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Bioassays utilizing water- and chloroform-based floral extracts provide insight into *Colletotrichum fioriniae* biology on blueberry and cranberry

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Attention: (REF: JoVE58880)

Review editor, Dr. Phillip Steindel and reviewers

Dear reviewers,

Please accept our responses and corrections to all comments, both editorial and reviewer based, individually outlined in a separate document "Waller Rebuttal Document for JoVE". The supplied revision has more protocol details, reasoning behind the use of said protocols, indications of limitations and important protocol steps as per comments. Overall, the comments did not fall into the "Major Concerns" category, however, did call for major modifications that warranted updating virtually every section of the manuscript. The revision now reads much more like a methods protocol than a manuscript detailing the lifecycle of *Colletotrichum fioriniae*. In agreement with this the title has also been modified, "Bioassays utilizing water- and chloroform-based floral extracts provide insight into *Colletotrichum fioriniae* biology on blueberry and cranberry".

Included in this submission: revised manuscript, 6 figures (.emf (vector)), 8 supplemental photos (.jpg), 3 supplemental videos (.mp4), and an updated materials list (.xlsx). Supplemental materials have been renamed in the text (Supplemental Figure # or Supplemental Movie #). The large video files were originally uploaded via the "JoVE File Uploader for Article 58253" and their new more appropriate file names should be as follows: "active-FEextraction.mp4" is now "Sup movie 1.mp4", "pass-FEextraction.mp4" is now "Sup movie 2.mp4", "CB rw-FE deployed.mp4" is now "Sup movie 3.mp4". Each of the supplemental photos and movies have an appropriate caption and notation in-text. Individual .tif files are available upon request for the figures that are composed of collaged microscope photos.

Protocol sections 6 and 7 (floral rainwater devices and collection) could be creatively filmed together. The reason we included these two devices was to demonstrate the flexibility of working with varying crop inflorescence architectures, with the aim of appealing to other cropping systems/applications.

We greatly appreciate the time taken to provide peer-review and are excited that our peers await this manuscript / video package. Thank you.

Respectfully, on behalf of the authors,



Timothy James Waller

TITLE:

***Colletotrichum fioriniae* Development in Water and Chloroform-based Blueberry and Cranberry Floral Extracts**

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KEYWORDS:

Colletotrichum; pathogen biology; floral stimulation; floral extracts; bloom; chloroform extraction; coverslip bioassay; rainwater monitoring; surface waxes; secondary conidiation; appressoria; decision making tools

SUMMARY:

Here, bioassays designed to monitor the development of a fungal pathogen, *Colletotrichum fioriniae*, in the presence of blueberry or cranberry floral extracts on glass coverslips are described. Water-, chloroform-, and field rainwater- based floral extraction techniques are detailed as well as insight into how this information can be applied.

ABSTRACT:

To accurately monitor the phenology of the bloom period and the temporal dynamics of floral chemical cues on fungal fruit rotting pathogens, floral extraction methods and coverslip bioassays were developed utilizing *Colletotrichum fioriniae*. In blueberry and cranberry, this pathogen is optimally controlled by applying fungicides during the bloom period because of the role flowers play in the initial stages of infection. The protocol detailed here describes how floral extracts (FE) were obtained using water-, chloroform-, and field rainwater-based methods for later use in corresponding glass coverslip bioassays. Each FE served to provide a different set of information: response of *C. fioriniae* to mobilized floral chemical cues in water (water-based), pathogen response to flower and fruit surface waxes (chloroform-based), and field-based monitoring of collected floral rainwater, moving *in vitro* observations to an agricultural setting. The FE is broadly described as either water- or chloroform-based, with an appropriate bioassay described to compensate for the inherent differences between these two materials. Rainwater that had run off flowers was collected in unique devices for each crop, alluding to the flexibility and application of this approach for other crop systems. The bioassays are quick, inexpensive, simple, and provide the ability to generate spatiotemporal and site-specific information about the presence of

stimulatory floral compounds from various sources. This information will ultimately better inform disease management strategies, as FE decrease the time needed for infection to occur, thus providing insight into changing risks for pathogen infection over the growing season.

INTRODUCTION:

Colletotrichum fioriniae causes a fruit rot of both Highbush Blueberry (*Vaccinium corymbosum* L.) and the large American Cranberry (*V. macrocarpon* Aiton)^{1,2}. This pathogen was recently delineated from the *C. acutatum* species complex³⁻⁶ and is a causal agent of blueberry anthracnose and a member of the cranberry fruit rot complex, in addition to causing numerous other plant diseases worldwide⁷. *C. fioriniae* has a latent, hemibiotrophic lifestyle⁸, with infections occurring during bloom and symptom development not becoming apparent until the fruit are in final stages of maturation⁹. In blueberry and cranberry, fruit rot is only adequately controlled with fungicide applications made during the bloom period. The pathogen overwinters in dormant blueberry floral bud scales¹⁰ and sporulates during bloom. Conidia are moved throughout the canopy via rain-splash dispersal^{11,12} and inoculum buildup has been strongly correlated to the bloom period¹³. Response of *Colletotrichum* species to host flowers is not unique to *Vaccinium*, as flowers are important components of citrus post bloom fruit drop (PFD)¹⁴ as well as strawberry anthracnose¹⁵, in both cases causing the pathogen to sporulate. All of these cases highlight the need for effective methods to evaluate the temporal dynamics of floral chemical cues on *C. fioriniae* and other pathogens that infect during bloom. The insights provided by the methods described here are becoming increasingly more valuable.

This protocol details methods of floral extract (FE) procurement and guides the evaluation of *C. fioriniae* responses to FE via glass coverslip bioassays^{15,16}. The floral extraction techniques are broken into two main types; water-based extractions (*active*-FE, *passive* (*pass*-FE), and field rainwater-based (rw-FE)), and chloroform-based (ch-FE)¹⁷ extractions. The water-based extractions allow for inspection of water mobilized floral chemical cues. These mobilized cues are likely important components of the infection court, since FE greatly increases the speed of infection¹⁶, in addition to providing the moisture required for the infection to occur. Additionally, they represent a more natural condition as floral stimulation can be washed throughout the canopy during wetting-events as previously observed in blueberry and other crop systems^{14,16}. Chloroform-based floral extractions (ch-FE) also provide valuable information pertaining to pathogen response to host surface waxes^{17,18}, elucidating the early growth stages of conidia once deposited onto susceptible host organs (*i.e.* flowers, ovaries and developing fruit). Pathogen response to seasonal changes in host surface waxes can also be monitored using this protocol. Accordingly, the bioassays are tailored to working with either water-based FE or chloroform-based FE to mitigate the inherent differences between these two materials.

The data generated from the bioassays revealed that water-based extractions stimulate higher levels of secondary conidiation than chloroform-based extractions where there was a definitive appressorial response, therefore implicating multiple compounds present in the FE. Interestingly, both of these growth responses were observed when using rainwater that had run off of blueberry and cranberry flowers, indicating multiple stimulatory compounds can be washed from

the surface of flowers. Thus, monitoring for floral stimulation will provide insight into the probability of pathogen success in an agricultural system.

The ultimate goal of this protocol is to provide a methodology for generating baseline biological information on fungal plant pathogens in response to floral chemical cues, as well as initiating methodologies that can utilize this floral information to aid in site-specific disease management and decision-making processes.

PROTOCOL:

1. Fungal Isolates and Spore Suspensions

1.1. Isolate *Colletotrichum fioriniae* from a naturally infected host¹⁹. Then, store the clean cultures on corn meal agar (CMA) slants. Place plug of culture (from CMA slant) onto a standard plastic cell culture dish (9 cm diameter) containing either clarified V8 juice agar (cV8A) (modified from Miller 1955), or non-clarified V8 juice agar (V8A)²⁰. When colonies begin to sporulate, streak conidia (orange conidial masses) onto another cV8A or V8A containing cell culture dish (with a standard sterile loop) to produce a high-density sporulating culture.

NOTE: Any protocol steps with fungi should be performed in a Laminar flow hood to reduce the possibility of contaminating fungal cultures and/or bioassays.

1.2. After 7 days of growth, using a standard sterile loop gather a small amount of conidia from the high-density culture (by lightly touching the loop to the conidial mass) and stir this into a 15 mL centrifuge tube containing 10 mL of sterile deionized water (SDW). Vortex this sample for 10 s, then using a standard pipette plunge up and down numerous times to further mix the sample.

1.3. Then, place a drop of the vortexed sample onto the hemocytometer and estimate spore concentrations. Count 5-10 fields on the hemocytometer, obtain the average, and multiply average by the appropriate dilution factor (*i.e.* 10,000), thus obtaining the conidial concentration per mL SDW.

1.4. Then using a volume (V)/concentration (C) equation ($V_1 \bullet [C_1] = V_2 \bullet [C_2]$) adjust (with SDW) to 1.0×10^5 conidia per mL of SDW with a 5 mL final volume. This is referred to as the spore suspension and is only made immediately prior to use in either bioassay.

