PROGRESS REPORT: July to December 2023

Pest Management Strategies: Non-chemical strategies for pest control

Evaluation of RNA-based biopesticide against *Botrytis cinerea*, the causative agent of Gray Mold of Brambles

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Background of the project:

"Gray Mold" disease is a significant problem in all blackberry cultivation regions. The disease is caused by the fungus *Botrytis cinerea* (*B. cinerea*). This pathogen has a wide host range and can overwinter on dead leaves, plant debris, and stems. Growers use a wide range of pesticides yearly to control *B. cinerea*. In this proposal, we would like to explore the possibility of using the spray-induced gene silencing (SIGS)strategy to control the severity of *B. cinerea disease in blackberry plants. The success of this project will significantly help bramble growers safeguard their crops against B. cinerea* without using pesticides, thus helping improve both consumer and environmental well-being. Further, the outcome of this project will enable researchers to develop alternative, greener solutions against other invading pathogens. With this background, the project was proposed with four study objectives over two years. They are:

1. *Selection of B. cinerea* target gene;
2. Development of hairpin RNA and *in vitro* studies on fungus culture;
3. Bioassay on blackberry plant in the greenhouse; and,
4. Selection of carrier for RNA and bioassay in the greenhouse.

Work done since July 2023:

Objective 1: *Selection of B. cinerea* target gene:

1. **Selection of target genes for RNA silencing:**
   To have an efficient control of *B. cinerea* through SIGS, the selection of a target gene is essential. Specifically, the target gene should play a crucial role in the pathogen's life cycle and should not overlap with the host gene sequence. Based on the literature survey and previous experimental data, five target genes were selected: VELVET complex, Ergosterol, lanosterol 14α-demethylase, chitin synthase 1, and *Elongation factor.*

   VELVET complex (BcVel) is a highly conserved group of proteins found in fungi that are important in reproduction and virulence on host plants. Ergosterol (BcERG) is vital in the fungal cell membrane and involved in several biological functions. Similarly, chitin synthase 1 (BcCHS1) is crucial in the cell wall formation of the *B. cinerea*. At the same time, the *Elongation factor (*BcEF*)* is a set of proteins that facilitates the translational elongation of fungal proteins. The lanosterol 14α-demethylase (BcERG11), is an ergosterol. BcERG11 is the primary target of triazole antifungal agents. Though the triazole class of compounds are very efficient antifungal agents, several pathogens have developed resistance against these compounds.

2. Isolation and pure culture of *B. cinerea*:

To obtain the pure culture of *B. cinerea*, the leaf sample collected from the blackberry plants infected with *B. cinerea* was brought to the laboratory and cultivated in potato dextrose agar (PDA) with chloramphenicol antibiotic. After sporulation, the gray mold was re-cultured twice to obtain the pure culture of the fungus (Figure 1). The fungus was identified based on cultural characteristics.

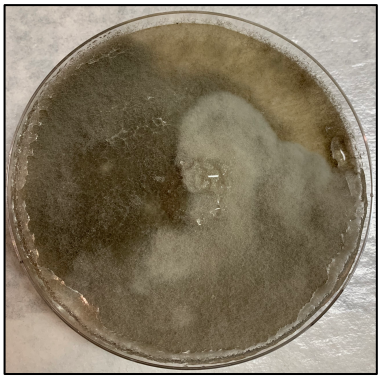

Figure 1. Pure culture of *B. cinerea* obtained from blackberry leaf sample.

Objective 2: Development of hairpin RNA and *in vitro* studies on fungus culture

1. Development of DNA constructs to induce RNA silencing:

DNA constructs of selected genes were developed using strategies to induce RNA silencing in fungi.

1. Hairpin construct targeting a single gene of *B. cinerea*:
   To this purpose, the 312-nt gene sequence selected from BcVel1 of *B. cinerea* was constructed in an inverted repeat (IR) separated by an intron region. Such obtained construct was sub-cloned in pBlueScript SK(+) vector and used for in vitro RNA synthesis using T7 promoter. The expected length of RNA is approx. 830-nt
2. Hairpin construct targeting multiple genes of *B. cinerea*:
   Here, partial gene sequences of BcERG11, BcCHS1, and BcEF were combined to construct an IR separated by an intron region. The obtained IR DNA was sub-cloned in the pBlueScript SK(+) vector and used for in vitro RNA synthesis using a T7 promoter. The expected length of RNA is approx. 1660-nt.
3. Single and Double-stranded RNA targeting multiple genes of ERG:
   The three ergosterol genes, viz., EGR1, ERG11, and EGR13, were combined to obtain a 769-nt long DNA construct. This DNA was amplified by two independent PCR to add a T7 promoter sequence at the beginning or end of the DNA. This strategy helps synthesize a sense strand RNA, anti-sense strand RNA, and produce a dsRNA. To create a dsRNA, sense-strand RNA and anti-sense strand

RNAs were mixed proportionally and cooled slowly after heating at 95^0C for 3 minutes.

