacy. Although efficacy was reduced in highly resistant isolates, the partial suppression observed indicates that RNAi-based strategies can still exert biological activity when fungicide effectiveness is diminished. This underscores the value of combining RNAi and chemical control as complementary tools, capable of reinforcing each other's action and extending protection across a broader range of resistance phenotypes. Importantly, this combined approach not only improved disease control while lowering fungicide use but also offers a sustainable alternative for integrated management.

This strategy is particularly relevant in the current context of global agriculture, where the number of available fungicide active substances is declining due to increasing regulatory restrictions and environmental concerns. According to the European Food Safety Authority (EFSA) and the European Commission, the number of approved fungicide actives in the EU has dropped from approximately 1,000 in the early 2000s to around 400 to 450 today (EFSA 2024; European Commission Pesticide Database 2024). This reduction, combined with the rapid emergence of fungicide-resistant pathogens, presents major challenges for effective plant disease management. In this scenario, innovative solutions such as combining RNAi, nanotechnology, and reduced-dose fungicides offer a promising way to maintain effective crop protection while reducing chemical input. Our study is the first to demonstrate that integrating nanoencapsulated dsRNA with fungicides can provide effective control of cucurbit powdery mildew. While previous research has explored the use of metal nanoparticles to enhance fungicide delivery against pathogens like *B. cinerea* and *A. alternata* (Malandrakis et al. 2020; Nami et al. 2017), our findings extend this concept to RNA-based disease management.

On the other hand, our results are also consistent with previous studies exploring RNAi applications for controlling other plant pathogens. *PxSdhC*-dsRNA selectively inhibits the growth of *B. cinerea*, with a significant reduction in colony diameter compared with controls, while having no effect on other fungal species. This specificity is aligned with the high sequence similarity observed between the *SdhC* gene of *B. cinerea* and *P. xanthii, SdhC*-dsRNA, supporting the hypothesis that RNAi efficacy is strongly dependent on sequence homology, as previously reported by Koch et al. (2016) and Wang et al. (2016). Our phylogenetic analysis, showing that *B. cinerea* clusters closer to *P. xanthii* than to the other tested fungi, provides a molecular explanation for the observed specificity and aligns with the current understanding of RNAi efficiency being determined by both gene target conservation and phylogenetic proximity. Cross-species silencing has been described in other fungal pathogens. For instance, McLoughlin et al. (2018) demonstrated that dsRNA designed to target virulence genes in *Sclerotinia sclerotiorum* also suppressed infection by *B. cinerea*, due to conserved orthologs. Similarly, Koch et al. (2016) showed that dsRNA targeting *CYP51* genes in *F. graminearum* inhibited fungal growth and could potentially be adapted to other fungi sharing conserved sterol biosynthesis pathways. These findings support the broader applicability of RNAi-based tools to control multiple fungal pathogens by targeting conserved gene families but also highlight the importance of precise sequence selection to avoid unintended effects on non-target organisms.

In summary, the results of this study offer new perspectives for controlling powdery mildew in cucurbits. The technique of SIGS using nanoencapsulated dsRNA combined with low doses of fungicides, targeting the same biological pathway, emerges as an effective and sustainable control strategy. By integrating these innovations, it may be possible to preserve existing active substances, reduce environmental impact, and delay the development of resistance. These approaches support the long-term protection of crops while complying with increasingly strict regulatory and sustainability requirements. To further validate and optimize this approach, future studies should include (i) open-field evaluations to confirm the effectiveness of SIGS-fungicide combinations under real agricultural conditions; (ii) efficacy trials using highly resistant isolates treated with field-rate fungicides in combination with dsRNA to assess the robustness of the strategy under strong resistance pressure; (iii) long-term experiments with sensitive and low-resistance isolates treated with repeated applications of low-dose fungicides plus dsRNA to monitor potential shifts in resistance levels over time; (iv) testing of different gene targets or their combinations to enhance durability; and (v) exploration of alternative nanoparticle carriers to improve dsRNA delivery, stability, and environmental safety. Such investigations will be essential to support the large-scale implementation of this integrated strategy for the sustainable management of powdery mildew in cucurbits.

