WASHINGTON BLUEBERRY COMMISSION

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Washington Blueberry Commission Research Proposal

Title: Optimizing a high throughput fungicide resistance screening assay for *Botrytis* management in blueberries

Year Initiated _2023_____ Current Year _2024____ Terminating Year _2024_ ___

Principal Investigator:
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Justification and Background:

The mild marine climate in northwestern Washington predisposes blueberry to diseases such as botrytis blight and fruit rot caused by *Botrytis* spp. These diseases result in serious pre- and post-harvest losses in over 200 economically important crops worldwide including blueberries (Naegele et al. 2021). Application of synthetic fungicide sprays is the primary management strategy for control of gray mold on blueberries and other small fruits.

There is limited information about fungicide resistance profiles of *Botrytis* spp. in blueberry fields from Washington, which could influence the choice of fungicide application programs. For example, searches of peer-reviewed scientific literature indicated only two studies conducted in four and five fields in 2012 and 2015, respectively that focused on *Botrytis* fungicide resistance in Washington State (Kozhar et al. 2020; Saito et al. 2016). Furthermore, these surveys did not include newer fungicide chemistries in FRAC7 (SDHI) class, which are currently available for gray mold management. To address this knowledge gap, we secured funding from WBC, Northwest Center for Small Fruit Research, and Washington Commission on Integrated Pest Management to sample several blueberry fields in Washington and Oregon in 2022-23. We obtained over 1,300 *Botrytis* spp. isolates which are currently being used for fungicide resistance screening.

Our prior efforts to use a semi-automated 96-well microplate reader to determine sensitivity of *Botrytis* isolates to site-specific fungicides were not successful. Hence, we are currently using conidial germination assays on fungicide-amended agar plates that are rated visually with the aid of a microscope. Using this procedure, we screened *Botrytis*

isolates (n=214 to 223 depending on the fungicide tested) obtained from 16 fields in WA (12 and 4 fields from Whatcom and Skagit Counties, respectively). Preliminary results showed varied frequency of isolates with reduced sensitivity/tolerance to FRAC7 fungicides. These frequencies were 0.68 ± 0.07 (boscalid; n=223), 0.52 ± 0.07 (fluopyram; n=223), 0.18 ± 0.04 (isofetamid; n=214), and 0.39 ± 0.06 (fluxapyroxad; n=214).

Resistance to FRAC 7 group fungicides is associated with multiple mutations such as H272Y, H272R, H272V, H272L, N230I, and P225F. Research has indicated that new FRAC7 fungicides do not exhibit cross resistance to boscalid-resistant strains (Amiri et al. 2014; Sierotzki and Scalliet 2013) and their efficacy depends on the type and frequency of mutation in the target gene (Kozhar et al. 2021). For example, no cross resistance was observed between fluopyram and boscalid when H272R mutation is present (Alzohairy et al. 2023). Hence, understanding the frequency and type of boscalid-resistant mutant strains in WA blueberry fields will provide growers needed information for prudent selection and deployment of fungicides for effective gray mold resistance management.

The purpose of this project is to continue investigating sensitivity of *Botrytis* isolates to site-specific fungicides and determine mutations that confer resistance to FRAC7 fungicides. A broader goal that will be achieved with this project is to develop standardized protocols that will facilitate fungicide resistance testing for samples obtained from growers. The outcomes from this research will enable blueberry growers with foundational knowledge for *Botrytis* management to promote fungicide stewardship.

Relationship to WBC Research Priority(s):

The proposal addresses the following research priority: "Disease pests including bacterial blight, *Alternaria*, anthracnose, *Botrytis*, mummy berry and root diseases such as *Armillaria*; incorporating resistance management strategies into disease control programs."

Objectives:

Although several fungicides exist to manage *Botrytis*, the pathogen is notorious for developing fungicide resistance. Hence, there is a need for profiling this reduced sensitivity, which will be accomplished through the following objectives:

1) Screen *Botrytis* isolates for reduced sensitivity to site-specific fungicides;
2) Evaluate ability of *Botrytis* isolates to infect fungicide-treated berries in vivo; and
3) Identify genetic mutations conferring reduced sensitivity to FRAC7 fungicides.

Procedures:

1) Screen Botrytis isolates for reduced sensitivity to site-specific fungicides

We will test sensitivity of *Botrytis* isolates collected from 22 WA fields in 2023 (early-, mid-, and late-season) to the following technical grade fungicides and associated FRAC classes: cyprodinil and pyrimethanil (FRAC9), fludioxonil (FRAC12), boscalid, fluopyram, isofetamid, and fluxapyroxad (FRAC7), and fenhexamid (FRAC17). We will

continue using the same discriminatory doses that were used for screening *Botrytis* isolates obtained in 2022 [boscalid (10 ppm), fluopyram (5 ppm), isofetamid (5 ppm), fluxapyroxad (10 ppm), fludioxonil (0.1 ppm), fenhexamid (1 ppm), Cyprodinil and Pyrimethanil (4 ppm and 25 ppm)] (Weber and Hahn, 2011).

