

Identification and Prevalence of Seedborne *Botrytis* spp. in Dry Pea, Lentil, and Chickpea in Montana

Swarnalatha Moparthy,^{1,2} Lipi P. Parikh,¹ Erin E. Gunnink Troth,¹ and Mary E. Burrows^{1,†}

¹ Department of Plant Sciences & Plant Pathology, Montana State University, Bozeman, MT 59717

² Department of Entomology and Plant Pathology, North Carolina State University, Raleigh, NC 27695-7613

Abstract

Botrytis spp. cause gray mold and are significant pathogens of pulse crops (dry pea, lentil, and chickpea). Seedling infection can result in plant stunting and death. In this study, 100 *Botrytis* isolates were recovered from pulse crop seed samples that were submitted to the Regional Pulse Crop Diagnostic Laboratory at Montana State University. Nine *Botrytis* spp. were found to be associated with pulse seeds in Montana based on a combination of cultural characteristics; the amplification of partial sequences of the *G3PDH*, *HSP60*, and *RPB2* genes; and phylogenetic analysis. *Botrytis cinerea* ($n = 54$) was the predominant species, followed by *B. euroamericana* ($n = 22$) and *B. prunorum* ($n = 11$). There were a few isolates of *B. mali* and five novel *Botrytis* spp. that included one cryptic species. To determine the pathogenicity and aggressiveness of the isolates, dry pea cultivar Lifter, lentil cultivar Richlea, and chickpea

cultivar Sierra, detached leaves were inoculated using mycelial plugs. Lesion diameter produced by *Botrytis* isolates on three hosts differed ($P < 0.05$). Aggressiveness of *B. cinerea* was high in all three hosts and varied among the tested isolates. Spore inoculations were conducted on greenhouse-grown dry pea, lentil and chickpea plants using one sporulating isolate each of *B. cinerea*, *B. prunorum*, and *Botrytis* sp. 1. Results indicated that these isolates were pathogenic on the tested hosts. This study illustrates that many species of *Botrytis* are associated with pulse crop seed in Montana and can be aggressive on multiple crops, which may have implications for disease management.

Keywords: *Botrytis cinerea*, *Botrytis euroamericana*, *Botrytis mali*, *Botrytis prunorum*, chickpea, dry pea, lentil, pulses, seedborne

Pulse seeds (dry pea, lentil, and chickpea) offer a significant source of protein and fiber for human consumption and animal feed. Acreage planted to pulse crops is on the increase in the United States. Montana is the leading producer of pulses in the United States. In 2020, 475,000 acres of pea (*Pisum sativum* L.), 360,000 acres of lentil (*Lens culinaris* Medikus), and 110,000 acres of chickpea (*Cicer arietinum*, L.) valued at \$130 million were harvested in Montana (USDA-NASS 2021).

For successful pulse crop production, planting high-quality seeds free of pathogens with a high germination rate is essential for crop yields and competitiveness against weeds. Seedborne diseases are important in the origination of within-field and long-distance spread of disease in pulse crop production (Dolas et al. 2018; Gullino et al. 2014; Nishikawa et al. 2006; Tivoli and Banniza 2007). Seedborne fungal pathogens may also lead to seed rot and a reduction in germination capacity (Kumar et al. 2020). Seedborne diseases are challenging to detect because seeds do not show symptoms of infection. Infected pulse seeds transport pathogens from one growing season to another and introduce pathogens to new geographic regions (Kumar et al. 2020).

In Montana, *Botrytis* spp. were the dominant fungal pathogens associated with chickpea seed in 2020 (E. Gunnink-Troth, unpublished data). Previous research has shown that *Botrytis cinerea* and *B. fabae* are the predominant pathogens associated with lentil seed (Lindbeck et al. 2009). *Botrytis* spp. persist on the infected plant debris and seed in the form of mycelia, conidia, or sclerotia

(Davidson et al. 2007). In leguminous crops, seedborne infections can manifest as seedling rot and stunting of the seedlings, and may eventually result in the death of the seedlings (Davidson et al. 2007). *B. cinerea* Pers. can survive up to 5 years in chickpea seed and up to 15 months on infested chickpea stubble (Davidson et al. 2007; Salam et al. 2011). Studies have shown that *B. cinerea* can be transmitted from the infected seed to seedlings even though symptoms are not evident in the seed (Barnes and Shaw 2003; Burgess et al. 1997; Legard et al. 2000; Malone and Musket 1964; Morrall 1997; Mukherjee and Mercer 1994; Sowley et al. 2010).