Acknowledgements

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Literature Cited

Álvarez, B. 1993. Epidemiología de *Sphaerotheca fuligine*a (Schlecht. ex. Fr.) Poll. en Melón. Ph.D. thesis. Universidad de Málaga, Málaga, Spain.

Álvarez, B., and Torés, J. A. 1997. Cultivo in vitro de *Sphaerotheca fuliginea* (Schlecht. ex Fr.), efecto de diferentes fuentes de carbono sobre su desarrollo. Bol. San. Veg. Plagas 23:283-288.

Bakhat, N., Jiménez-Sánchez, A., Ruiz-Jiménez, L., Padilla-Roji, I., Velasco, L., Pérez-García, A., and Fernández-Ortuño, D. 2025. Fungal effector genes involved in the suppression of chitin signaling as novel targets for the control of powdery mildew disease via a nontransgenic RNA interference approach. Pest Manag. Sci. 81:3452-3463.

Bellón-Gómez, D., Pérez-García, A., de Vicente, A., and Torés, J. A. 2012. Control integrado del oídio de las cucurbitáceas. Vida Rural 344:24-27.

Bellón-Gómez, D., Vela-Corcía, D., Pérez-García, A., and Torés, J. A. 2015. Sensitivity of *Podosphaera xanthii* populations to anti-powdery-mildew fungicides in Spain. Pest Manag. Sci. 71:1407-1413.

Braun, U., and Cook, R. T. A. 2012. Taxonomic Manual of the Erysiphales (Powdery Mildews). CBS Biodiversity Series 11. CBS-KNAW Fungal Biodiversity Centre, Utrecht, the Netherlands.

Ceballos Alcantarilla, E. 2017. Desarrollo de Métodos Inmunoquímicos Para el Análisis en Alimentos de Fungicidas Inhibidores de la Succinato Deshidrogenasa. Ph.D. thesis. Universitat de València, Valencia, Spain.

Chen, N., Dai, X., Hu, Q., Tan, H., Qiao, L., and Lu, L. 2023. Sprayable double-stranded RNA mediated RNA interference reduced enzymatic browning of fresh-cut potatoes. Postharvest Biol. Technol. 206:112563.

Degnan, R. M., McTaggart, A. R., Shuey, L. S., Pame, L. J. S., Smith, G. R., Gardiner, D. M., Nock, V., Soffe, R., Sale, S., Garrill, A., Carroll, B. J., Mitter, N., and Sawyer, A. 2023. Exogenous double-stranded RNA inhibits the infection physiology of rust fungi to reduce symptoms in planta. Mol. Plant Pathol. 24:191-207.

Delgado-Martín, J., Delgado-Olidén, A., and Velasco, L. 2022. Carbon dots boost dsRNA delivery in plants and increase local and systemic siRNA production. Int. J. Mol. Sci. 23:5338.

del Pino, D., Olalla, L., Pérez-García, A., Rivera, M. E., García, S., Moreno, R., de Vicente, A., and Torés, J. A. 2002. Occurrence of races and pathotypes of cucurbit powdery mildew in southeastern Spain. Phytoparasitica 30:459-466.

Duanis-Assaf, D., Galsurker, O., Davydov, O., Maurer, D., Feygenberg, O., Sagi, M., Poverenov, E., Fluhr, R., and Alkan, N. 2022. Double-stranded RNA targeting fungal ergosterol biosynthesis pathway controls *Botrytis cinerea* and postharvest grey mould. Plant Biotechnol. J. 20:226-237.

EFSA (European Food Safety Authority). 2024. The 2022 European Union report on pesticide residues in food. EFSA J. 22:e8753.

European Commission. 2022. Proposal for a Regulation of the European Parliament and of the Council on the Sustainable Use of Plant Protection Products and Amending Regulation (EU) 2021/2115. COM(2022) 305 final. Document 52022PC0305. Brussels, Belgium.