All isolates will be screened using technical grade fungicides individually. In addition, a subset of isolates will be screened with formulation grade fungicides that include two active ingredients (ex: Pristine, Luna Tranquility, Switch). For the conidial germination assay, 5 µl of the conidial suspension (1×10^5 conidia per ml) will be pipetted onto plates with or without fungicide-amended media. Conidial germination will be assessed visually using a microscope 14-16 hours post incubation at 22°C in the dark. While screening boscalid, fluopyram, isofetamid, fluxapyroxad, fludioxonil, fenhexamid, *Botrytis* isolates will be categorized as sensitive (S; 0-20% growth rate of control at the discriminatory dose), Moderate (>20 to <50% growth rate of control at the discriminatory dose; "M" rating only seen for FRAC 7 group fungicides) and reduced sensitive/tolerant (R; >50% growth rate of control at the discriminatory dose). For cyprodinil and pyrimethanil, two doses will be used (4 ppm and 25 ppm), and the isolates will be categorized as Sensitive (S = <50% growth rate of control at 4 ppm and no growth at 25 ppm), Moderate (M = >50% growth rate of control at 4 ppm and up to 20% growth at 25 ppm), or reduced sensitive/tolerant (R = >50% growth rate of control at 4 ppm and >20% growth at 25 ppm) (Fernández-Ortuño et al. 2013). All experiments will be conducted twice with two replications.

Botrytis isolates will then be categorized (nCCR; number of chemical class resistances) according to the number of FRAC classes to which they were resistant. ANOVA will be used to compare average nCCR of *Botrytis* isolates at a single time point and location with other locations and time points. Multiple comparisons of average nCCR will be done using Tukey's honest significance difference test. All results will be tied back to grower field history so we can understand how fungicide applications affect development of reduced sensitivity in *Botrytis*.

2) *Evaluate ability of* Botrytis *isolates to infect fungicide-treated berries in vivo.*

Organically grown blueberry fruit will be used to assess fungicidal efficacy on fruit inoculated with *Botrytis* isolates (n=10) with different resistance phenotypes (determined from Objective 1) to various FRAC groups using methods developed by Saito et al. (2016). Fruits will be surface sanitized with 0.5% sodium hypochlorite for 2 min, rinsed with sterile water three times, and allowed to air dry in a fume hood. The fruits will be sprayed with fungicides (Luna Tranquility, Switch, Pristine with and without Captan 80WDG) at label rates recommended for blueberry using a hand mister. Non-fungicide treated control fruits will be sprayed with sterile distilled water. After drying, fruit will be wound-inoculated to deliver 1 µl of *Botrytis* conidial suspensions ($1 \text{ X } 10^5$ conidia/ml). Inoculated fruit will be placed in a humid chamber and incubated at 22°C in the dark. Five days post inoculation, disease incidence and severity will be estimated. Disease severity will be rated visually on a 0-4 scale (0 = no symptoms; 1 = <25%, 2 = 25% to 50%,

3 = 50% to 75%, 4 = >75% of the blueberry fruit with surface decay). Average score will be calculated for each replicate. Means for each treatment and isolate will be separated by Tukey's honest significance test. Each treatment will have ten fruits with three replications. The experiment will be conducted twice.

3) Identify genetic mutations conferring reduced sensitivity to FRAC7 fungicides

Previous research by Dr. Peever (Kozhar et al. 2020) showed 62%of boscalid-resistant *Botrytis* isolates to possess H272Y mutation in the *sdhB* gene and 32% with H272R mutation. In this objective, we will screen boscalid-resistant strains identified in Objective 1 for H272Y and H272R mutations using PCR protocols used by Kozhar et al. (2020). We will also determine the presence of other mutations (H272V, H272L, N230I, P225F, P225H) in the target gene (*sdhB*) of bosaclid-resistant *Botrytis* isolates (from Objective 1; n=50). Briefly, DNA will be extracted from 7–10-day old cultures and ~950 base pair of the *sdhB* gene will be amplified using primers and protocols developed by Leroux et al. (2010). PCR products will be purified and sequenced. Nucleotide sequences will be analyzed using multiple sequence alignment in MEGA software with appropriate reference sequences, and mutations at amino acid positions 225, 230, and 272 will be identified. Frequency of such mutations will be correlated with sampling year, sampling location and grower management practices.

Describe how this research will benefit Washington blueberry growers:

The proposed research will result in understanding field-scale prevalence of *Botrytis* spp. isolates with single or multiple resistance to different FRAC groups of fungicides. Knowledge obtained from this research will enable growers to select fungicide chemistries to which the pathogen is sensitive, alternate fungicides with different modes of action, and/or use fungicide mixtures to mitigate resistance and disease. We will disseminate research results to growers through publishing an article in the Whatcom Ag Monthly newsletter and delivering a talk at the WA Small Fruit Conference & Ag Show. It is to be noted that based on our research results presented at the 2023 WA Small Fruit Conference & Ag Show, small fruit growers, technical experts, and industry representatives met on December 11, 2023 (audience size = 29) to discuss future *Botrytis* resistance management strategies.

References:

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8. Saito, S., et al. 2016. Plant Dis.100:2087-2093.
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Budget:

	2024	**2025**	**2026**
Salaries	$18,980	$	$
Time-Slip	$	$	$
Operations (goods & services)	$	$	$
Travel[1/]	$983	$	$
Meetings	$	$	$
Other[2/]	$6,750	$	$
Equipment	$	$	$
Benefits	$8,287	$	$
Total	**$35,000**	**$**	**$**

1/ Travel to grower fields for obtaining samples and disease monitoring (15 trips @ 100 miles at 0.655/mile).
2/ Goods and services: Growth media and Petri plates ($3,000); molecular biology reagents and sequencing ($2,500); consumables for PCR and pathogenicity assays ($1,250).
Salary and benefits ($27,267) for a lab technician for 10 months at 50%FTE.

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