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Identification of a Universal Marker for Detecting Possible Mutation in *Botrytis cinerea* Isolates Associated with Virulence

Moytri RoyChowdhury[1,2]*, Jake R. Erickson[1] , Peter Rafael Ferrer[2], Brian Foley[3], Shannon Piele[2], James Titius[2], Kshitij Shrestha[1] and Caleb Fiedor[2]

[1]*Department of Biological Sciences, Idaho State University, 650 Memorial Dr. Gale Life Sciences Building, Pocatello, ID 83209, USA*
[2]*Department of Biology, Fresno Pacific University, 1717 S Chestnut Ave, Fresno, CA 93702, USA*
[3]*Los Alamos National Laboratory, Santa Fe, NM 87545, USA*

Abstract

Botrytis cinerea sporadically infects plants in Mediterranean climates and contributes to a significant crop loss every year. Diseases caused by *B. cinerea* can affect many crops and are of particular concern to strawberry growers in California, which is the major state for fresh strawberry production in the United States. This study looks at genetic mutations, and the subsequent phenotypical changes, of several strains of *B. cinerea* obtained from plant tissues. It was found that strains with four nucleotide insertions were more virulent than their wild type counterparts. Strains with single nucleotide polymorphisms had conidia smaller or similar in size to the wild type strains and exhibited similar virulent properties as the wild type. We also observed identical mutations of fungal samples obtained from different plant tissues from Asia.

Keywords: *B. cinerea*; Isolates; Virulence; Strawberries; Mutation

Introduction

B. cinerea Pers.: Fr and related *Botrytis* species are common and economically important pathogens affecting a wide variety of vegetables and ornamental crops [1]. While there are 25-30 necrotropic plant pathogens closely related to *B. cinerea* Pers.: Fr (teleomorph *Botryotinia fuckelian* (de Bary) Whetzel, *B. cinerea* is the most comprehensively studied [2,3]. The added attention is due to the vast economic effects caused by the fungus's widespread host range, infecting over 200 plant species, and by its involvement in pre- and post-harvest crop losses worldwide, which cause vast economic effects [2,4]. The ability to infect diverse plant species and tissues under a wide-range of environmental conditions is aided by *B. cinerea's* ability to survive in topsoil, which cause it to be very persistent in nature. It is also aided by dispersed airborne conidia or ascospores, which allow *B. cineria* to infect host tissue and sexually reproduce [5].

The conidia, which are commonly found in large quantities in necrotophic host tissue, are the main sources of infection by *B. cinerea* [2]. In the presence of high levels of humidity (>93%), germination is activated by physical and chemical signals. This activation is a cAMP-dependent pathway and MAP kinase cascade, which are involved in signal transductions that lead to germination [2]. Because of their low degree of differentiation, the ability of *B. cinerea* appressoria to physically penetrate intact host tissue seems to be limited and probably needs to be supported by secreted lytic enzymes [6]. *B. cinerea* is found to secrete a number of endopolygalacturonases, which are used to soften cell walls [7]. Although, the ability to penetrate the cell wall of host tissues might need the action of specific chitin synthase isoenzymes, which is shown to be necessary for pathogenicity [8,9]. Once penetration has occurred, it is typically followed by the prompt death of the host cell.

The fungus is phenotypically characterized by grey mold on the tissue that it infects [10]. The pattern of growth can vary in color and density, but in general the pathogen has gray mycelium that differentiates it from other fungi [11]. It occurs sporadically in Mediterranean climates and contributes to a significant crop loss every year, making *B. cinerea* an extreme nuisance in California [12].

Out of all the Californian crops that *B. cinerea* is known to infect, it has one of the most undesirable effects on its sixth most valuable crop, the field strawberry (*Fragaria ananassa)*. California is the major state for strawberry production in the U.S., accounting for over 80% of the fresh market product grown [13]. Second to grapes, strawberries are the most important cultivated fruit of the berry family worldwide with 3.82 million tons produced per year [14]. *B. cinerea* causes one of the most prominent post-harvest molds that cause quality deterioration of strawberries and other fruits in the field and during refrigeration storage [15,16]. Gray mold can cause up to a 30% to 40% loss of the harvest where chemical methods of pest management are not practiced. This loss can reach 50% to 60% in areas of acute infestations, in which the economic loss will be up to 100% [15].

Botryticides and fungicides are currently used to combat these losses, but both come with their own sets of problems. The use of botryticides, despite their latest availability, results in continued crop loss, while the use of fungicides is becoming increasingly restricted or forbidden because of their unfavorable effects on ecosystem and human health [12]. Fungicides are still used for disease control, but the fungi are adapting quickly to these drugs *via* mutations, including the insertion of retroposon sequences into gene promoters [17,18]. As a result of recurring fungicide treatments, resistant strains are quickly developing [19]. Three types of increasing multidrug resistance populations of *B. cinerea* have appeared since the mid 1990's in Europe [17]. After studying the immerging resistant strains, a few distinctive fungicide resistance mechanisms have been identified in both field and greenhouse environments [20,21]. It has been anticipated that by continually selecting multi-resistant strains, chemical control of *B. cinerea* in the field will become increasingly difficult [17].

With the increased resistance and concern for human health in the last decade, there is a need for a greater understanding of the development of this resistance epidemic in order to create resilient and

***Corresponding author:** Moytri RoyChowdhury, Department of Biological Sciences, Idaho State University, 650 Memorial Dr. Gale Life Sciences Building, Pocatello, ID 83209, USA, Tel: +2082822149; E-mail: roycmoyt@isu.edu