2. Active, Water-based Floral Extracts (active-FE)^{15,16}

Note: See Supplemental Figure 1, Supplemental Figure 2, Supplemental Figure 3, and Supplemental Movie 1.

2.1. Carefully hand-collect blueberry (April-May) and cranberry (June-July) flowers during their respective peak blooms in the field (Supplemental Figure 1). Wear and change nitrile gloves between sampling locations (cultivars/varieties/physical locations) to inhibit human skin oil

contamination as well as cross contamination between floral varieties. Place either blueberry or cranberry flowers (from a single source) into plastic bags (fill a 100 x 150 mm bag) and immediately refrigerate at 4 ° C once flowers are collected.

NOTE: The *active*- extraction is modified from Leandro *et al.* (2003)¹⁶.

2.2. Prior to extraction, remove any deteriorated, damaged, diseased flowers, then using healthy flowers remove the ovaries, sepals and peduncles with curved forceps (45° tweezers) and discard. Use only the remaining organs (corolla, stigma, style and stamen) for *active*- extractions. Cut cheesecloth (150 x 150 mm) and place within a 7 x 7 mm funnel, place into a clean 50 mL centrifuge tube, and set aside.

2.3. Combine 1 part processed flowers to 9 parts SDW (1 wt: 9 vol ratio) in a mortar. Gently grind with a pestle for 30 s (take care to not completely pulverize the samples). Strain resulting pulp through prepared cheesecloth/funnel and collect in centrifuge tube (50 mL). Clean or use new mortar/pestle between each extraction with 95% EtOH and warm running water.

2.4. Prepare a vacuum filtration apparatus: gather Buchner funnel, vacuum filter Erlenmeyer flask (up to 1000 mL capacity), 55 mm circle filter paper, and vacuum hose/source. Add filter paper to an appropriate sized Buchner funnel and place atop the flask, then connect apparatus to a water/suction source via vacuum hose and set aside.

2.5. Further clarify the blueberry floral extracts by centrifuging for 10 min at 8055 x g. Pour off supernatant into prepared vacuum filter apparatus, turn on vacuum source, filter supernatant, then pour flask contents into a new centrifuge tube (50 mL). Clean all apparatus components between each filtration with 95% EtOH and warm running water. Further filter through a syringe adapted with a 0.22 µm pore size, acetate sterilizing, filter into a new centrifuge tube (50 mL).

2.5.1. For the cranberry floral extracts, only vacuum filter through filter paper and pour flask contents into a new centrifuge tube (50 mL).

2.6. Resulting preparation is referred to as *active*- floral extract (*active*-FE). Store all water-based floral extracts (*active*-, *passive*-, rainwater collections) at -20° C in 5-50 mL aliquots until experimental use. Repeat extractions with multiple samples (at least 3 extractions per sample type) to provide replicates.

3. *Passive*, Water-based Floral Extracts (*pass*-FE)¹⁶

Note: See **Supplemental Movie 2**.

3.1. Hand collect whole blueberry flowers (50 g) as above, and refrigerate after collecting. Prior to extraction, remove any deteriorated, damaged, diseased flowers and remove only the peduncles from intact flower. Refrigerate prepared samples until the *passive*- extraction system, described below, is in place.

3.2. Rinse all components prior to use with 95% ethanol (EtOH) and warm running water to prevent contamination (plastic mesh sheet, two plastic mesh baskets, glass bread pan, and pump mist bottle). Also prepare a 4 layer cheesecloth/funnel/50 mL centrifuge tube for each extraction as described above (in step 2.2), and set aside.

3.3. Prepare the sieve: place a plastic mesh sheet (aka clear bar matting) into one plastic mesh basket (114 x 102 mm). Place a second, identical, mesh basket upside down (inverted) into a glass bread-pan (127 x 229 mm) (to avoid flowers sitting in SDW) and place the mesh sheet containing basket atop. Add 50 g of prepared flowers into the sieve.

NOTE: Alternatively, small test tube [cleansing] baskets can be used in place of the originally used plastic mesh baskets (old, green, strawberry mesh pint baskets are often difficult to source).

3.4. Evenly mist flowers with 250 mL using a pump mist bottle and capture runoff in the glass bread-pan. Then strain filtrate through the prepared cheesecloth/funnel into a clean centrifuge tube. Resulting preparation is referred to as *passive*-floral extract (*pass*-FE); store as per above (step 2.6).

3.5. Clean all components between each extraction with 95% EtOH and warm running water. Repeat extractions with multiple samples to provide replicates (at least 3 per sample type).

4. Chloroform-based Floral Extracts (ch-FE)¹⁷

4.1. Collect blueberry and cranberry flowers as per above (step 2.1), and prepare flowers for extraction (step 2.2, without cheesecloth/funnel preparation). Keep prepared flowers refrigerated until prior to use.

4.1.1. Any work performed with chloroform must be performed in a fume hood for safety reasons. This includes preparation of materials / glassware, extraction procedures, and bioassay conductance (steps using ch-FE).

4.2. Clean all components with 95% EtOH twice, then twice with chloroform to prevent contamination for each extraction: threaded glass culture tubes, 2 glass beakers, small stainless steel screen, and a [glass] graduate cylinder. Set aside to dry upside-down. Rinse polytetrafluoroethylene (PTFE) lined caps twice with 95% EtOH only (chloroform will damage the outer materials of the cap) and set aside to dry.

4.3. Combine 1 part processed flowers to 9 parts chloroform (1 wt: 9 vol ratio) in a beaker (flowers then chloroform), gently swirl for 30 s, and strain through a stainless steel screen into the second beaker. Pour ch-FE from the second beaker into the glass culture tube (10 - 15 mL) and affix the PTFE cap. Wrap the cap with parafilm to prevent evaporation.

4.4. This preparation is the chloroform-based floral extract (ch-FE). Store sample in darkness (to reduce light degradation) at 4 ° C until experimental use. Repeat extractions with multiple samples to provide replicates (at least 3 per sample type).

5. Collection of Rainwater from Blueberry Flowers (BB rw-FE)¹⁶

NOTE: The blueberry floral rainwater collection device consists of an air spray gun disposable paint spray cup with connection adapter (cup: female thread, adapter: male to male thread), 50 mL centrifuge tubes (polypropylene), parafilm, and plastic coated wires (standard telephone wire, individual internal wire strand contents).

5.1. Select multiple locations within a blueberry bush to capture rainwater run off of flowers prior to creating collection devices. These include directly under inflorescences (flower) to the very base of the bush (crown). Record diameter of the stems (ranging from 1-5 cm) at selected locations, as this will dictate the size of holes used for device attachment, described below.

5.2. For each selected location create a collection device. First drill a hole at the bottom of a spray cup (where the cup curves towards the threaded opening) to the corresponding stem diameter, with a step-bit attached to a drill press. Then, cut a straight line from the top of the hole to the mouth of the spray cup. Drill 4 equidistant holes (large enough to thread the plastic coated wire), at the mouth of the spray cup, and attach one end of the plastic coated wires leaving one end free.

5.3. Drill a hole large enough to thread the paint sprayer cup adapter into a 50 mL centrifuge cap lid. Seal adapter threads with parafilm to prevent leaking. Attach this to both the centrifuge cap and threaded portion of the sprayer cup. Attach the mated 50 mL centrifuge tube.

5.4. Repeat steps 5.3-5.4 to create multiple devices as per selected locations, a minimum of 4 collection devices per sampling location.

5.5. Deploy devices at selected locations by flexing sprayer cups to fit onto stems. Orient the cut-side of the spray cup upwards using the plastic-coated wires attached to other stems (to insure water passing over flowers is captured). Affix parafilm any openings that could leak rainwater. (Supplemental Figure 3, Supplemental Figure 4, Supplemental Figure 5, and Supplemental Figure 6).

5.6. Label collection tubes with deployment date / time. After a rain-event, remove and replace the bottom (tube) portion of the centrifuge tube (label date / time of collection). Bring runoff collections (referred to as blueberry rainwater floral extract (BB rw-FE)) inside and vacuum filter (filtration described in steps 2.4-2.5). Store as above (step 2.6), until used in a water-based bioassay.

6. Collection of Rainwater from Cranberry Flowers (CB rw-FE)

6.1. The floral rainwater collection device in cranberry consists of a 7 X 7 cm polypropylene funnel, 50 mL centrifuge tubes (polypropylene), parafilm, and 4 standard, plastic coated twist ties (per device).

6.2. First, heat puncture 8 equidistant holes (diameter of twist ties) around the mouth of the funnel using a metal probe. Insert twist ties into 4 holes. Attach the other ends to that holes' opposite location, forming a neat crossing pattern. Wrap funnel down-stem with parafilm and set aside.

6.3. Drill a hole large enough to insert the funnel down-stem into a 50 mL centrifuge cap with a step-bit. Insert prepared funnel into the centrifuge cap. Repeat steps 6.2-6.3 to create multiple devices, a minimum of 4 collection devices per sampling location.