European Commission. 2024. EU Pesticides Database: Active substances. Directorate-General for Health and Food Safety (DG SANTE), European Commission, Brussels, Belgium. https://food.ec.europa.eu/plants/pesticides/eu-pesticides-database_en

Fernández-Ortuño, D., Pérez-García, A., López-Ruiz, F., Romero, D., de Vicente, A., and Torés, J. A. 2006. Occurrence and distribution of resistance to QoI fungicides in populations of *Podosphaera fusca* in south central Spain. Eur. J. Plant Pathol. 115:215-222.

Fernández-Ortuño, D., Torés, J. A., de Vicente, A., and Pérez-García, A. 2007. Multiple displacement amplification, a powerful tool for molecular genetic analysis of powdery mildew fungi. Curr. Genet. 51:209-219.

Fisher, M. C., Hawkins, N. J., Sanglard, D., and Gurr, S. J. 2018. Worldwide emergence of resistance to antifungal drugs challenges human health and food security. Science 360:739-742.

Fletcher, S. J., Reeves, P. T., Hoang, B. T., and Mitter, N. 2020. A perspective on RNAi-based biopesticides. Front. Plant Sci. 11:51.

FRAC. 2019. Pathogen risk list. Fungicide Resistance Action Committee. www.frac.info/media/qluf3unn/frac-pathogen-list-2019.pdf. (accessed 19 September 2025).

Gilardi, G., Demarchi, S., Garibaldi, A., and Gullino, M. L. 2013. Management of downy mildew of sweet basil (*Ocimum basilicum*) caused by *Peronospora belbahrii* by means of resistance inducers, fungicides, biocontrol agents and natural products. Phytoparasitica 41:59-72.

Godfrey, D., Zhang, Z., Saalbach, G., and Thordal-Christensen, H. 2009. A proteomics study of barley powdery mildew haustoria. Proteomics 9:3222-3232.

Hoang, N. H., Le Thanh, T., Sangpueak, R., Treekoon, J., Saengchan, C., Thepbandit, W., Papathoti, N. K., Kamkaew, A., and Buensanteai, N. K. 2022. Chitosan nanoparticles-based ionic gelation method: A promising candidate for plant disease management. Polymers 14:662.

Hu, D., Chen, Z.-Y., Zhang, C., and Ganiger, M. 2020. Reduction of *Phakopsora pachyrhizi* infection on soybean through host- and spray-induced gene silencing. Mol. Plant Pathol. 21:794-807.

Iroha, N. B., Oluchi, E. C., Christiana, C. N., and Abeng, F. E. 2023. Chapter 14: Carbon dots in protein and nucleic acid delivery. Pages 313-324 in: Carbon Dots in Biology. De Gruyter, Berlin, Germany.

Karny, A., Zinger, A., Kajal, A., Shainsky-Roitman, J., and Schroeder, A. 2018. Therapeutic nanoparticles penetrate leaves and deliver nutrients to agricultural crops. Sci. Rep. 8:7589.

Koch, A., Biedenkopf, D., Furch, A., Weber, L., Rossbach, O., Abdellatef, E., Linicus, L., Johannsmeier, J., Jelonek, L., Goesmann, A., Cardoza, V., McMillan, J., Mentzel, T., and Kogel, K.-H. 2016. An RNAi-based control of *Fusarium graminearum* infections through spraying of long dsRNAs involves a plant passage and is controlled by the fungal silencing machinery. PLoS Pathog. 12:e1005901.

Koch, A., and Wassenegger, M. 2021. Host-induced gene silencing – mechanisms and applications. New Phytol. 231:54-59.

Koressaar, T., and Remm, M. 2007. Enhancements and modifications of primer design program Primer3. Bioinformatics 23:1289-1291.

López-Ruiz, F. J., Pérez-García, A., Fernández-Ortuño, D., Romero, D., García, E., de Vicente, A., Brown, J. K. M., and Torés, J. A. 2010. Sensitivities to DMI fungicides in populations of *Podosphaera fusca* in south central Spain. Pest Manag. Sci. 66:801-808.