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Isolate number	Isolated from	Type of infection	Area	Ranch	Date	Note
BOT01	Strawberry	postharvest	Oxnard	04.2156	Feb. 2008	Fruit
BOT02	Strawberry	postharvest	Oxnard	04.2156	Feb. 2008	Fruit
BOT03	Strawberry	postharvest	Oxnard	04.2156	Feb. 2008	Fruit
BOT04	Strawberry	postharvest	Oxnard	04.2156	Feb. 2008	Fruit
BOT05	Strawberry	postharvest	Oxnard	04.2156	Feb. 2008	Fruit
BOT06	Strawberry	postharvest	Oxnard	04.2156	Feb. 2008	Fruit
BOT07	Strawberry	postharvest	Oxnard	04.2156	Feb. 2008	Fruit
BOT08	Strawberry	postharvest	Oxnard	04.2156	Feb. 2008	Fruit
BOT09	Strawberry, El Dorado	field	Oxnard	Sammis	Mar.2008	Fruit
BOT10	Strawberry, El Dorado	field	Oxnard	Lennox	Mar.2008	Fruit
BOT11	Strawberry, El Dorado	field	Oxnard	Sammis	Mar.2008	Fruit
BOT12	Strawberry, El Dorado	field	Oxnard	Lennox	Mar.2008	Fruit
BOT13	Strawberry, El Dorado	field	Oxnard	Beardsley	Mar.2008	Fruit
BOT14	Strawberry, El Dorado	field	Oxnard	Sammis	Mar.2008	Fruit
BOT15	Strawberry, El Dorado	field	Oxnard	Lennox	Mar.2008	Fruit
BOT16	Strawberry, El Dorado	field	Oxnard	Sammis	Mar.2008	Fruit
BOT17	Strawberry, El Dorado	field	Oxnard	Sammis	Mar.2008	Fruit
BOT18	Strawberry, El Dorado	field	Oxnard	Sammis	Mar.2008	Fruit
BOT19	Strawberry, El Dorado	field	Oxnard	Beardsley	Mar.2008	Fruit
BOT20	Strawberry, El Dorado	field	Oxnard	Sammis	Mar.2008	Fruit
BOT21	Strawberry, El Dorado	field	Oxnard	Lennox	Mar.2008	Fruit
BOT22	Strawberry, El Dorado	field	Oxnard	Lennox	Mar.2008	Fruit
BOT23	Strawberry, El Dorado	field	Oxnard	Beardsley	Mar.2008	Fruit
BOT24	Strawberry, El Dorado	field	Oxnard	Beardsley	Mar.2008	Fruit
BOT25	Strawberry, El Dorado	field	Oxnard	Beardsley	Mar.2008	Fruit
BOT26	Strawberry, El Dorado	field	Oxnard	Beardsley	Mar.2008	Fruit
BOT27	Strawberry, El Dorado	field	Oxnard	Beardsley	Mar.2008	Fruit
BOT28	Strawberry, El Dorado	field	Oxnard	Beardsley	Mar.2008	Fruit
BOT29	Strawberry, El Dorado	field	Oxnard	Beardsley	Mar.2008	Fruit
BOT30	Strawberry, El Dorado	field	Oxnard	Beardsley	Mar.2008	Fruit
BOT31	Strawberry, El Dorado	field	Oxnard	Sammis	Mar.2008	Fruit
BOT32	Strawberry, El Dorado	field	Oxnard	Sammis	Mar.2008	Fruit
BOT33	Strawberry, El Dorado	field	Oxnard	Lennox	Mar.2008	Fruit
BOT34	Raspberry, Pacifica	field	Oxnard	Lennox	Mar.2008	Fruit
BOT35	Raspberry, Pacifica	field	Oxnard	Lennox	Mar.2008	Fruit
BOT36	Raspberry, Pacifica	field	Oxnard	Lennox	Mar.2008	Fruit
BOT37	Raspberry, Pacifica	field	Oxnard	Sammis	Mar.2008	Fruit
BOT38	Raspberry, Pacifica	field	Oxnard	Beardsley	Mar.2008	Fruit
BOT39	Raspberry, Pacifica	field	Watsonville		May.2008	Cane infection
BOT40	Raspberry, Pacifica	field	Watsonville		May.2008	Cane infection
BOT41	Raspberry, Pacifica	field	Watsonville		May. 2008	Cane infection
BOT42	Raspberry, Pacifica	field	Watsonville		May. 2008	Cane infection
BOT43	Raspberry, Pacifica	field	Watsonville		May. 2008	Cane infection
BOT44	Raspberry, Pacifica	field	Watsonville		May.2008	Cane infection
BOT45	Raspberry, Pacifica	field	Watsonville		May.2008	Cane infection
BOT46	Strawberry	field	Watsonville		May. 2008	Cane infection
BOT47	Strawberry	field	Watsonville		May. 2008	Cane infection
BOT48	Strawberry	field	Watsonville		May. 2008	Cane infection
BOT49	Strawberry	field	Watsonville		May. 2008	Cane infection
BOT50	Strawberry	field	Salinas	Davis	Jun. 2008	Fruit
BOT51	Strawberry	field	Watsonville	TCR	Jun. 2008	Fruit
BOT52	Strawberry	field	Salinas	Davis	Jun. 2008	Fruit
BOT53	Strawberry	field	Salinas	Davis	Jun. 2008	Fruit
BOT54	Strawberry	field	Salinas	Davis	Jun. 2008	Fruit
BOT55	Strawberry	field	Salinas	Davis	Jun. 2008	Fruit
BOT56	Strawberry	field	Salinas	Davis	Jun. 2008	Fruit
BOT57	Strawberry	field	Watsonville	TCR	Jun. 2008	Fruit
BOT58	Strawberry	field	Salinas	Davis	Jun. 2008	Fruit
BOT59	Strawberry	field	Salinas	Davis	Jun. 2008	Fruit
BOT60	Strawberry	field	Salinas	Davis	Jun. 2008	Fruit

BOT61	Strawberry	field	Salinas	Davis	Jun. 2008	Fruit
BOT62	Strawberry	field	Salinas	Davis	Jun. 2008	Fruit
BOT63	Strawberry	field	Salinas	Davis	Jun. 2008	Fruit
BOT64	Strawberry	field	Watsonville	TCR	Jun. 2008	Fruit
BOT65	Strawberry	field	Watsonville	TCR	Jun. 2008	Fruit
BOT66	Strawberry	field	Watsonville	TCR	Jun. 2008	Fruit
BOT67	Strawberry	field	Watsonville	TCR	Jun. 2008	Fruit
BOT68	Strawberry	field	Salinas	Davis	Jun. 2008	Fruit
BOT69	Strawberry	field	Salinas	Davis	Jun. 2008	Fruit
BOT70	Strawberry	field	Salinas	Davis	Jun. 2008	Fruit
BOT71	Strawberry	field	Salinas	Davis	Jun. 2008	Fruit
BOT72	Strawberry	field	Salinas	Davis	Jun. 2008	Fruit
BOT73	Strawberry	field	Salinas	Davis	Jun. 2008	Fruit
BOT74	Strawberry	field	Watsonville	TCR	Jun. 2008	Fruit
BOT75	Strawberry	field	Watsonville	TCR	Jun. 2008	Fruit
BOT76	Strawberry	field	Watsonville	TCR	Jun. 2008	Fruit
BOT77	Strawberry	field	Watsonville	TCR	Jun. 2008	Fruit
BOT78	Blackberry	field	Watsonville	TCR	7/11/2014	Leaf Petiole
BOT79	Strawberry	field	Nursery	Tule Lake	6/19/2014	Fruit

Table 1: List of isolates collected and used for morphological, phylogenetic, and pathogenic analysis.

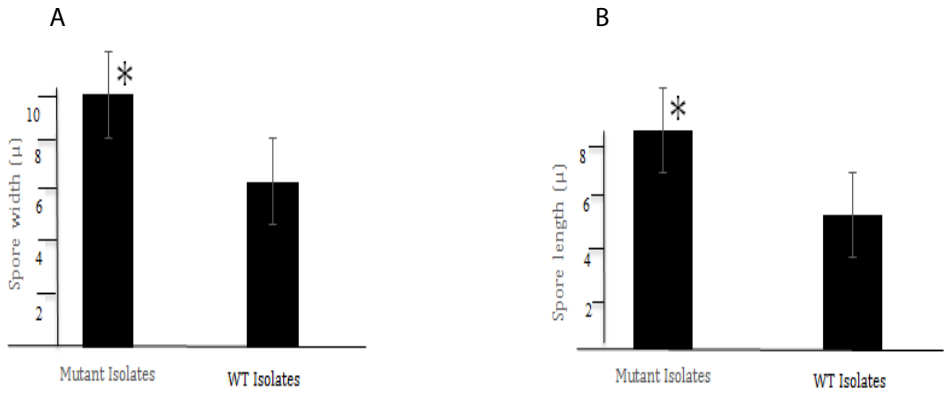

Figure 1: Graphical representation of mutant versus wild type isolates.
Legend: (A) refers to the conidia width of mutant and non-mutant isolates, (B) refers to the conidia length of mutant and non-mutant isolates. The star denotes the average mutant, which is statistically significant when compared to non-mutant. The p values were <0.05, 9.33×10^{-6} for length and 5.33×10^{-6} for width. Allowing for the rejection of the null hypothesis, therefore the mutant conidia sizes are larger than the non-mutants.
The mean conidia size of all isolates displaying wild type characteristics was 4.74 µm × 6.04 µm. For isolates displaying SNPs, the mean conidia size was 5.02 µm × 5.85 µm. And with isolates displaying a 4-nucleotide insertion, the mean conidia size was 6.25 µm × 7.95 µm (Table 2).

maintainable disease control methods [12]. A rapid and convenient method to detect resistant mutations is vital for understanding and managing fungicide resistance [22]. In using genomics to continue the study of mutations in *B. cinerea,* it is likely that the identification of resistance causing mutations can lead to effective disease control methods. Therefore, the objectives of this research were to: i) identify genetic diversity of *Botrytis* isolates obtained from strawberries in California using sequencing analysis, ii) identify morphological and phylogenetic diversity of California's population of *Botrytis* in strawberries, iii) use pathogenicity assay to detect if the diversity has an impact on virulence. This study was undertaken to characterize and identify the different species of *Botrytis* using sequencing analysis and morphology. In addition, pathogenicity studies were undertaken to understand the role of these species in fruit decay [23].