6.4. Deploy labeled (date/time) devices into selected cranberry bogs. Neatly tuck two flower bearing inflorescences (known as uprights) under the crossed plastic twist ties. Then vertically orient the device by piercing the centrifuge tube into the cranberry canopy (**Supplemental Figures 7-8, Supplemental Movie 3**).

6.5. After rainfall or overhead irrigation remove and replace the bottom (tube) portion of the centrifuge tube (label date / time of collection). Bring runoff (referred to as cranberry rainwater floral extract (CB rw-FE)) inside and vacuum filter (filtration described in steps 2.4-2.5). Store as above (step 2.6), until used in a water-based bioassay.

7. Bioassay using Water Based Floral Extracts^{15,16} (active-FE, pass-FE, rw-FE)

Note: See **Figure 1**.

7.1. This bioassay is prepared in a Laminar flow hood. Prepare materials: triple rinse glass coverslips with 95% EtOH and air dry, then put aside. Cut paper towel disks to the internal diameter of a standard plastic cell culture dishes (9 cm). Place 2 layers of paper disks within culture dishes and soak with 2 mL SDW.

7.2. Prepare at least 5 mL of a 1.0×10^5 conidia per mL of SDW spore suspension (*C. fioriniae*) as per above (step 1.2-1.4), set aside. Next, mix equal volumes of SDW and water-based floral extract in 2 mL microcentrifuge tubes. Then, add an equal volume of the spore suspension to the prepared 2 mL microcentrifuge tubes (SDW plus FE); the resulting preparation is referred to as an aqueous treatment mixture. For the control, omit FE portion and replace with SDW, to keep conidial concentrations consistent.

NOTE: portion size is dependent on number of replicates and time-points. Typically, portions do not exceed 500 μ L. Once conidia and FE are combined the bioassay has begun, 0 h post-inoculation.

7.3. Place the pre-cleaned glass coverslips on top of the soaked paper towels within the cell culture dish. Place a 40 μ L droplet of aqueous treatment mixture onto the center of a coverslip. Repeat for desired treatments, including the control, close the cell culture dish. Repeat in separate cell culture dishes for replications (at least 3) and time points (each dish is for 1 time point). Once all treatments and replicates have been dispensed, place all replicated cell culture dishes into a sealed plastic container (30 x 13 x 7 mm) and incubate at 25° C in the dark.

7.4. At predetermined time-points, add 10 μ L of a fixative like lactophenol cotton-blue (20.0 g phenol crystals, 20.0 mL 2.5% lactic acid, 40.0 mL glycerol, 0.05 g cotton blue) to the droplets, stopping growth and semi-preserving the mount.

7.4.1. Wait 2 h to collect the 0 h time point as this allows conidial adhesion to the glass surface but is not long enough for conidial germination. For all other time points, add fixative at corresponding time.

7.5. Once a fixative has been added, carefully invert the coverslips, placing them droplet side down on a glass microscope slide to facilitate microscopic examination (1 coverslip per slide).

When all coverslips are on the glass microscope slides, leave them to settle and partially dry in the flow hood for 20 min.

7.6. Count all conidia (total conidia) present on the coverslip (primary conidia, germinated conidia, and newly formed secondary conidia) as well as appressoria at either 400X magnification (counting 16 fields) or 200X (counting 4 fields), totaling an area of 3.808 mm². Replicate entire bioassay for statistical analysis. For assays using water-based FE, analyze data as described in Waller *et al.* 2017¹⁶, typically displaying as mean count/mm² (total conidia or appressoria).

8. Bioassay using Chloroform-based Floral Extracts (ch-FE)¹⁷

Note: See **Figure 2**.

8.1. Prepare materials and cell culture dishes as per above (steps 7.1). Additionally, rinse an equal number of Van Tieghem cells (VanT. cells) (8 mm OD, 6 mm ID) to coverslips, as well as a glass pipette (1 mL with 1 μ L increments) within a fume hood, (twice with 95% EtOH then twice with chloroform), and set aside.

8.2. In laminar flow hood, using a 2 mL microcentrifuge tube add two equal volumes (at least 500 μ L portions) of SDW and 1 volume of spore suspension, then set aside. This is the aqueous treatment mixture for ch-FE bioassays.

8.3. In a fume hood, place a VanT. cell onto a glass coverslip within the prepared plastic cell culture dish. Dispense (with glass pipette) 33 μ L of desired ch-FE into the center of the Van T. cell (do not touch the walls of the VanT. Cell; for the control treatment, add virgin chloroform), and **allow to dry**. Repeat in separate cell culture dishes for replications (at least 3).

8.4. Once ch-FE has dried, dispense 99 μ L of the prepared aqueous treatment mixture with a standard pipette into the center of the VanT. cell, then close all cell culture dishes. Once the aqueous treatment mixture has come in contact with the dried ch-FE treatments, the bioassay has begun, 0 h post-inoculation.

8.5. Once all treatments and replicates have been dispensed, place all (closed) cell culture dishes into a sealed plastic container (30 x 13 x 7 mm) and incubate at 25° C in the dark.

8.6. At predetermined time-points, add 15 μ L of a fixative (lactophenol cotton-blue) to the VanT. cell and let sit for at least 5 min to insure adequate fungal staining. After that time, carefully remove the VanT. cell and follow coverslip inversion and data acquisition steps above (steps 7.5-7.6). For data analysis, follow methods described in Gager 2015¹⁷, typically displaying data as *appressorium formation*, which is the ratio of total conidia to appressoria counted in the area observed (3.808 mm²).

9. Cranberry Phenology-based Extractions¹⁷

9.1. Hand collect cranberry flowers (in June; 100 g), immature fruit (twice; July and August; 200 g), and mature cranberry fruit at harvest (October; 200 g), place into appropriately sized plastic bags and refrigerate at 4° C immediately after collection. Use the contamination precautions outlined above (step 2.1).

NOTE: There will be extra plant material collected at each time but these amounts guard against extracting obviously fungal infected ovaries and fruit (showing symptoms and signs of disease).

9.2. Prior to extraction, remove any deteriorated, damaged, diseased flowers, then using only healthy flowers, remove the sepals, peduncles, corollas, stigmas, styles and stamens with curved forceps (45° tweezers) and discard all but the ovaries. Once ovaries are collected, perform the chloroform-based extraction at a 1 wt: 9 vol ratio (detailed in steps 4.2-4.4, using only ovaries). Store samples until all other extractions are complete, *i.e.* until bioassay conductance.

9.3. Once fruit are collected, perform a chloroform-based extraction (steps 4.2-4.4, using collected fruit instead of flowers), but add 10 g of fruit to 90 mL of chloroform and once extracted, allow the solution to evaporate to 9 mL (resulting in a 10 g: 9 mL wt: vol solution).

9.4. Make fruit extractions immediately after collecting in July, August, and October. Store as per above (step 4.4) until all extractions are collected. After the last chloroform-based fruit extraction, subject all phenology-based chloroform extracts collected for this assay to the chloroform-based bioassay and analyze accordingly (steps 8.1-8.6).

REPRESENTATIVE RESULTS:

The results presented here are a few examples of the many assays that can be performed using this methodology. **Figure 1** is an illustrated guide to the water-based FE bioassay, and is

supplemented by **Figure 2** which follows on to the chloroform-based FE bioassay. **Figure 3** provides a visual guide to what can be expected upon microscopic evaluation of *C. fioriniae* at 24 h, in both water- and chloroform-based bioassays (compared SDW controls). **Figure 4** details a 24 h time-course study with *C. fioriniae* in the presence of the cranberry variety ‘Stevens’ ch-FE, and gives visual reference to an important result of this research: FE decreased the time needed to form infection structures compared to SDW. **Figure 5** provides an example of data collected from a coverslip bioassay using cranberry floral rainwater runoff (CB “Flower” rw-FE). **Figure 6** represents another important result: floral ovary ch-FE was much more stimulatory than fruit ch-FE, indicating the importance of bloom in the lifecycle of *C. fioriniae*. The supplemental photos and movies provide important visuals of the flowers used in the extractions and floral rainwater collection devices/deployment, in addition to movies that visualize the *active*- and *passive*-extraction (water-based) processes.

FIGURE AND TABLE LEGENDS:

Figure 1: General overview of the water-based floral extract (FE) bioassay. This assay was utilized for water-based floral extracts with both blueberry and cranberry flowers: *active*-floral extracts (*active*-FE), *passive*-floral extracts (*pass*-FE) and floral rainwater runoff (rw-FE). The -FE portion typically constitutes the experimental/variable factor. Conversely the -FE portion can remain constant and time points/hours post-inoculation can be evaluated. Preference has been made towards 4 field analysis at 200X magnification. Abbreviations: Sterile deionized water, SDW; Area per field of view, A.