Malandrakis, A. A., Kavroulakis, N., and Chrysikopoulos, C. V. 2020. Synergy between Cu-NPs and fungicides against *Botrytis cinerea*. Sci. Total Environ. 703:135557.

Mandel, M., and Higa, A. 1970. Calcium-dependent bacteriophage DNA infection. J. Mol. Biol. 53:159-162.

Martínez-Cruz, J., Romero, D., de la Torre, F. N., Fernández-Ortuño, D., Torés, J. A., de Vicente, A., and Pérez-García, A. 2018. The functional characterization of *Podosphaera xanthii* candidate effector genes reveals novel target functions for fungal pathogenicity. Mol. Plant-Microbe Interact. 31:914-931.

Martínez-Cruz, J., Romero, D., Hierrezuelo, J., Thon, M., de Vicente, A., and Pérez-García, A. 2021. Effectors with chitinase activity (EWCAs), a family of conserved, secreted fungal chitinases that suppress chitin-triggered immunity. Plant Cell 33:1319-1340.

Martínez-Cruz, J. M., Polonio, Á., Zanni, R., Romero, D., Gálvez, J., Fernández-Ortuño, D., and Pérez-García, A. 2021. Chitin deacetylase, a novel target for the design of agricultural fungicides. J. Fungi 7:1009.

McLoughlin, A. G., Wytinck, N., Walker, P. L., Girard, I. J., Rashid, K. Y., de Kievit, T., Fernando, W. G. D., Whyard, S., and Belmonte, M. F. 2018. Identification and application of exogenous dsRNA confers plant protection against *Sclerotinia sclerotiorum* and *Botrytis cinerea*. Sci. Rep. 8:7320.

McRae, A. G., Taneja, J., Yee, K., Shi, X., Haridas, S., LaButti, K., Singan, V., Grigoriev, I. V., and Wildermuth, M. C. 2023. Spray-induced gene silencing to identify powdery mildew gene targets and processes for powdery mildew control. Mol. Plant Pathol. 24:1168-1183.

Mitter, N., Worrall, E. A., Robinson, K. E., Li, P., Jain, R. G., Taochy, C., Fletcher, S. J., Carroll, B. J., Lu, G. Q., and Xu, Z. P. 2017. Clay nanosheets for topical delivery of RNAi for sustained protection against plant viruses. Nat. Plants 3:16207.

Miyamoto, T., Hayashi, K., Okada, R., Wari, D., and Ogawara, T. 2020. Resistance to succinate dehydrogenase inhibitors in field isolates of *Podosphaera xanthii* on cucumber: Monitoring, cross-resistance patterns and molecular characterization. Pestic. Biochem. Physiol. 169:104646.

Mosa, M. A., and Youssef, K. 2021. Topical delivery of host induced RNAi silencing by layered double hydroxide nanosheets: An efficient tool to decipher pathogenicity gene function of *Fusarium* crown and root rot in tomato. Physiol. Mol. Plant Pathol. 115:101684.

Mumbanza, F. M., Kiggundu, A., Tusiime, G., Tushemereirwe, W. K., Niblett, C., and Bailey, A. 2013. In vitro antifungal activity of synthetic dsRNA molecules against two pathogens of banana, *Fusarium oxysporum* f. sp. *cubense* and *Mycosphaerella fijiensis*. Pest Manag. Sci. 69:1155-1162.

Nami, S., Baradaran, B., Mansoori, B., Kordbacheh, P., Rezaie, S., Falahati, M., Mohamed Khosroshahi, L., Safara, M., and Zaini, F. 2017. The utilization of RNA silencing technology to mitigate the voriconazole resistance of *Aspergillus flavus*; Lipofectamine-based delivery. Adv. Pharm. Bull. 7:53-59.