Materials and Methods

Fungal isolate collection

We obtained a total of 79 pure culture isolates from Driscoll's Strawberry Associates, Watsonville, California (Table 1). The isolates were grown on Potato Dextrose Agar (PDA) with 0.01% tetracycline at 27°C for a period of 21 days. Plugs (0.6 cm diameter) of developed hyphae were placed in the center of a PDA plate and the cultures were routinely transferred every 3 week.

Microscopy

Microscopy was done using a phase contrast microscope (M4000-D Swift Instruments Inc, Japan). Measurements were taken at 1000 x magnification using oil immersion. Mycelia used in this study were 21 days old. Mycelia were placed on a slide and stained with methylene blue prior to observation under the microscope. We measured the conidia length and width for all 79 isolates, each with three replicates. Measurements were done using the ocular micrometer after calibrating the microscope with the stage micrometer. Photographs were taken with 8-megapixel iSight camera with 1.5 u pixels.

DNA extraction

21-day-old Mycelium was used to extract DNA from each of the isolates. DNA was extracted from the fungal isolates using PrepMan

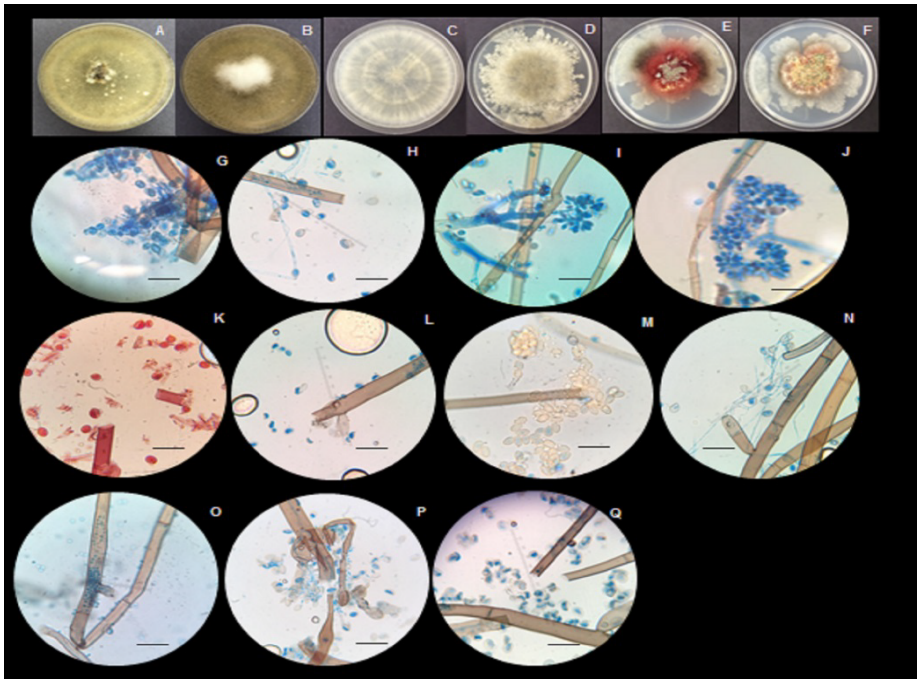

Figure 2: Morphological diversity of *Botrytis*.

Legend: A: Isolate 49 (Possible mutant) hyaline pattern in middle with spores dispersing only in center. Black center with dark yellow mycelium; B: Isolate 68 (mutant) powdery center with light brown mycelium in a circular form; C: Isolate 15 hyaline patter, radiating in circular growth from the center outward, very light yellow and white in color; D: Isolate 61 (mutant) visible growth of branching hyphae, brown in the middle and white/light yellow; E: Isolate 70 same branching patter as isolate 61 with very dark brown and bright red center; F: Isolate 58 similar to 70, but with less growth of mycelium outward, hints of red, green, and yellow; G isolate 30 (4 nucleotide insertion mutant) measurements 7.87 µm × 8.59 µm; H isolate 33 (4 nucleotide insertion mutant) measurements 6.35 µm × 10.96 µm; I isolate 35 (4 nucleotide insertion mutant) measurements 8.71 µm × 9.29 µm; J isolate 39 (4 nucleotide insertion mutant) measurements 7.92 µm × 10.03 µm; K isolate 49 (4 nucleotide insertion mutant) measurements 8.63 µm × 9.71 µm; L isolate 61 (3 SNP's) measurements 5.07 µm × 6.36 µm; M isolate 68 (4 nucleotide insertion mutant) measurements 8.42 µm × 10.75 µm; N isolate 77 (4 nucleotide insertion mutant) measurements 8.62 µm × 9.26 µm; O isolate 71 (wild type) measurements 4.94 µm × 6.2 µm; P isolate 62 (wild type) measurements 5.03 µm × 6.91 µm; Q isolate 44 (4 nucleotide insertion mutant) measurements 5.33 µm × 7.11 µm. All spore images are at 100 x magnification. Scale at 1 µm.

™ Ultra from Applied Biosystems by Life Technologies, Carlsbad, California [24,25]. The DNA was quantified using a nanodrop 2000c UV-Vis Spectrophotometer.

PCR and sequencing

PCR was done using a MJ Mini ™ Personal Thermal Cycler from Bio-Rad. Amplification was done as follows: (1 cycle for 1 min at 95°C, 35 cycles of 45 sec at 94°C, 45 sec at 50°C, 45 sec for extension at 72°C; 1 cycle of final extension for 5 min at 72°C). Primers 729+(5'AGCTCGAGAGAGATCTCTGA3', bases 788,925–788,944) and 729-(5'CTGCAATGTTCTGCGTGGAA3', complement of bases 789,634–789,653), that are specific for *B. cinerea,* were used for amplification [26]. These primers amplify a region on chromosome 13 (bases 788,945–789,633), which is a part of the complete genome sequence of *B. cinerea* B05.10 (http://www.ncbi.nlm.nih.gov/bioproject/PRJNA264284). A single band of 0.7 kb that is specific to *B. cinerea* was amplified in our isolates. We also used a second set of primers: BC108+(5'-ACCCGCACCTAATTCGTCAAC-3', bases 789,015–789,035) and BC563–(5' GGGTCTTCGATACGGGAGAA-3', complement of bases 789,470–789,489). These new primers were used to amplify a DNA fragment of 0.48 kb to 0.36 kb in case there is a possible deletion of 0.12 kb that could not be detected with the primers C729+/– [27]. Upon verification of desired band sizes through gel electrophoresis, the PCR products were purified using the USB®EXOSAP-IT® reagent (USB Corporation, Cleveland Ohio) [28]. The purified PCR products were sequenced at UC Davis sequencing facility. Nucleotide sequences (bases 789,012 to 789,488 of chromosome 13) were analyzed and aligned using Vector NTi software version 8 (Life Technologies, Pleasanton, California).

Sequence analysis

The 476 bases (bases 789,012 to 789,488 of chromosome 13) and 688 bases (bases 788,945 to 789,633 of chromosome 13), which were sequenced and aligned to each other for analysis, are too small for accurate phylogenetic analysis. These gene regions contain a few polymorphic sites that are useful for distinguishing several genotypes. Thus, they are also useful for estimating population diversity on chromosome 13 among the isolates. We used the Highlighter tool to compress the sequence information into a figure that illustrates the diversity (Figure 1). (http://www.hiv.lanl.gov/content/sequence/HIGHLIGHT/HIGHLIGHT_XYPLOT/highlighter.html).