Figure 2: General overview of the chloroform-based floral extract (ch-FE) bioassay. This assay was utilized for both blueberry and cranberry (flowers, ovaries and fruit). Only one type of aqueous treatment mixture was used in this assay, 1 part spore suspension to 2 parts SDW (to keep conidial concentration consistent due to ch-FE evaporation). This assay can be used to compare multiple ch-FE (waxes from various plant surfaces), or multiple time points/hours post-inoculation using a single ch-FE. Abbreviations: Sterile deionized water, SDW; Van Tieghem [glass] cells, VanT. cell.

Figure 3: Visual comparison of *Colletotrichum fioriniae* in the presence of water-based FE and ch-FE. In this assay blueberry ‘Bluecrop’ (*active*-FE, water-based) (fungal isolate: BB#10) and cranberry ‘Stevens’ chloroform-based (ch-FE) (fungal isolate: CB-PMAP182) floral extracts were compared to SDW controls. A dramatic increase in secondary conidiation (rings) and appressorium formation (arrowheads) were observed when comparing conidia in the presence of SDW (control) **(A)** to *active*-FE **(B)** at 24h post-inoculation. However, secondary conidiation was not as apparent when comparing the chloroform bioassay SDW control **(C)** to ch-FE **(D)**; rather, *C. fioriniae* growth shifted towards appressorial formation. Shown is a common response to each extraction type, water-based and chloroform-based, regardless of host/floral species described.

Figure 4: Time-course study (24 h) with *Colletotrichum fioriniae* in the presence of ch-FE. In this assay, an SDW control **(A-D)** and cranberry ‘Stevens’ ch-FE **(E-F)** were visually inspected at 0, 6, 12, and 24 h post-inoculation (an example of variable time points instead of comparing multiple

FE). Appressorium formation (arrowheads) began at 6 h in the ch-FE and steadily increased throughout subsequent time points. This results eludes to an important factor of pathogen biology during the bloom period: flowers reduce the time needed to form infection structures.

Figure 5: Graphical display of data collected using rw-FE in a bioassay. Rainwater run off of cranberry flowers (CB “Flower” rw-FE) and virgin rainwater that had not touched any cranberry plant tissues (“Ground” rw-FE) from a single wetting-event plus a standard *active*, cranberry water-based floral extract (CB *active*-FE) (positive control) and SDW (negative control) were subjected to a water-based coverslip bioassay and evaluated for *C. fioriniae* growth. CB “Flower” rw-FE had the same level of secondary conidiation and appressorium formation as the standard CB *active*-FE at 24 h post-inoculation, indicating that the collection devices were effective in capturing floral stimulants released during a wetting-event. Total conidia is comprised of primary (deposited), conidia and newly formed secondary conidia. Letters indicate significant differences at $p < 0.05$ according to Fischer’s Least Significant Difference test (LSD); uppercase, total conidia; lowercase, appressoria.

Figure 6: Cranberry phenology based ch-FE bioassay, visual inspection. Disease management for fruit rotting fungi often involves bloom time fungicide applications. Here, cranberry chloroform-based extracts (ch-FE) from multiple growth stages of cranberry (‘Stevens’) were visually evaluated for the effect of surface waxes on *C. fioriniae* at 24 h post-inoculation. Ovaries collected in June (**A**), immature fruit collected in July and August (**B, C**), harvested fruit collected in October (**D**), and an SDW control (**E**) were inspected for appressorial formation (arrowheads). Ovary ch-FE had the greatest magnitude of appressorial formation, indicating that this plant phenology (bloom) is critically important to the lifecycle of *C. fioriniae*.

Supplemental Figure 1: Blueberry inflorescence. Blueberry flowers were collected for extractions during full bloom (April-May in New Jersey, USA) (shown ‘Bluecrop’). Note the overlap of corollas/ovaries from adjacent flowers and the overall architecture of the inflorescence compared to **Supplemental Figure 2** (cranberry upright).

Supplemental Figure 2: Cranberry upright. Cranberry flowers were collected for extractions during full bloom (June-July in New Jersey, USA) (shown ‘Stevens’). Note the varied flower stages on a single cranberry inflorescence (upright), and the hooked, water droplet retaining the shape of the corolla.

Supplemental Figure 3: Blueberry rainwater deployment (flower). Completed blueberry floral rainwater collection device, placed directly under a cluster of inflorescences. Note the plastic-coated wire used to vertically orient the device.

Supplemental Figure 4: Blueberry rainwater deployment (stem). Completed blueberry floral rainwater collection device, placed half way down the stem between an inflorescence and the crown of the bush.

Supplemental Figure 5: Blueberry rainwater deployment (crown). Completed blueberry floral

rainwater collection device, placed at the base of the bush (crown). Note plastic coated wires can be removed if not necessary.

Supplemental Figure 6: Blueberry rainwater deployment (ground). Completed virgin rainwater collection device, placed adjacent to blueberry bushes.

Supplemental Figure 7: Cranberry rainwater deployment (close-up). Completed cranberry floral rainwater collection device, with two uprights tucked under the neatly crossed wire ties.

Supplemental Figure 8: Cranberry rainwater deployment. Multiple completed cranberry floral rainwater devices deployed in a bog.

Supplemental Movie 1: Active, water-based floral extracts (*active*-FE). Supplemental video support following steps 2.3-2.5.1. Blueberry 'Bluecrop' flowers were used.

Supplemental Movie 2: *Passive*, water-based floral extracts (*pass*-FE). Supplemental video support following steps 3.3-3.4. Blueberry 'Bluecrop' flowers were used.

Supplemental Movie 3: Deployment of cranberry floral rainwater collection devices. Supplemental video support following step 6.4.

DISCUSSION:

The bioassays detecting the *C. fioriniae* response to floral extracts (FEs) were developed for the blueberry and cranberry fruit rot pathosystems but can be readily adapted to other horticultural crops. The protocol detailed above has been valuable in acquiring many important data sets including, but not limited to: FE effects on multiple isolates of numerous pathogens, time-course information pertaining to fungal growth stages in the presence of various FEs, comparison of extraction techniques, inspection of individual chemicals on *C. fioriniae* growth and differentiation, evaluation of individual flower organ extracts, effects of temperature on *C. fioriniae* while in the presence of FE, effects of phenology dependent wax extractions, and floral rainwater effects. Through the use of these techniques, data generated has also provided a much clearer understanding of *C. fioriniae* life stages and partially elucidates why the bloom period is so critical to the control of many fruit rotting pathogens.

Initially, all flowers were processed identically to the *active*-FE, but the extraction process has moved towards using whole flowers. Floral dissection was time consuming and had very little effect on the bioactivity of the resulting FEs. However, individual floral organs can and have been evaluated using this protocol, but great care must be taken to not completely macerate the floral tissues (**Supplemental Movie 1**, with precautions detailed in step 2.3), as this may result in released fungi-toxic/static compounds into the FE that could distort the microscopic evaluations. Less invasive extractions such as *pass*-FE (**Supplemental Movie 2**) and rw-FE are now more favorable due to their ease of acquisition. Additionally, these extraction techniques require only vacuum filtration to acquire biologically active floral chemical cues.

The flowers utilized in all extractions were typically refrigerated for 0-3 days prior to extract preparation. A challenge of this protocol is time management of FE turnover (field collection through storage of extracts). This was exacerbated by numerous samples from multiple sources and dates. Frozen flowers have not been evaluated to any real extent, as thawed flowers appear deteriorated and discolored. However, once the water-based FEs have been prepared, repeated freezing and thawing has shown no effect on the bioactivity of the FE, so as long as the FE are quickly refrozen after bioassay preparation (viable 3 year old FE).

Chloroform-based extraction enables the investigation of pathogen responses to three-dimensional floral/fruit surface waxes in a two-dimensional plane via ch-FE evaporation on glass coverslips. However, it is unlikely that the actual crystalline structures of waxes deposited from the ch-FE are identical to the surface from which they were collected. Meaning, supplemental techniques should be implemented if fungal response to specific wax structures *in vivo* are the main focus of investigation. Chloroform-based extracts need more storage maintenance than the water-based extractions. In addition to keeping the ch-FE extracts in the dark, the PTFE lined cell culture tube caps and parafilm sealing wrap need to be regularly checked for evaporative leaks and replaced as necessary.

The concept of monitoring floral rainwater runoff is rooted in the idea of advancing site-specific disease monitoring tools. The rainwater collection devices can be adapted to many other plant architectures, so long as the collection device captures rainwater that has run off of flowers. This approach provides information on whether or not floral stimulation is present in the field at any given time and can be monitored throughout the season. Alternatively, collection devices can be deployed at multiple canopy locations to determine how far floral cues have been washed during any given wetting-event. In future experiments, rw-FE will dictate when fungicide applications should begin and when they can safely end. Additionally, by monitoring phenology dependent wax extractions (protocol section 9), the importance of the bloom period to pathogen biology has become even more evident. That section was also included to demonstrate the flexibility of these bioassays, providing methods that allow for side-by-side comparison of host surface waxes that are temporally separated. The data generated using the floral extraction techniques and bioassays represent tangible indicators of pathogen stimulation, specific chemical classes important to pathogen biology, and targets for future control strategies.