Nowara, D., Gay, A., Lacomme, C., Shaw, J., Ridout, C., Douchkov, D., Hensel, G., Kumlehn, J., and Schweizer, P. 2010. HIGS: Host-induced gene silencing in the obligate biotrophic fungal pathogen *Blumeria graminis*. Plant Cell 22:3130-3141.

Nunes, C. C., and Dean, R. A. 2012. Host-induced gene silencing: A tool for understanding fungal host interaction and for developing novel disease control strategies. Mol. Plant Pathol. 13:519-529.

Ons, L., Bylemans, D., Thevissen, K., and Cammue, B. P. A. 2020. Combining biocontrol agents with chemical fungicides for integrated plant fungal disease control. Microorganisms 8:1930.

Padilla-Roji, I., Ruiz-Jiménez, L., Bakhat, N., Vielba-Fernández, A., Pérez-García, A., and Fernández-Ortuño, D. 2023. RNAi technology: A new path for the research and management of obligate biotrophic phytopathogenic fungi. Int. J. Mol. Sci. 24:9082.

Pérez-García, A., Mingorance, E., Rivera, M. E., Del Pino, D., Romero, D., Torés, J. A., and De Vicente, A. 2006. Long-term preservation of *Podosphaera fusca* using silica gel. J. Phytopathol. 154:190-192.

Pérez-García, A., Romero, D., Fernández-Ortuño, D., López-Ruiz, F., De Vicente, A., and Torés, J. A. 2009. The powdery mildew fungus *Podosphaera fusca* (synonym *Podosphaera xanthii*), a constant threat to cucurbits. Mol. Plant Pathol. 10:153-160.

Polonio, Á., Díaz-Martínez, L., Fernández-Ortuño, D., de Vicente, A., Romero, D., López-Ruiz, F. J., and Pérez-García, A. 2021a. A hybrid genome assembly resource for *Podosphaera xanthii,* the main causal agent of powdery mildew disease in cucurbits. Mol. Plant-Microbe Interact. 34:319-324.

Polonio, Á., Fernández-Ortuño, D., de Vicente, A., and Pérez-García, A. 2021b. A haustorial-expressed lytic polysaccharide monooxygenase from the cucurbit powdery mildew pathogen *Podosphaera xanthii* contributes to the suppression of chitin-triggered immunity. Mol. Plant Pathol. 22:580-601.

Polonio, Á., Pineda, M., Bautista, R., Martínez-Cruz, J., Pérez-Bueno, M. L., Barón, M., and Pérez-García, A. 2019. RNA-seq analysis and fluorescence imaging of melon powdery mildew disease reveal an orchestrated reprogramming of host physiology. Sci. Rep. 9:7978.

Qiao, L., Lan, C., Capriotti, L., Ah-Fong, A., Nino Sanchez, J., Hamby, R., Heller, J., Zhao, H., Glass, N. L., Judelson, H. S., Mezzetti, B., Niu, D., and Jin, H. 2021. Spray-induced gene silencing for disease control is dependent on the efficiency of pathogen RNA uptake. Plant Biotechnol. J. 19:1756-1768.

Ray, P., Sahu, D., Aminedi, R., and Chandran, D. 2022. Concepts and considerations for enhancing RNAi efficiency in phytopathogenic fungi for RNAi-based crop protection using nanocarrier-mediated dsRNA delivery systems. Front. Fungal Biol. 3:977502.

Romero, D., Eugenia Rivera, M., Cazorla, F. M., Codina, J. C., Fernández-Ortuño, D., Torés, J. A., Pérez-García, A., and de Vicente, A. 2008. Comparative histochemical analyses of oxidative burst and cell wall reinforcement in compatible and incompatible melon–powdery mildew (*Podosphaera fusca*) interactions. J. Plant Physiol. 165:1895-1905.

Rueden, C. T., Schindelin, J., Hiner, M. C., DeZonia, B. E., Walter, A. E., Arena, E. T., and Eliceiri, K. W. 2017. ImageJ2: ImageJ for the next generation of scientific image data. BMC Bioinf. 18:529.