Pathogenicity assay

Isolates were grown on PDA plates at 27°C. Plugs (0.6 cm diameter) of developed hyphae were placed in the center of a PDA plate. The plates were grown for 21 days.

To produce inoculum, the spores were washed from the plates with 4 ml KH_2PO_4 glucose solution, filtered with 50 l m-mesh sieves, and adjusted to a concentration of 10^7 conidia mL^{-1} using Bright-Line haemocytometer (Cambridge Instruments Inc. USA) [3,29]. Before inoculation, the spore suspensions were vortexed thoroughly to ensure the homogeneous dispersal of conidia in the solution.

Whole *Fragaria ananassa*, the common field strawberry, were

obtained commercially and used for inoculation. Uniform and undamaged strawberries were selected for experimentation. The fruits were surface-sterilized with 2% sodium hypochlorite (NaClO) for a period of 2 min, rinsed with sterile water, and then air dried on a clean bench for approximately 1 h [3]. Once dried, the fruits were injected with the inoculum (approximately 1-2 mm in depth) at the strawberry's equator with a sterile syringe [30]. The fruits were also inoculated with double distilled H_2O as a negative control.

Inoculations were done in egg cartons. The cartons were placed in a cardboard box and incubated at 20°C for 6 days. The egg cartons were randomized and their positions changed within the cardboard box. The fruits were monitored for lesion size every 12 h for a period of 5 days.

Results

Surveys

The host range and geographic distribution of *B.cinerea* in California are summarized in Table 1. There were 79 isolates collected from various locations in California and 63 of those isolates were sequenced. Agricultural crop hosts in this study on which *B. cinerea* were identified included: strawberry (*F. ananassa*) collected from Oxnard, Watsonville, Salinas, and Tule Lake; blackberry (*Rubus fruticosus*) from Watsonville; and raspberry (*Rubus idaeus*) from Oxnard and Watsonville. There were a total of 66 isolates collected from strawberries, 12 isolates collected from raspberries, and 1 from blackberry. *B. cinerea* samples were collected from infected areas of raspberry and strawberry crops. *B. cinerea* was collected from leaf petiole in the blackberry sample.

Morphology-pattern of growth

Various kinds of growth patterns were observed on the PDA agar plates at 27°C under light. Mycelia were produced in numerous patterns that displayed powdery, cottony, and radial characteristics (Figure 2). The colonies were varied, from white, dirty white or grayish in color to reddish, brown and black. Many displayed a hyaline pattern in the middle but as it dispersed it became light gray to dark brown. The pattern of growth was similar to the observations recorded by others [31].

Microscopy

Studies were conducted to measure the length and width of the conidia in the 79 studied isolates. We observed larger conidia sizes in the mutant isolates, identified through genomic studies, using sequencing analysis (Table 2) [32,33]. The larger conidia size was observed only in those mutant lines that had 4 nucleotide insertions (Table 3). However, isolates 04, 16, 59, 61, 63, and 74, which only had single nucleotide polymorphisms (SNPs), showed smaller/similar conidia size compared

		Spore Width (um)					Spore Length (um)			
Isolate number	Replicate 1	Replicate 2	Replicate 3	Mean	Standard Error	Replicate 1	Replicate 2	Replicate 3	Mean	Standard Error
BOT01	5.00	5.10	5.12	5.07	0.037	6.00	5.80	5.99	5.93	0.065
BOT02	5.00	5.20	5.11	5.10	0.058	6.14	6.10	6.20	6.15	0.029
BOT03	5.00	5.00	5.00	5.00	0.00	6.14	6.14	6.26	6.18	0.040
BOT04	4.71	4.70	4.74	4.72	0.012	6.86	6.82	6.65	6.78	0.064
BOT05	5.00	5.16	5.20	5.12	0.061	6.43	6.30	6.50	6.41	0.059
BOT06	4.71	4.69	4.68	4.69	0.0088	6.43	6.21	6.51	6.38	0.090
BOT07	5.00	5.10	5.00	5.03	0.033	5.00	4.97	5.20	5.06	0.072
BOT08	4.71	4.72	4.64	4.69	0.025	6.43	6.22	6.25	6.30	0.066
BOT09	5.00	5.30	5.20	5.17	0.088	6.43	6.43	6.49	6.45	0.020
BOT10	5.00	5.16	5.23	5.13	0.068	7.14	7.15	6.92	7.07	0.075
BOT11	4.71	4.66	4.69	4.69	0.015	5.71	5.66	5.55	5.64	0.047
BOT12	4.43	4.39	4.41	4.41	0.012	6.43	6.22	6.35	6.33	0.061
BOT13	5.00	5.11	5.16	5.09	0.047	6.43	6.70	6.55	6.56	0.078
BOT14	4.28	4.30	4.24	4.27	0.018	6.43	6.20	6.34	6.32	0.067
BOT15	4.43	4.41	4.42	4.42	0.0058	5.86	6.00	5.70	5.85	0.087
BOT16	5.00	4.90	5.20	5.03	0.088	5.00	5.23	5.13	5.12	0.067
BOT17	5.00	5.10	5.00	5.03	0.033	6.43	6.24	6.51	6.39	0.080
BOT18	4.71	4.71	4.83	4.75	0.040	6.14	6.00	6.20	6.11	0.059
BOT19	4.71	4.72	4.63	4.69	0.028	6.43	6.31	6.23	6.32	0.058
BOT20	5.00	5.18	5.20	5.13	0.064	6.43	6.59	6.55	6.52	0.048
BOT21	5.00	5.20	5.26	5.15	0.079	5.00	5.20	5.10	5.10	0.058
BOT22	5.00	5.01	5.14	5.05	0.045	5.00	5.00	5.00	5.00	0.00
BOT23	4.28	4.30	4.28	4.29	0.007	6.43	6.21	6.20	6.28	0.075
BOT24	5.00	5.10	4.90	5.00	0.058	6.13	6.31	6.13	6.19	0.060
BOT25	5.00	5.20	5.12	5.10	0.058	6.14	6.00	6.26	6.13	0.075
BOT26	5.00	5.10	5.10	5.07	0.033	6.00	6.19	6.12	6.10	0.055
BOT27	5.86	5.89	5.83	5.86	0.017	7.43	7.38	7.25	6.35	0.054
BOT28	5.00	5.00	5.00	5.00	0.00	5.90	6.00	5.99	5.96	0.032
BOT29	5.67	5.60	5.40	5.56	0.081	7.86	7.73	7.70	7.76	0.049
BOT30	7.86	7.90	7.86	7.87	0.013	8.57	8.65	8.56	8.59	0.028
BOT31	4.71	4.70	4.90	4.77	0.065	5.00	5.30	5.22	5.17	0.090
BOT32	4.43	4.40	4.23	4.35	0.062	5.28	5.25	5.20	5.24	0.023
BOT33	6.43	6.39	6.25	6.35	0.055	11.0	10.8	11.1	11.0	0.086
BOT34	4.71	4.69	4.88	4.76	0.060	6.14	6.80	6.50	6.48	0.19
BOT35	8.57	8.68	8.88	8.71	0.091	9.28	9.25	9.36	9.30	0.033