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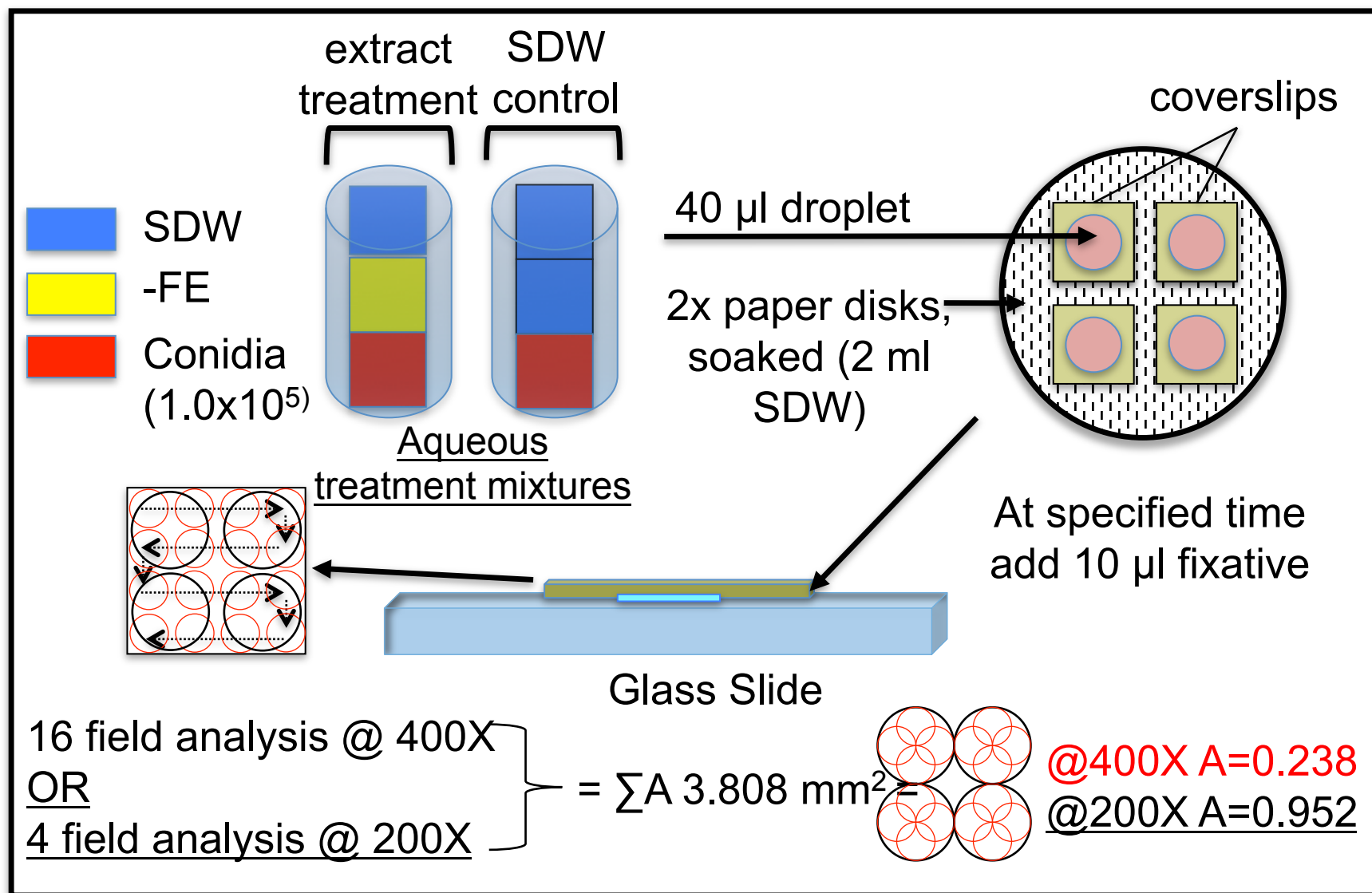
DISCLOSURES:

The authors have nothing to disclose.