Ruiz-Jiménez, L., Polonio, Á., Vielba-Fernández, A., Pérez-García, A., and Fernández-Ortuño, D. 2021. Gene mining for conserved, non-annotated proteins of *Podosphaera xanthii* identifies novel target candidates for controlling powdery mildews by spray-induced gene silencing. J. Fungi 7:735.

Schwartz, S. H., Hendrix, B., Hoffer, P., Sanders, R. A., and Zheng, W. 2020. Carbon dots for efficient small interfering RNA delivery and gene silencing in plants. Plant Physiol. 184:647-657.

Sierotzki, H., and Scalliet, G. 2013. A review of current knowledge of resistance aspects for the next-generation succinate dehydrogenase inhibitor fungicides. Phytopathology 103:880-887.

van der Oost, J., Swarts, D. C., and Jore, M. M. 2014. Prokaryotic Argonautes – Variations on the RNA interference theme. Microb. Cell 1:158-159.

Vela-Corcía, D., Bellón-Gómez, D., López-Ruiz, F., Torés, J. A., and Perez-Garcia, A. 2014. The *Podosphaera fusca TUB2* gene, a molecular "Swiss

Army knife" with multiple applications in powdery mildew research. Fungal Biol. 118:228-241.
Vela-Corcía, D., Romero, D., Torés, J. A., De Vicente, A., and Pérez-García, A. 2015. Transient transformation of *Podosphaera xanthii* by electroporation of conidia. BMC Microbiol. 15:20.
Verma, K., and Modgil, M. 2024. RNA interference (RNAi) mediated technique for combating plant diseases: Harnessing nanoparticles for effective delivery and enhanced efficacy. Plant Cell Tiss. Organ Cult. 157:53.
Vielba-Fernández, A., Bellón-Gómez, D., Torés, J. A., de Vicente, A., Pérez-García, A., and Fernández-Ortuño, D. 2018. Heteroplasmy for the cytochrome *b* gene in *Podosphaera xanthii* and its role in resistance to QoI fungicides in Spain. Plant Dis. 102:1599-1605.
Vielba-Fernández, A., de Vicente, A., Pérez-García, A., and Fernández-Ortuño, D. 2019. Monitoring methyl benzimidazole carbamate-resistant isolates of the cucurbit powdery mildew pathogen, *Podosphaera xanthii*, using loop-mediated isothermal amplification. Plant Dis. 103:1515-1524.
Vielba-Fernández, A., Polonio, Á., Ruiz-Jiménez, L., de Vicente, A., Pérez-García, A., and Fernández-Ortuño, D. 2021. Resistance to the SDHI fungicides boscalid and fluopyram in *Podosphaera xanthii* populations from commercial cucurbit fields in Spain. J. Fungi 7:733.
Wang, M., and Jin, H. 2017. Spray-induced gene silencing: A powerful innovative strategy for crop protection. Trends Microbiol. 25:4-6.
Wang, M., Weiberg, A., Lin, F.-M., Thomma, B. P. H. J., Huang, H.-D., and Jin, H. 2016. Bidirectional cross-kingdom RNAi and fungal uptake of external RNAs confer plant protection. Nat. Plants 2:16151.
Wang, Y., Yan, Q., Lan, C., Tang, T., Wang, K., Shen, J., and Niu, D. 2023. Nanoparticle carriers enhance RNA stability and uptake efficiency and prolong the protection against *Rhizoctonia solani*. Phytopathol. Res. 5:2.
Yang, W., Wang, B., Lei, G., Chen, G., and Liu, D. 2022. Advances in nanocarriers to improve the stability of dsRNA in the environment. Front. Bioeng. Biotechnol. 10:974646.
Zand Karimi, H., and Innes, R. W. 2022. Molecular mechanisms underlying host-induced gene silencing. Plant Cell 34:3183-3199.
Zhang, H., Zhang, H., Demirer, G. S., González-Grandío, E., Fan, C., and Landry, M. P. 2020. Engineering DNA nanostructures for siRNA delivery in plants. Nat. Protoc. 15:3064-3087.