BOT36	5.00	5.10	5.00	5.03	0.033	5.71	5.63	5.80	5.71	0.049
BOT37	5.20	5.12	5.00	5.11	0.058	6.14	6.00	6.30	6.15	0.087
BOT38	5.20	5.14	5.16	5.17	0.018	6.43	6.40	6.58	6.47	0.056
BOT39	7.86	7.90	8.00	7.92	0.042	10.0	9.89	10.2	10.0	0.091
BOT40	5.20	5.18	5.00	5.13	0.064	6.57	6.50	6.60	6.56	0.030
BOT41	5.10	5.00	5.20	5.10	0.056	5.71	5.41	5.60	5.57	0.088
BOT42	5.89	5.82	5.86	5.86	0.020	7.14	7.26	7.30	7.23	0.048
BOT43	4.00	4.90	4.89	4.60	0.30	6.43	6.23	6.35	6.34	0.058
BOT44	5.00	5.89	5.10	5.33	0.28	7.14	7.00	7.21	7.12	0.062
BOT45	5.00	4.87	5.00	4.96	0.043	5.96	6.00	5.90	5.95	0.029
BOT46	5.00	4.90	5.10	5.00	0.058	6.43	6.16	6.16	6.25	0.090
BOT47	5.00	5.10	5.30	5.13	0.088	6.23	6.20	6.23	6.22	0.010
BOT48	5.00	5.20	5.20	5.13	0.067	6.00	6.00	6.11	6.04	0.037
BOT49	8.57	8.68	8.64	8.63	0.032	9.28	9.23	9.00	9.17	0.086
BOT50	4.98	5.19	5.19	5.12	0.07	6.43	6.40	6.50	6.44	0.030
BOT51	5.43	5.39	5.58	5.47	0.058	7.00	7.00	7.20	7.07	0.067
BOT52	0.00	0.00	0.00	0.00	0.00	6.76	6.70	6.72	6.73	0.018
BOT53	5.43	5.49	5.50	5.47	0.022	7.86	7.73	7.59	7.73	0.078
BOT54	5.00	5.20	5.00	5.07	0.067	5.42	5.86	5.80	5.69	0.14
BOT55	5.00	5.10	5.30	5.13	0.088	5.00	5.02	5.00	5.01	0.0067
BOT56	5.00	4.90	5.16	5.02	0.076	6.29	6.30	6.30	6.30	0.0033
BOT57	5.00	4.96	5.21	5.06	0.078	6.43	6.40	6.21	6.35	0.069
BOT58	5.00	4.89	5.10	5.00	0.061	6.43	6.12	6.25	6.27	0.090
BOT59	5.00	5.10	5.30	5.13	0.088	5.00	5.00	5.30	5.10	0.10
BOT60	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
BOT61	5.00	5.23	5.00	5.08	0.077	6.43	6.23	6.42	6.36	0.065
BOT62	5.00	5.11	5.00	5.04	0.037	6.86	6.89	7.00	6.92	0.043
BOT63	5.00	5.29	5.14	5.14	0.083	6.43	6.42	6.59	6.48	0.055
BOT64	4.43	5.39	4.58	4.80	0.30	6.00	6.02	6.23	6.08	0.074
BOT65	5.00	4.20	4.90	4.70	0.25	6.43	6.20	6.28	6.30	0.067
BOT66	5.00	4.80	5.00	4.93	0.067	6.23	6.20	6.23	6.22	0.010
BOT67	5.89	5.90	5.90	5.90	0.003	6.89	6.86	6.86	6.87	0.010
BOT68	8.57	8.20	8.50	8.42	0.11	10.7	10.8	10.8	10.8	0.030
BOT69	4.71	4.65	4.60	4.65	0.032	6.43	6.59	6.52	6.51	0.046
BOT70	5.00	4.89	5.10	5.00	0.061	6.14	6.10	6.11	6.11	0.012
BOT71	5.00	4.99	4.85	4.95	0.048	6.14	6.30	6.16	6.20	0.050
BOT72	5.00	5.29	5.39	5.23	0.12	7.86	7.88	7.85	7.86	0.0088
BOT73	4.43	4.49	4.50	4.47	0.022	6.30	6.28	6.24	6.27	0.018
BOT74	5.00	4.96	5.12	5.03	0.048	5.00	5.00	5.00	5.00	0.00
BOT75	4.90	4.97	5.09	4.99	0.055	7.00	6.42	6.42	6.61	0.19
BOT76	5.71	5.65	5.60	5.65	0.032	7.14	7.14	7.19	7.16	0.017
BOT77	8.57	8.60	8.70	8.62	0.039	9.28	9.20	9.30	9.26	0.031
BOT78	5.00	5.00	4.80	4.93	0.067	6.43	6.20	6.35	6.33	0.067
BOT79	5.20	5.20	5.10	5.17	0.033	6.14	6.00	6.10	6.08	0.042

Table 2: Measurements of width and length of conidial spores found in each isolate.

to the non-mutant lines. A t-test of unequal variance on the length and width of the conidia was done to show the significance of the size of the conidia for possible mutant isolates compared to the non-mutant isolates; p-values were <0.05 for length and width of the conidia. Results showed a p–value of (5.33×10^{-6}) for length and (9.33×10^{-6}) for width (Figures 1A and 1B). Thus, the size of mutant conidia with a 4-nucleotide addition was significantly larger than the non-mutant lines; this can be seen in the graph (Figures 3A-3C). Conidia with SNPs did not show significant difference when compared with wild type isolates (Figures 3D and 3E).

PCR amplification

PCR primers amplified a 0.7 kb intergenic sequence unique to *B. cinerea* [26]. A secondary set of primers amplified an internal region of the 720 bp, identified by C729+/- primer sets in order to identify all strains as *B. cinerea*. Because of the specificity of primer C729 +/- [26], we were able to get PCR bands for all our 79 samples. We were also able to obtain sequencing results for 65 samples. However, since it was reported that C729+/- primers were not able to identify some of the *Botrytis* species due to a deletion [27], we also used another set of primers: BC108/563 [26]. This was done in an effort to make sure that all our desired products would be identified correctly. Both sets of primers were also used to for each of the 79 samples to assure accuracy. Using the two sets of primers successfully amplified all of the samples, but it was impossible to sequence all of the samples. A possible explanation for 14 samples not having sequencing information is, a PCR reaction is exponential and therefore amplification can go beyond the linear part of the reaction, meaning if we had inefficient primers it will produce products similar to those of very efficient primers. However, sequencing is linear. Therefore, inefficient primers can produce weak

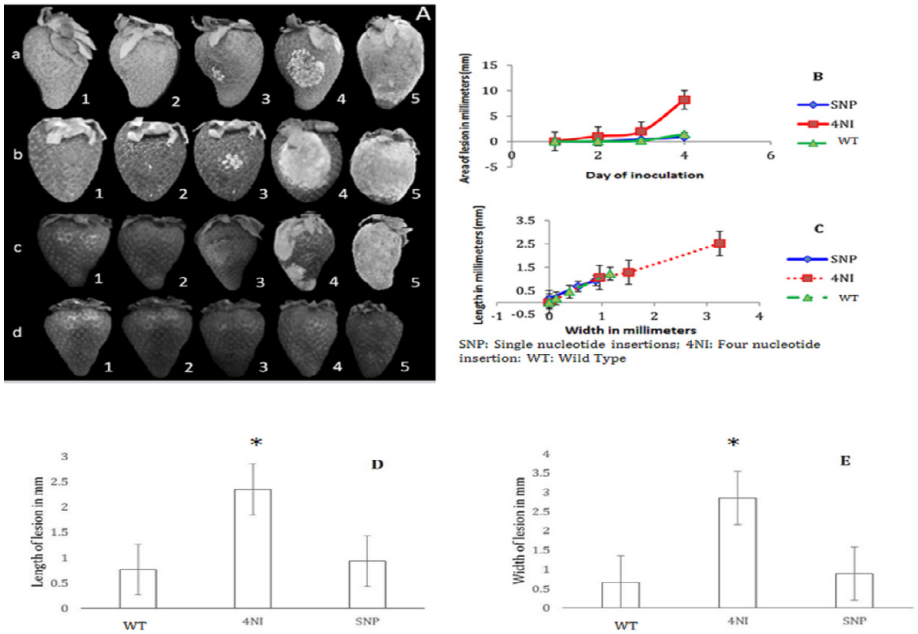

Figure 3: Virulence pattern of wild type and mutant isolates of *B. cinerea*.