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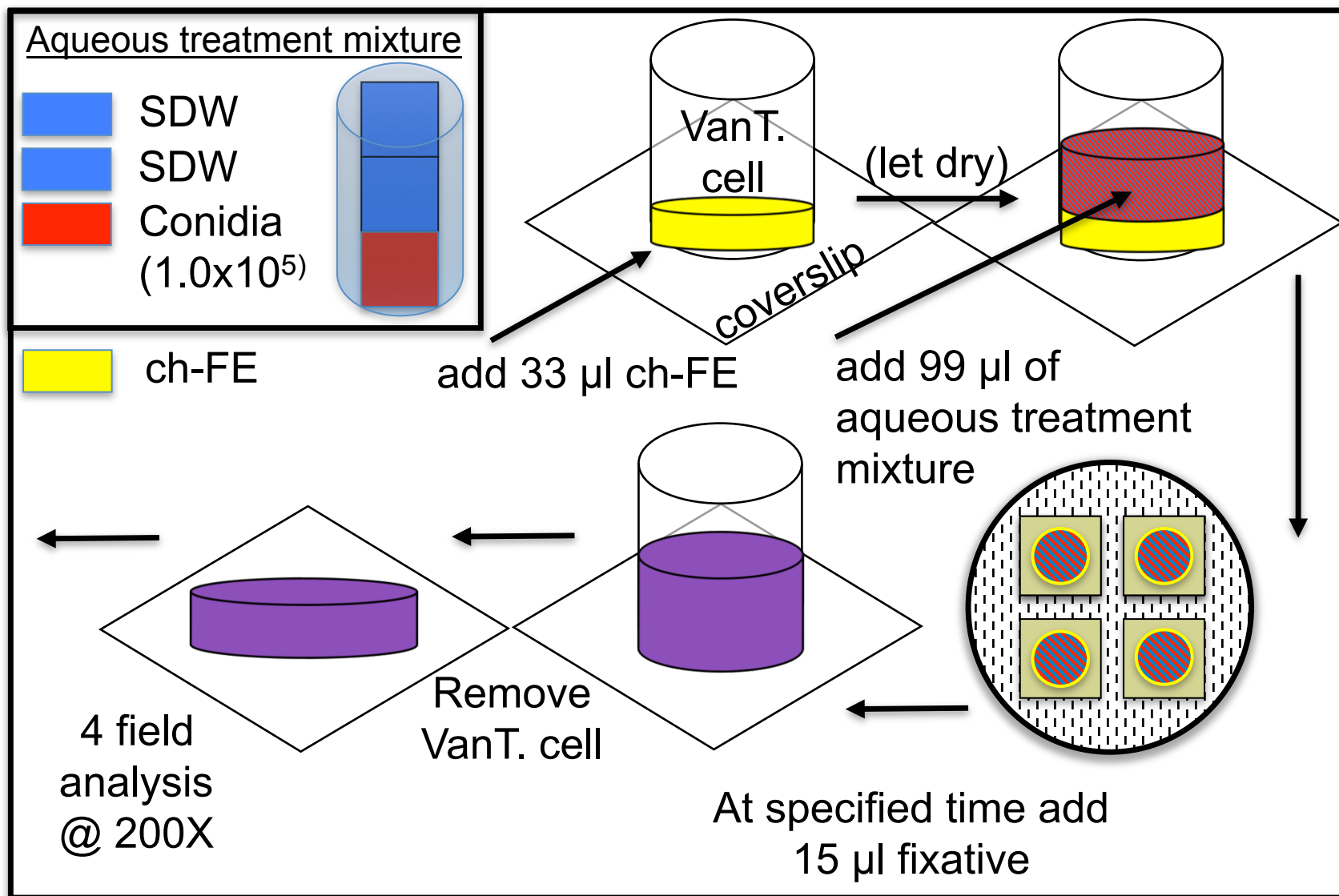
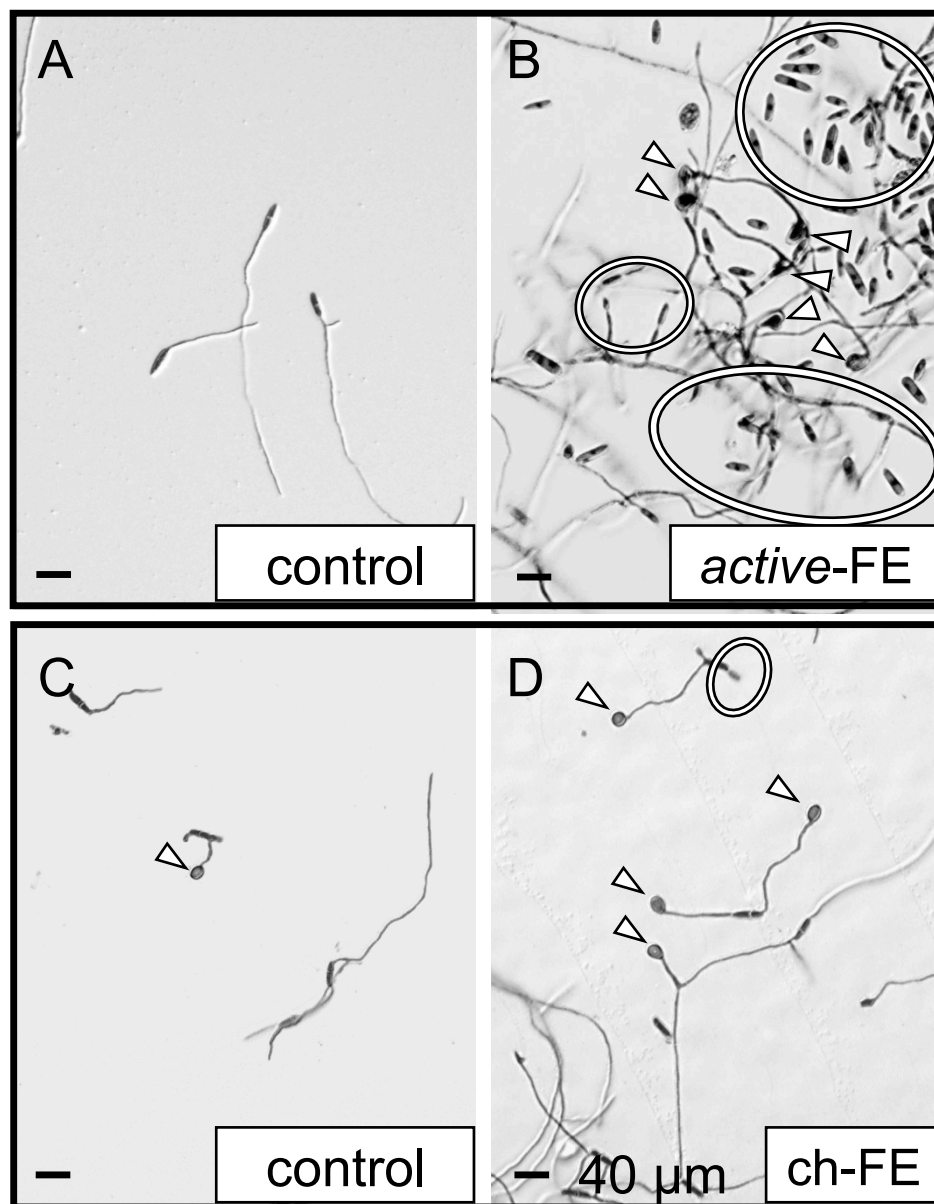
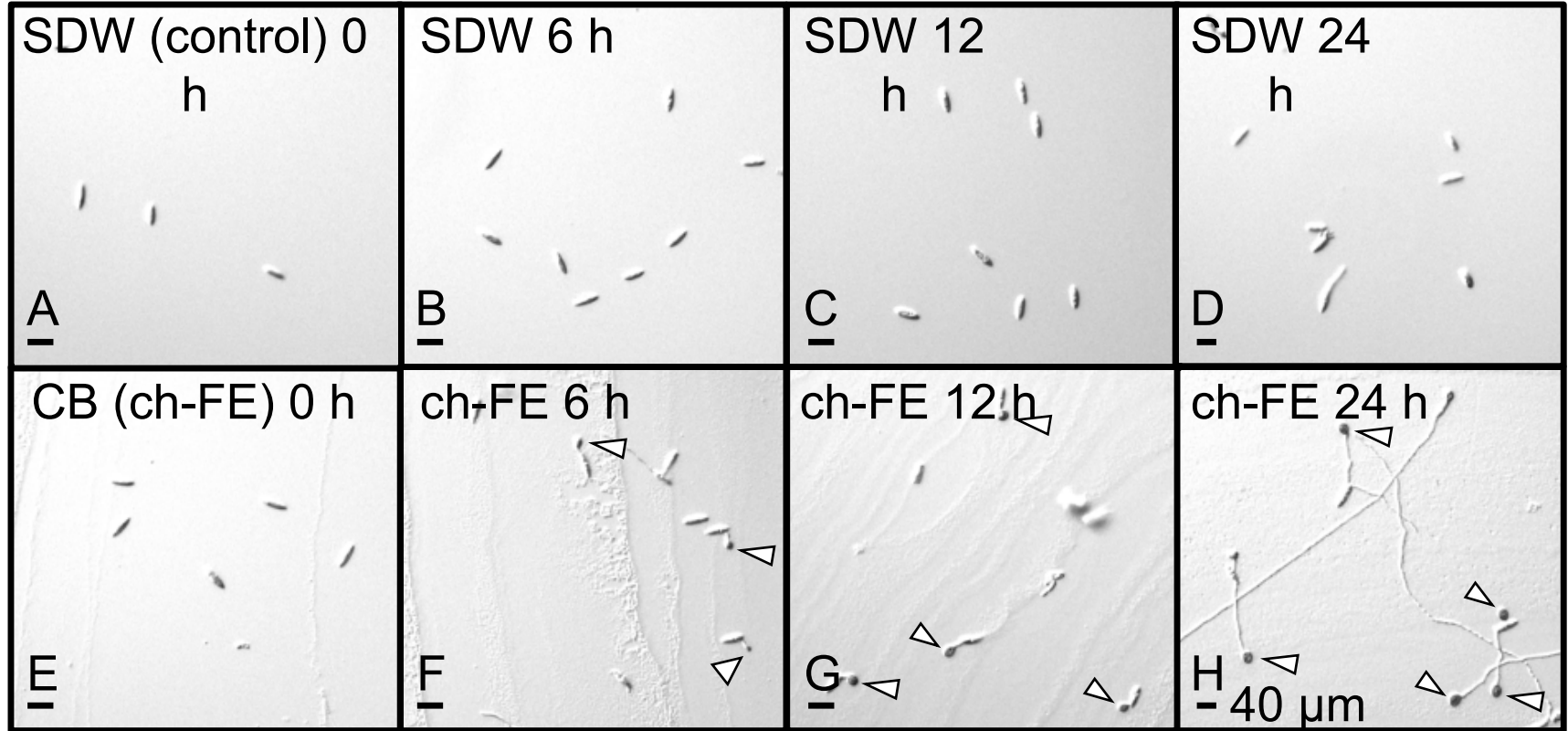
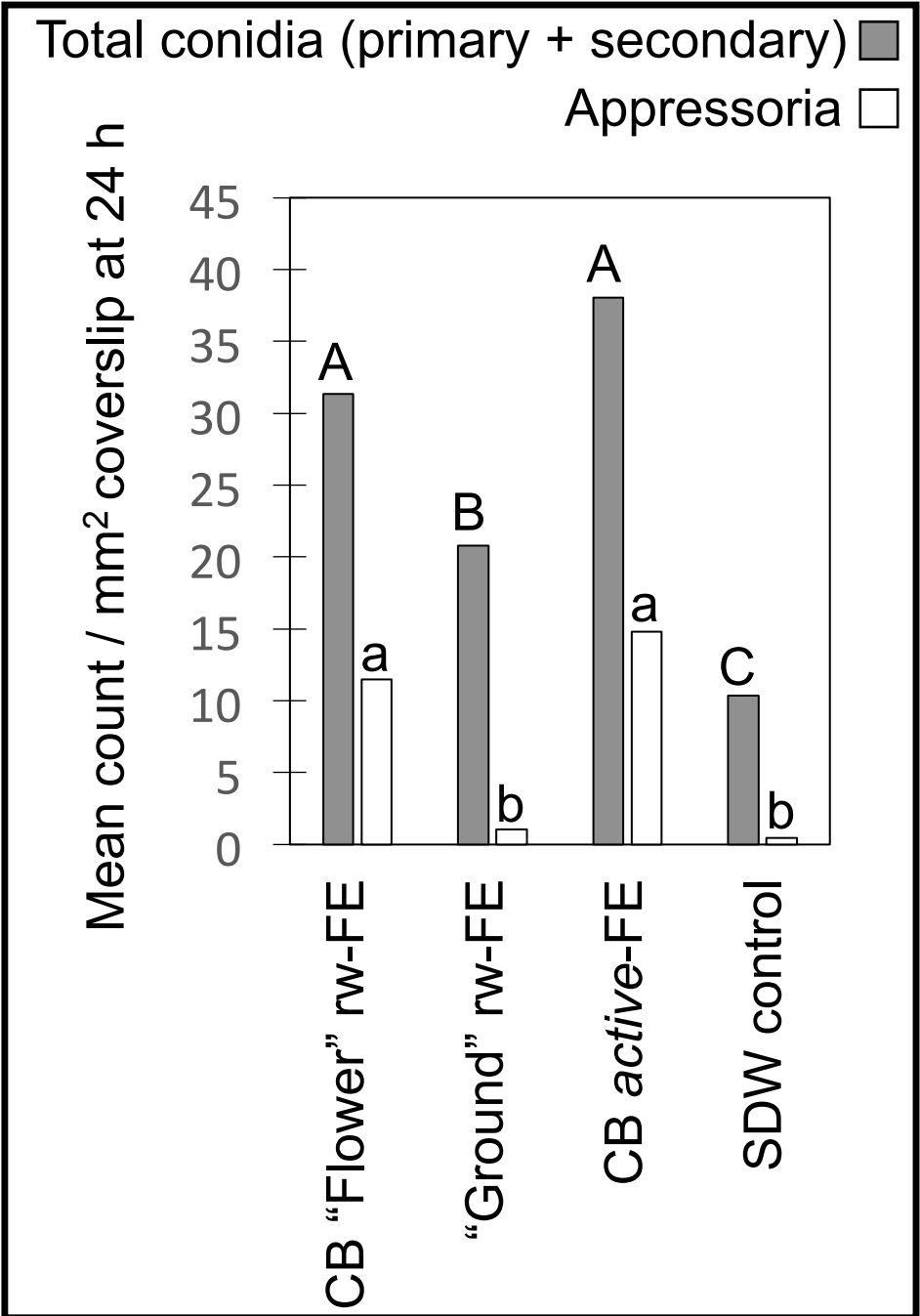
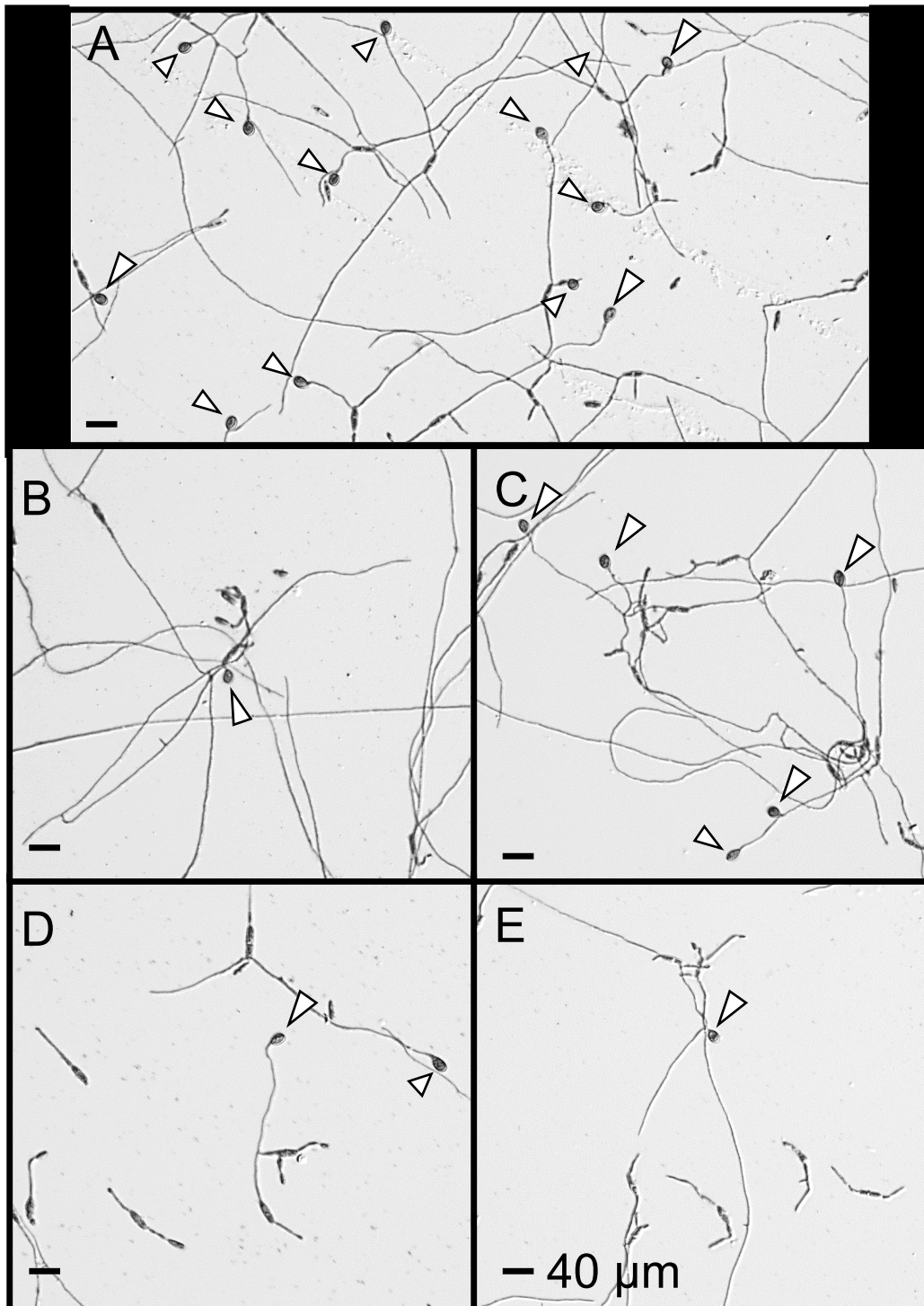