2. ***In-vitro* studies on fungus culture:**
To study the effect of RNA silencing of different target genes on *B. cinerea,* the 10ug of *in-vitro* synthesized RNA was mixed with 30µL of fungal spores. 10 µL of such mixture was transferred to the center of PDA culture plates containing chloramphenicol. The PDA culture plates were incubated at room temperature for ten days.

As shown in Figure 2, targeting the BcVel1 gene had the most negligible effect on the growth of *B. cinerea* (Figure 2A). On the other hand, targeting multiple genes (BcERG11, BcCHS1, BCEF) had the highest effect on fungal growth (Figure 2B), followed by dsRNA targeting multiple ERG genes (Figure 2E). Further, treating fungal spores with single-stranded RNA (ssRNA) targeting multiple ERG genes showed reduced fungal growth (Figures 2C and 2D). However, this effect is less than the dsRNA targeting the same gene (Figure 2E). Further, the fungal spores treated with RNA against multiple genes (BcERG11, BcCHS1, BCEF) and multiple ERG showed reduced fungal biomass compared to the control.

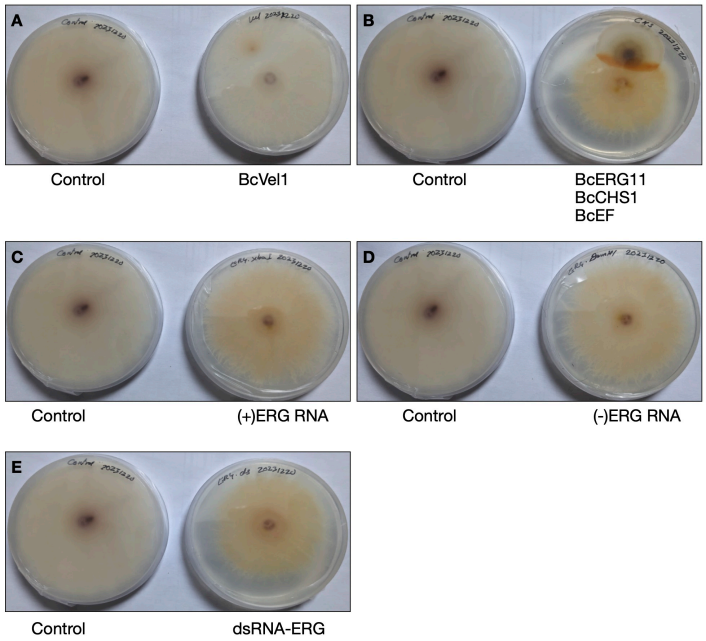

Figure 2. Effect of RNA silencing of different genes on B. cinerea growth. In the figure, control indicates, that fungal spores are treated with sterile water; BcVel1 indicates, fungal spores are treated with in vitro synthesized hairpin RNA targeting BcVel1 of *B. cinerea;* BcERG11, BcCHS1, BCEF indicates, fungal spores are treated with in vitro synthesized hairpin RNA targeting BcERG11, BcCHS1, and BcEF genes of *B. cinerea;*, (+)ERG indicates, fungal

spores are treated with *in vitro* synthesized sense RNA targeting multiple ERG genes of *B. cinerea;* (-)ERG indicates, fungal spores are treated with *in vitro* synthesized anti-sense RNA targeting multiple ERG genes of *B. cinerea; and,* dsRNA-ERG indicates, fungal spores are treated with *in vitro* synthesized double-stranded RNA targeting multiple ERG genes of *B. cinerea.* All the experiments repeated three times.

Future work plan:
So far, we have completed the proposal's first two objectives. From the results obtained so far, it is clear that RNA silencing effectively reduces the growth of *B. cinerea*. These results are very promising. Since dsRNA targeting multiple ERG genes and hairpin RNA against the multiple genes (BcERG11, BcCHS1, and BcEF) were found compelling, in the future, these RNAs will be used for future assays.

Though we successfully reduced fungal growth, our experimentation failed to eliminate fungus. Here, it is worth noting that we performed only one RNA treatment and RNA is less stable. Therefore, it is possible that RNA was degraded before inducing RNA silencing in all the all spores. This indicates the requirement of (i) carrier molecule to increase the stability of RNA and/or (ii) the requirement of multiple treatments. Hence, we would like to perform Objective 4 (selection of carrier molecule) before performing Objective 3 (*Bioassay on blackberry plants*). This re-organization will help us to stabilize the RNA which is required for effective field application.

In a nutshell, we successfully selected at least six different target gene sequence targets (four ERG, CHS1, and EF) that were found effective in controlling the growth of *B. cinerea*. The increasing stability of RNA using a carrier molecule and an additional RNA treatment will further reduce the fungal growth and its eventual use under field conditions. This opens an alternative non-chemical strategy to control the gray mold in blackberry.

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