Legend: A. Isolate 27 (4 nucleotide insertion) a1. Day 0, a2. Day 01, a3. Day 02, a4. Day 03, a5. Day 04; Isolate 49 (4-nucleotide insertion) b1. Day 0, b2 Day 01, b3. Day 02, b4. Day 03, b5. Day 04; Isolate 61 SNP (3- G → A) c1. Day 0, c2. Day 01, c3. Day 02, c4. Day 03, c5. Day 04; Control (H_2O) d1. Day 0, d2. Day 01, d3. Day 02, d4. Day 03, d5. Day 04; B Lesion growths in millimeters taken over a period of 4 days; C Length/Width growth over time (4 days); D T-test for unequal variance for the length and the width of lesion size. P-value<0.05, so the null hypothesis can be rejected. Rate of growth of mutants is greater than that of non-mutants. P-value for length 0.018, p-value for width 0.0025.

There were statistically significant differences in the virulence between our 15 isolates with the 4-nucleotide insertion than the other isolates without this insertion, as seen through lesion development (Figure 3A). The p-values were <0.05 for length and width of the lesion size, and the growth can be viewed in Figures 3D and 3E. Differences were not statistically significant between the other isolates (Figures 3D and 3E) when compared to the wild type sequence without the 4-base insertion. It was also noticed that the pattern of lesion development was similar to the wild type isolates (Figures 3D and 3E). These lesions were smaller in size than the lesions obtained from the 4-base nucleotide mutants.

or undetectable bands, which might have been the reason for the 14 samples with no sequencing information. Thus, inefficient primers can lead to failed sequencing although the PCR reaction was a success.

Sequence analysis

A total of sixteen (Table 3) of our isolate sequences shared the same 4-base insertion (GGGA at columns 282-285), which was also shared by the *Botrytis* identified from *Gladiolus* sp. of India. The 4-base insertion was seen in twelve of the isolates (27, 29, 30, 33, 35, 42, 44, 49, 51, 53, 68, and 72 when using both the BC108/563 and the C729+/- primers. Three isolates 67, 76, and 77 showed the 4-base insertion only when sequenced using the BC108/563 primers. Isolate 39 showed the 4-base insertion only when sequenced using the C729+/- primer (Table 3) (Figures 4A and 4B). Microscopic studies showed that each of the sixteen mutants shared identical phenotypic changes. The highlighter plot (Figures 4A and 4B) shows that there is as much variability within the population of *Botrytis* sampled in Californian strawberry fields, as there is between isolates collected in other areas of the world. For example, the 4-base insertion GGGA columns 282-285 in our alignment is shared with an isolate found on Gladiolus flowers from India with Gene Bank accession number KP141796 (as yet unpublished). The authors who submitted this isolate have yet to publish their phenotypic findings, thus we have yet to see if the 4 bp insertion corresponds with increased virulence in the Indian isolate of Gladiolus. The majority of our isolates had the same 4 single-base differences from the complete genome sequence (http://www.ncbi.nlm.nih.gov/bioproject/PRJNA264284 chromosome 13) used as the "MASTER" or reference sequence for this figure.

Pathogenicity

To evaluate the virulence of different *B. cinerea*, artificial inoculation experiments with the strawberry (*F. ananassa*) was done. Disease symptoms were always greater after inoculation of mutant isolates with 4 nucleotide insertion compared to the wild type and mutant isolates with SNPs in all our three replicates (Figures 3A, 3B, and 3C).

Surprisingly, individual fruits of the negative controls also showed slight disease symptoms 4 days after inoculation. It is to be noted here that *B. cinerea* (grey mold) appears on the surface of a strawberry fruit, only when the fruiting body of the pathogen is visible. However, it is to be noted that the fungus spreads quickly and can exist in a non-fruiting stage in the fruit (non-visible stage). It is quite possible that the strawberries selected for analysis may have the pathogen in the non-fruiting stage. Therefore, we made sure our control fruits, and the other fruits, went through the same sterilization technique to maintain uniformity. The graph indicated that the infection rate was three times higher than the wild type isolates, and five times higher than the negative control (Figures 3B and 3C).

Discussion

Considering the importance of *B. cinerea* and its significant damage to a wide variety of crops, its control management is necessary. The first step to manage this pathogen is to identify it [27]. Identification has traditionally been dependent on morphological and cultural characteristics coupled with host specificity [34]. Morphological characteristics are often influenced by environmental conditions, which are variable and therefore do not always provide accurate

Isolate number	Cultivar	Type of infection	Isolate origin	Primers C729+/-	Primers BC108+/563-	Type of mutation
BOT01	strawberry	postharvest	Oxnard	(-)	na	
BOT02	strawberry	postharvest	Oxnard	na	na	
BOT03	strawberry	postharvest	Oxnard	na	na	
BOT04	strawberry	postharvest	Oxnard	(+)	na	Single bp change (T→C)
BOT05	strawberry	postharvest	Oxnard	(-)	na	
BOT06	strawberry	postharvest	Oxnard	na	na	
BOT07	strawberry	postharvest	Oxnard	(-)	(-)	
BOT08	strawberry	postharvest	Oxnard	(-)	(-)	
BOT09	strawberry, El Dorado	field	Oxnard	(-)	(-)	
BOT10	strawberry, El Dorado	field	Oxnard	na	na	
BOT11	strawberry, El Dorado	field	Oxnard	(-)	na	
BOT12	strawberry, El Dorado	field	Oxnard	(-)	(-)	
BOT13	strawberry, El Dorado	field	Oxnard	(-)	(-)	
BOT14	strawberry, El Dorado	field	Oxnard	(-)	(-)	
BOT15	strawberry, El Dorado	field	Oxnard	(-)	(-)	
BOT16	strawberry, El Dorado	field	Oxnard	(+)	(+)	Single bp change (G-->A)
BOT17	strawberry, El Dorado	field	Oxnard	(-)	(-)	
BOT18	strawberry, El Dorado	field	Oxnard	(-)	(-)	
BOT19	strawberry, El Dorado	field	Oxnard	(-)	(-)	
BOT20	strawberry, El Dorado	field	Oxnard	(-)	na	
BOT21	strawberry, El Dorado	field	Oxnard	na	na	
BOT22	strawberry, El Dorado	field	Oxnard	(-)	(-)	
BOT23	strawberry, El Dorado	field	Oxnard	(-)	(-)	
BOT24	strawberry, El Dorado	field	Oxnard	(-)	(-)	
BOT25	strawberry, El Dorado	field	Oxnard	na	na	
BOT26	strawberry, El Dorado	field	Oxnard	(-)	(-)	
BOT27	strawberry, El Dorado	field	Oxnard	(+)	(+)	4 nucleotide insertion (GGGA)
BOT28	strawberry, El Dorado	field	Oxnard	(-)	na	
BOT29	strawberry, El Dorado	field	Oxnard	(+)	(+)	4 nucleotide insertion (GGGA)
BOT30	strawberry, El Dorado	field	Oxnard	(+)	(+)	4 nucleotide insertion (GGGA)
BOT31	strawberry, El Dorado	field	Oxnard	(-)	(-)	
BOT32	strawberry, El Dorado	field	Oxnard	(-)	(-)	
BOT33	strawberry, El Dorado	field	Oxnard	(+)	(+)	4 nucleotide insertion (GGGA)
BOT34	strawberry, El Dorado	field	Oxnard	(-)	na	
BOT35	strawberry, El Dorado	field	Oxnard	(+)	(+)	4 nucleotide insertion (GGGA)
BPT36	strawberry, El Dorado	field	Oxnard	(-)	(-)	
BOT37	strawberry, El Dorado	field	Oxnard	(-)	(-)	
BOT38	strawberry, El Dorado	field	Oxnard	(-)	(-)	
BOT39	raspberry, Pacifica	field	Watsonville	(+)	na	4 nucleotide insertion (GGGA)
BOT40	raspberry, Pacifica	field	Watsonville	(-)	(-)	
BOT41	raspberry, Pacifica	field	Watsonville	(-)	(-)	
BOT42	raspberry, Pacifica	field	Watsonville	(+)	(+)	4 nucleotide insertion (GGGA)
BOT43	raspberry, Pacifica	field	Watsonville	na	na	
BOT44	raspberry, Pacifica	field	Watsonville	(+)	(+)	4 nucleotide insertion (GGGA)
BOT45	raspberry, Pacifica	field	Watsonville	(-)	(-)	
BOT46	raspberry, Pacifica	field	Watsonville	(-)	na	
BOT47	raspberry, Pacifica	field	Watsonville	(-)	(-)	
BOT48	raspberry, Pacifica	field	Watsonville	(-)	na	
BOT49	raspberry, Pacifica	field	Watsonville	(+)	(+)	4 nucleotide insertion(GGGA)
BOT50	strawberry	field	Salinas	(-)	(-)	
BOT51	strawberry	field	Watsonville	(+)	(+)	4 nucleotide insertion (GGGA)
BOT52	strawberry	field	Salinas	(-)	(-)	
BOT53	strawberry	field	Salinas	(+)	(+)	4 nucleotide insertion (GGGA)
BOT54	strawberry	field	Salinas	(-)	(-)	
BOT55	strawberry	field	Salinas	(-)	(-)	
BOT56	strawberry	field	Salinas	(-)	(-)	
BOT57	strawberry	field	Watsonville	na	na	
BOT58	strawberry	field	Salinas	na	na	
BOT59	strawberry	field	Salinas	na	(+)	SNP (1) A→G
BOT60	strawberry	field	Salinas	na	na	