Figure 3























Name of Material/ Equipment	Company	Catalog Number	Comments/Description
0.22 µm pore size, acetate sterilizing filter	VWR	101102-280	Blueberry floral extract (FE) clarification
200-1000 µl pipette with tips	-	-	Equipment, any make within range will be adequate
40-200 µl pipette with tips	-	-	Equipment, any make within range will be adequate
5-40 µl pipette with tips	-	-	Equipment, any make within range will be adequate
Air spray gun disposable paint spray cup with connection adapter	Harbor Freight	97098	Blueberry rainwater (rw-)FE collection
Autoclave	Amsco	3011	Equipment, media preparation
Bar mesh matting (plastic mesh sheet)	Winco	BL-240	Passive (pass)-FE collection
Benchtop timer	Fisher Scientific	06-662-47	Equipment, FE preparation
Black pressure/vacuum hose	VWR	62994-795	Vacuum filter component
Buchner funnel	Coors USA	60240	Vacuum filter component, accepts 55 mm filter paper disks
Bunsen burner	-	-	Equipment
Calcium carbonate	Fisher Scientific	C64-500	Media component
Centrifuge	Sorvall	RC 5B Plus	Equipment
Centrifuge tubes (15 ml)	Fisher Scientific	05-527-90	Equipment
Centrifuge tubes (50 ml)	VWR	10025-694	Equipment, rw-FE collection
Cheesecloth (grade 50)	Fisher Scientific	AS240	Equipment, FE preparation
Chloroform	VWR	JT9175-3	Chemical, trichloromethane: assay grade, ≥ 99% pure, for molecular biology, peroxide-free
Corn Meal Agar (CMA)	Fisher Scientific	811132	Pre-mix media, isolate storage on slants
Cotton-blue stain	Sigma-Aldrich	61335	Lactophenol cotton-blue stain
Curved forceps (45°)	Fisher Scientific	10-270	Equipment, flower processing and coverslip inversion
Difco Agar	VWR	90004-032	Media component
Drill-press	Delta	-	Equipment, rw-FE collection
EASypure LF Ultrapure water	Barnstead	D738	Equipment, deionized water source
Ethanol (95%)	-	-	Chemical
Filter flask (500 ml)	Pyrex	No. 5340	Vacuum filter component
Freezer (set to -20° C)	-	-	Equipment, storage of active-FE, pass-FE, rw-FE
Fume hood	Hamilton	-	Equipment, chloroform usage
Funnel (7 X 7 cm)	VWR	60820-110	Cranberry rw-FE collection, FE preparation
Generic glass slide	Fisher Scientific	22-038-101	Bioassay conductance
Generic plastic pump spray bottle	VWR	16126-454	pass-FE collection, at least 250 ml capacity
Glass cell culture tubes	-	-	Storage of ch-FE
Glass coverslips (22 x 22 mm)	Fisher Scientific	12-542B	Bioassay conductance
Glass Van Tieghem cells (hand cut glass tubes)	-	-	Chloroform (ch)-FE bioassay, (8 mm OD 6 mm ID)
Glass-pipette (1-100 µl)	Hamilton Co. Inc.	#710	ch-FE bioassay
Glycerol	Sigma-Aldrich	G5516	Lactophenol cotton-blue stain
Hemocytometer	Bright-Line	5971R10	Equipment
Incubator (set to 25° C, dark)	Percival	50036	Equipment, bioassay conductance
Lactic acid	Sigma-Aldrich	W261106	Lactophenol cotton-blue stain
Laminar flow hood	Labconco	3730400	Equipment, sterile work environment
Metal probe (generic)	-	-	Equipment
Microcentrifuge tubes (2 ml)	Fisher Scientific	05-408-138	Aqueous treatment mixture storage and preparation
Microscope, Leica DMLB	Leica	020-519.010	Equipment
Mortar (ceramic)	Coors USA	60313	Vacuum filter component
Nitrile gloves	Fisher Scientific	19-130-1597D	Flower collection
Paper disks (cut paper towels)	Office Basics	KCC01510	humidity control in bioassay
Parafilm	Bemis	PM-996	Plastic paraffin film
Pestle (ceramic)	Coors USA	60314	Vacuum filter component
Phenol crystals	Fisher Scientific	A92-100	Lactophenol cotton-blue stain
Plastic bags (~100 mm X 152 mm)	Uline	51294	Equipment, flower refrigeration
Plastic cell culture dishes (9 cm diameter)	Fisher Scientific	FB0875712	(Petri dish), bioassay conductance
Polytetrafluoroethylene (PTFE) lined caps	VWR	60927-228	Storage of ch-FE
Pyrex beakers (100 ml)	Pyrex	No. 1000	Preparation of ch-FE
Pyrex bread-pan	-	-	pass-FE collection
Pyrex graduated cylinder	-	-	Equipment, FE preparation
Refrigerator (set to 4° C)	-	-	Equipment, storage of ch-FE
Sealed plastic container (30 mm X 13 mm X 7 mm)	-	-	Bioassay conductance
Sharp-pointed dissecting scissors	Fisher Scientific	8940	Equipment, to cut cheese-cloth and paper disks
Stainless steel mesh strainer	VWR	470149-756	Preparation of ch-FE
Step drill bit (step-bit)	Dewalt	-	Equipment, rw-FE collection
Sterile loop (combi-loop)	Fisher Scientific	22-363-602	Culture preparation
Telephone wire (internal wires)	-	-	Blueberry rw-FE collection
Test tube basket	VWR	470137-792	Readily available substitution for plastic mesh [strawberry] basket
V8 Juice	Campbell's Soup Company	-	Fungal media component
Vintage plastic mesh [strawberry] baskets	Donation	-	pass-FE collection, can substitute for test tube basket (470137-792)
Vortex Genie (Vortex)	Fisher Scientific	12-812	Spore suspension preparation
Whatman No. 1 Qualitative 55 mm circles	Whatman	1001-055	Vacuum filter component
White plastic twist ties (100 mm)	Uline	S-566W	Cranberry rw-FE collection