BOT61	strawberry	field	Salinas	(-)	(+)	SNP (3) G→A
BOT62	strawberry	field	Salinas	(-)	(-)	
BOT63	strawberry	field	Salinas	(+)	(-)	SNP (2) T→C; A→G
BOT64	strawberry	field	Watsonville	(-)	(-)	
BOT65	strawberry	field	Watsonville	(-)	na	
BOT66	strawberry	field	Watsonville	na	na	
BOT67	strawberry	field	Watsonville	na	(+)	4 nucleotide insertion (GGGA)
BOT68	strawberry	field	Salinas	(+)	(+)	4 nucleotide insertion (GGGA)
BOT69	strawberry	field	Watsonville	na	na	
BOT70	strawberry	field	Watsonville	na	na	
BOT71	strawberry	field	Salinas	(-)	na	
BOT72	strawberry	field	Salinas	(+)	na	4 nucleotide insertion (GGGA)
BOT73	strawberry	field	Watsonville	na	na	
BOT74	strawberry	field	Watsonville	(+)	(-)	SNP (3) C→T; C→T; A→G
BOT75	strawberry	field	Watsonville	na	(-)	
BOT76	strawberry	field	Watsonville	na	(+)	4 nucleotide insertion (GGGA)
BOT77	strawberry	field	Watsonville	na	(+)	4 nucleotide insertion (GGGA)
BOT78	Blackberry		Watsonville	(-)	(-)	
BOT79	Strawberry	Nursery	Tule Lake	na	na	

Table 3: List of all isolates and primers used to determine mutation in sequencing.

Legend: (+) denotes mutation using the specific primer. (-) denotes no mutation or 100% match with NCBI original sequence of *Botrytis cinerea*. The far-right column displays the type of mutation referring to the (+) samples showed according to sequencing. (T → C) means that a base "t" was replaced with "c." (A → G) means that base "a" was replaced with "g." The 4-nucleotide insertion mutation was present with bases (GGGA). SNP refers to the number of single nucleotide polymorphisms present for that isolate.

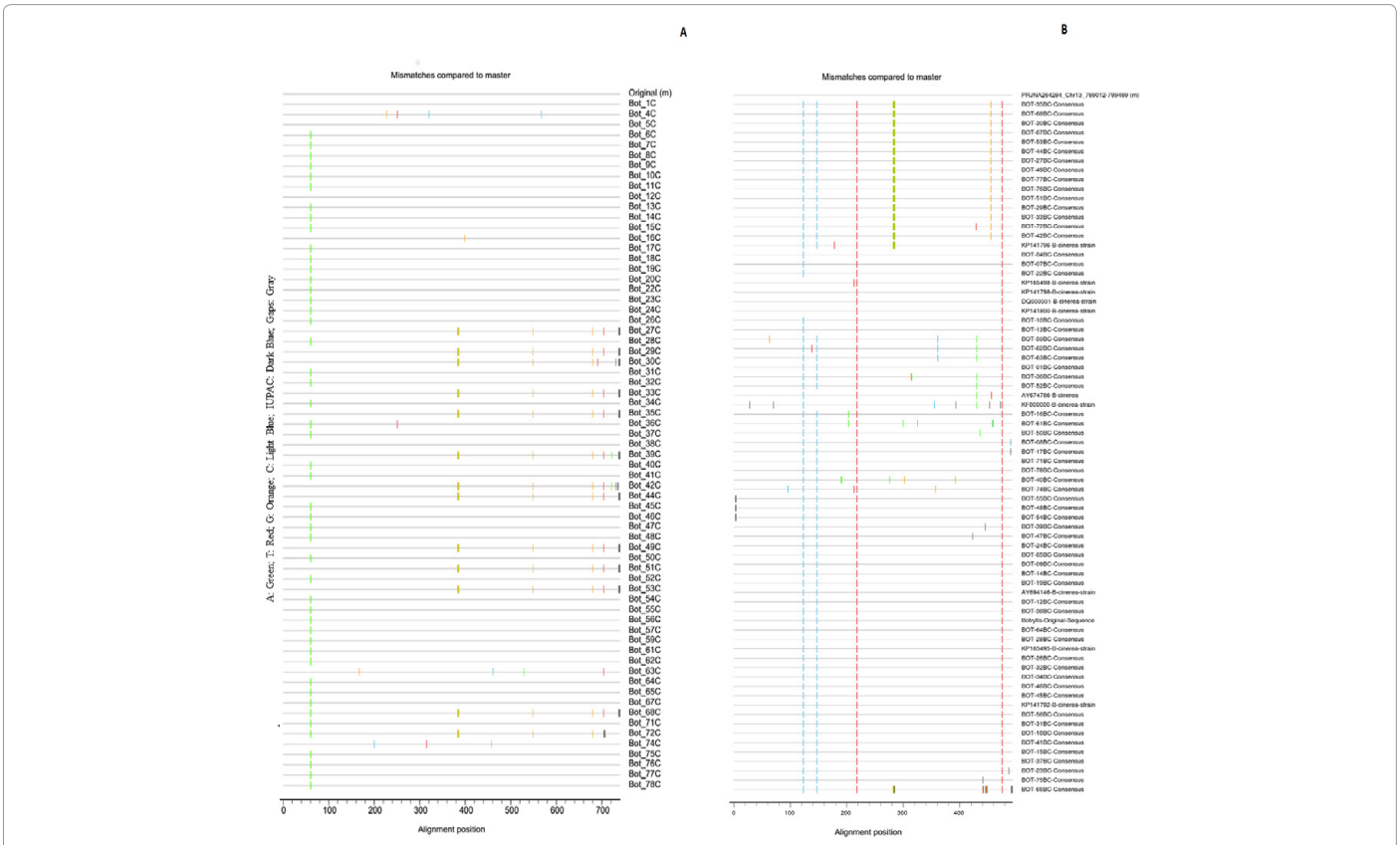

Figure 4: Nucleotide sequence comparison using highlighter plot.