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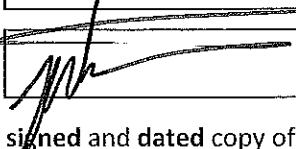
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Rebuttal Document for JoVE (manuscript #: JoVE58880)

Original* Title: Insight into *Colletotrichum fioriniae* biology via bioassays utilizing floral extracts from blueberry and cranberry

Modified Title:

Bioassays utilizing water- and chloroform-based floral extracts provide insight into *Colletotrichum fioriniae* biology on blueberry and cranberry

Order of Authors:

Timothy James Waller (Email/communications corresponding author)

Joshua D. Gager (Significant methods contributions)

Peter V. Oudemans (Official manuscript corresponding author)

Please note:

There was a numbering error early on in the original manuscript, a “Protocol # 3” was in place without any methods, skewing the numbering system. This has been corrected throughout the revised manuscript and the responses to the comments reflect this change. For example the original step 6 is now, appropriately, step 5.

An additional note refers to the original communications with Jaydev Upponi. Due to the ephemeral nature of flowers, some of the protocols were filmed prior to completing the editorial process of submission/review. Photos and videos of the pre-filmed steps have been included as supplemental photos and videos with associated captions in the manuscript text. Originally the plan was to include this footage in the main video production but due to the complexity of this protocol, we believe it better to leave as supplemental figures/photos and movies, so to include more filmable protocol steps.

In the revised submission all of the in-text modifications have been tracked. As per broad editorial and reviewer comments such as “add more detail to protocols, focus the summary/abstract/introduction more towards the protocols, and better tailor the discussion to JoVE requirements”, a large portion of the manuscript has been edited. Below, *italicized* text denotes reviewer comment, with responses to the comments shown as bullet points.

Rebuttal to editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

- The manuscript has been carefully read through and should not contain any spelling or grammatical errors.

2. Please rephrase the Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: “Here, we present a protocol to ...”

- A new summary has been written to more adequately address the purpose of the protocol.
- New Summary: (highlighted in red in the manuscript)

- (50 words) Here, bioassays designed to monitor the development of a fungal pathogen, *Colletotrichum fioriniae*, in the presence of blueberry or cranberry floral extracts on glass coverslips are described. Water-, chloroform-, and field rainwater- based floral extractions techniques are detailed as well as insight into how this information can be applied.

3. Please spell out each abbreviation the first time it is used.

- This has been corrected throughout the manuscript, figures, and Table of Materials.

4. Please use SI abbreviations for all units: L, mL, μ L, h, min, s, etc.

- This has been corrected throughout the manuscript, figures and Table of Materials.

5. Please include a space between all numerical values and their corresponding units: 15 mL, 37 °C; etc.

- This has been corrected throughout the manuscript, figures and tables.

6. Please revise the protocol to contain only action items that direct the reader to do something (e.g., "Do this," "Ensure that," etc.).

- This has been corrected throughout the manuscript, any non-imperative terms have been restricted to notes or other text areas.
- Safety procedures have been inserted at appropriate locations throughout the manuscript.

7. Please revise the Protocol steps so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step. Use sub-steps as necessary.

- In many cases steps have been either broken down, simplified, or joined with appropriate sub-steps/notes added.

8. Please add more details to your protocol steps.

- This has been carefully evaluated throughout the text and the tracked changes reflect the incorporation of more protocol detail. Almost the entire methods section has been updated as per editorial comment.

See examples below:

- Specific examples have been addressed (below) in addition to others that fall within the bounds of this editorial comment.

1.1: How large is the culture dish?

- Step 1.1 has been clarified to describe the diameter of the cell culture dish, (9 cm diameter). Additionally the size has been adjusted within the Table of Materials.

3.1: How many flowers are collected and placed into a plastic bag? Are blueberry and cranberry flowers placed into separate bags? Please specify.

- Step 2.1 has been clarified to denote when to separately pick each crop as well as how much to gather, “Place either blueberry or cranberry flowers into plastic bags (fill 100 mm X 150 mm bag) and immediately refrigerate at 4° C once flowers are collected”.

6.1: Please describe how to construct the collection device.

- The protocol for both blueberry and cranberry collection devices have been extensively modified. .

9. Step 2: Please describe any actions being performed here. Citation only is not sufficient.

- Additional information has been added.

10. Line 101, 131, etc.: Please number the video files as Movie 1, Movie 2, etc., and reference them in the protocol. Please also name the different images mentioned as Figure 1, Figure 2, or supplemental figures, etc.

- All additional photos and videos have been noted as supplemental materials. Two extraction methods, *active*- extraction and *passive*- extraction as well as the cranberry rainwater collection device deployment have been noted as supplemental movies. Due to the length of this protocol the additional videos are favored as standalone supplemental resources. A caption has been added for all supplemental materials as well as in-text notation.

11. There is a 2.75 page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

- Highlighted text (yellow) has been corrected to meet the requirements; at ~40 lines per page * 2.75 pages = ~110 lines, we have selected ~72 lines (to allow for spaces and headings) of filmable text. We have also included 3 supplemental videos, which should be left out of the main video production.

12. Figures, Table of Materials: Please use SI abbreviations for all units (mL, μ L, etc.) and include a space between all numerical values and their corresponding units (15 mL, 33 μ L, 24 h, etc.).

- Spaces have been added throughout the figures and within the Table of Materials. Abbreviations have also been announced if not commonly used.

13. Figures 3, 4, 6: Please include a space between the number and the units of the scale bar (40 μ m).

- A space has been added between the number and the units of the scale bar in figures 3, 4, and 6.

14. JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please revise the Discussion.

- The discussion has been significantly updated to be more aligned to the practices of a JoVE manuscript discussion section.

Rebuttal to Reviewer #1 comments:

Although the methods are the main focus, I suggest changing the title to give the method focus, e.g. Floral extract bioassays give insight to C.f. biology on blueberry and cranberry. Summary, abstract and introduction could also be rewritten to put more focus on the methods than on the pathogen.

- The title has been modified to address this concern and more accurately represent the body of work as described.
- New Title:
 - Bioassays utilizing water- and chloroform-based floral extracts provide insight into *Colletotrichum fioriniae* biology on blueberry and cranberry

Minor Concerns: *More focus on Methods in summary, abstract and introduction may improve the article.*

- This has been carefully evaluated throughout the text, the tracked changes reflect this.

Rebuttal to Reviewer #2 comments:

I am not sure that this will be very effective for use in disease forecasting.

- This suggestion has been removed until our research group can more substantially validate this claim.

Minor Concerns: *Comments on the pdf as sticky notes.*

- Sticky note comments have been addressed throughout the manuscript. Examples of word-choice suggestions are detailed below.
 - Replacing the word “inoculate” when describing the action of dispensing the treatment mixtures containing conidia on the glass coverslips has been modified.
 - Replacing the word “tissues” when describing the parts of the flower has been changed to “organs” and tissues has been omitted.
 - Numerous other editorial-based comments have been addressed as well throughout the manuscript.