Legend: Highlighter plot of nucleotide differences between the *Botritis cinerea* B05.10 complete genome record PRJNA264284 (http://www.ncbi.nlm.nih.gov/nuccore/CP009817.1) and sequences of the same genomic regions sequenced from isolates in Table 2 and other isolate sequences in GenBank (with accession numbers). Blank space equals identity to the master or reference sequence, while different colored ticks indicate single base changes or polymorphisms (SNPs) in each sequence. A: alignment sequence using primers C729+/-. B: alignment sequence using primers BC108/563.

information [35]. The taxonomy of *Botrytis* species has been used to show how cultural conditions could considerably modify taxonomic characters such as dimension and shape of conidia [35]. Conidia size, form, and colony characters are temperature and media dependent

and are often reversible [27,36]. Therefore, to eliminate variability, the use of molecular markers is a good alternative for identification [32]. Species-specific primers that had been used in *B. cinerea* detection [26] revealed a 4-nucleotide insertion and a single base pair substitution in sixteen isolates. Of the sixteen isolates, the isolates 67, 76, and 77 revealed the 4-nucleotide insertion only when using the BC108/563 primers. This is possibly because of a deletion that could not be detected by the C729+/- primers, as described previously [26]. However, the 4-nucleotide insertion for isolate 39 was only detected using C729+/- primers. This could be because of a failed sequencing reaction. From our experimental findings, we observed that these possible mutated isolates have a larger conidia size when compared to the wild type isolates in our study (Table 2). Virulence, as observed by lesion size on strawberries, was also significantly higher when these isolates were used to inoculate strawberries in the laboratory (Figures 3B and 3C). The question is, does mutation cause a change in conidia size and does this affect virulence? If so, is the mutation responsible for genetic diversity? Does this genetic diversity make epidemiology of the fungus difficult? Mutation is a change in the DNA at a particular locus in an organism. It is the ultimate source of new alleles in plant pathogen populations. It also is the source of new alleles that create new genotypes (such as new pathotypes) within clonal lineages. Small populations have fewer alleles. This is due to genetic drift, and due to fewer mutations generated in small populations. Old populations have more neutral alleles than new populations [16]. Therefore, the center of gene diversity for a species is most often the center of origin for a species. Plants and pathogens have coevolved for the longest time, leading to selection for a diversity of resistance alleles in the plant population [36]. This is why plant breeders seek resistant germplasm at centers of diversity. If the pathogen coevolved with its plant host at the center of origin, we predict that the pathogen population will also exhibit maximum diversity at the center of origin.

Mutation plays an important role in evolution. The ultimate source of all genetic variation is mutation. Mutation is an important first step of evolution because it creates a new DNA sequence for a particular gene, creating a new allele [37]. Recombination can also create a new DNA sequence (a new allele) for a specific gene through intragenic recombination [38]. Mutation acting as an evolutionary force by itself has the potential to cause significant changes in allele frequencies over very long periods of time. But, if mutation were the only force acting on pathogen populations, then evolution would occur at a rate that we could not observe [39,40].

In plant pathology, we are most often concerned with mutations that affect pathogen virulence, sensitivity to fungicides, or sensitivity to antibiotics. In pathogens that show a gene-for-gene interaction with plants, we are especially interested in the mutation from non-virulence to virulence, because this is the mutation that leads to pathogenicity [18,41]. Having that said, mutations from fungicide sensitivity to fungicide resistance are also important in agroecosystems, as are any mutations that affect fitness [42,43].

The marker we sequenced on chromosome 13 is not noted to be closely linked to any pathogenicity genes, so we have no explanation for why strains with one genotype at this locus would be extra pathogenic. The NEP2 pathogenicity gene is on chromosome 2, and the Gluconurodase pathogenicity gene is on chromosome 14.

Why are larger conidia more virulent? In a similar study on a zygomycetes fungus, it was reported that the short or absent isotropic growth period for larger spores, compared to the long phase observed prior to germ tube emergence for smaller spores, could be involved in the differences in virulence [23]. The larger spores are likely poised to undergo rapid invasive hyphal growth compared to the smaller spores. The extended isotropic growth phase of the smaller spores [27,44] results in slow germ tube formation, a block, or a delay in germ tube emergence, and could reduce virulence in the host. The response of macrophages to spores further supported their hypothesis, where larger spores engulfed by macrophages were still able to send germ tubes [45]. In a recent study, it was observed that the mutation of a *Botrytis* gene has altered the size of conidia and altered virulence [44]. Since we were not able to see similar results in our wild type isolates, we can conclude that the mutation can be considered as a possible reason for larger conidia size and virulence. It has also been reported that the larger conidia are usually fungicide resistant, have a greater life span, and are more virulent in *Botrytis* [46]. Since there is currently no efficient alternative to control *Botrytis*, the fungal pathogen is recognized by the Fungicide Resistance Action Committee (FRAC) as a pathogen at high risk of fungicide resistance development. The risk of resistance is also due to numerous characteristics including: large population size, long distance dissemination of conidia by air currents, high genetic variability, the ability to reproduce sexually and asexually, abundant sporulation, polycyclic disease cycle, and wide host range [29]. The development of resistance threatens high yields and crop quality. Thus, it is a serious issue for growers, scientists, and manufacturers. It not only reduces growers' and manufacturers' income, but it also has consequences for the environment and human health [46]. Therefore, using a genomic approach to identify diversity [47] within the fungal species, then connecting that diversity to morphological variation and virulence, will provide the initial knowledge needed to better understand the pathogen. This initial knowledge on the pathogenicity factors is essential for fungal infections and is very important because it gives researchers targets in the fight against this pathogen [48].

The isolates with larger conidia had an identical 4-nucleotide insertion, and were observed both in strawberries and raspberries (Tables 2 and 3). Based on the available information of fungicide resistance in *B. cinerea*, we can possibly assume that the mutation was a result of fungicide resistance [17]. There is also evidence for long-distance migration of mutant strains of pathogens [17].

Conclusion

Our findings have provided valuable genotypic and phenotypic information on the *B. cinerea* field isolates. We were able to provide information on the pathogenicity of these field isolates subjected to several fungicides not known in a laboratory setting. It is well known that application of fungicides can result in mutations in a pathogen and therefore contribute to resistance. The genotypic results obtained in our study can therefore be used for obtaining basic knowledge on fungicide resistance of this pathogen, and can also indicate appropriate resistance management strategies to ensure continued effectiveness for *Botrytis* control. In addition, we have identified a genetic marker for detection of *Botrytis* across a wide variety of plant species.

Sequence Submission

Sequence data from this article have been deposited with the NCBI gene bank under accession numbers: KU145342-KU145392, KX772771-KX772776, KX781161-KX781167.

Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Author Contribution

J.RE. was responsible for sequence analysis, submission of sequences

to NCBI gene bank, PCR, and manuscript preparation and submission. B.F. created the highlighter plot in Figure 1 and with bioinformatics software identified homology of the pathogen sequence in other plant species. S.P. performed microscopy analysis on all isolates used in this study. J.T. created Figure 3A and assisted with sequence analysis and inoculation. P.R.F. created Figures 3B, 3C and 3D, designed and conducted inoculation experiments. C.F. assisted with DNA extraction and PCR. K.S. edited and organized the manuscript and figures for manuscript submission. M.R.C. supervised the research project, wrote the first draft of the manuscript, designed experiments, and assisted with sequence analysis and submission.

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