Phylogenetic analysis of glyceraldehyde 3-phosphate dehydrogenase (*G3PDH*), heat-shock protein 60 (*HSP60*), and DNA-dependent RNA polymerase II (*RPB2*) genes has delineated various *Botrytis* spp. (Staats et al. 2005). Genetic diversity in *Botrytis* spp. can be studied by the evaluation of transposable elements (TEs) in a given population (Muñoz et al. 2002). Fungal isolates were divided into four groups based on the TEs they carry: *boty* isolates carry only *boty* TE, *flipper* isolates carry only *flipper* TE, *transposa* contain both *boty* and *flipper*, and *vacuma* lack both *boty* and *flipper* (Diolez et al. 1995; Giraud et al. 1997; Levis et al. 1997).

A molecular marker was developed based on the variation in *HhaI* restriction enzyme digestion patterns on a PCR-amplified *Bc-hch* gene, and *B. cinerea* was divided into group I and group II phylogenetic cryptic species (Fournier et al. 2005). Several *Botrytis* spp., including *B. pseudocinerea*, *B. californica*, *B. sinoviticola*, and *B. medusae*, belong to group I (Harper et al. 2019; Saito et al. 2016; Zhang et al. 2018; Zhou et al. 2014), whereas group II is considered as *B. cinerea* sensu stricto (Fournier et al. 2005).

In this study, 100 *Botrytis* isolates were collected from pulse seeds that were submitted to the Regional Pulse Crop Diagnostic Laboratory (RPCDL) between 2016 and 2020. Based on colony morphology and color on potato dextrose agar (PDA) (Alpha Biosciences Inc., Baltimore, MD, U.S.A.), *Botrytis* isolates from the collection comprised several species, the majority of which showed in vitro characteristics similar to *B. cinerea*. However, some of the isolates were morphologically distinct. This study aimed to (i) identify the *Botrytis* spp. associated with pulse seed in Montana using morphological, molecular, and phylogenetic techniques; (ii) determine the phylogenetic relationships of the identified *Botrytis* spp.;

[†]Corresponding author: M. E. Burrows; mburrows@montana.edu

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and (iii) validate the pathogenicity of the isolated *Botrytis* spp. on pulse crops.

Materials and Methods

Isolation of *Botrytis* spp. from pulse seed

From 2016 to 2020, cultures with *Botrytis* morphology were recovered from pulse seed samples submitted to the RPCDL at Montana State University for testing (International Rules for Seed Testing 2019). Seed lots (chickpea $n = 500$, dry pea and lentil $n = 400$) were disinfested by soaking in 1% sodium hypochlorite for 10 min, drained, and air dried before plating 10 seeds per PDA plate (Alpha Biosciences Inc.). The plates were incubated at 20°C for 7 days under cool white fluorescent light (12 h of light followed by 12 h of darkness). The plates were observed for fungal growth, and representative isolates with *Botrytis* morphology were hyphal-tipped twice on quarter-strength PDA plates to generate a pure culture. Pure cultures were stored in 15% glycerol at -80°C as mycelial plugs for long-term use; for short-term use, the mycelial plugs were placed in sterile distilled water and stored at 4°C. The collection source of the isolates used in this study is listed in Supplementary Table S1.

Cultural and morphological characteristics of *Botrytis* spp.

An agar plug from a stored culture (5 mm in diameter) was placed in the center of a 9-cm-diameter Petri dish containing PDA for cultural characterization. Three replicates were used for each isolate, and the plates were incubated at 20°C for 7 days in a daily cycle of 12 h of darkness and 12 h of light. Isolates were classified visually as conidial producers and nonconidial producers.

To promote conidial production ability in vitro (for the cultures that did not produce conidia on PDA), agar plugs containing actively growing mycelium were placed on each of the following: malt extract agar (Alpha Biosciences Inc.), pea agar containing 16 g of ground peameal and 25 g of agar powder (Alpha Biosciences Inc.) per liter, and hay agar. Hay agar was composed of 50% *Poa annua* (dry leaves at 25 g/liter) and 50% of *Trifolium pratense* (dry leaves at 25 g/liter). An infusion was prepared from the dry hay leaves by autoclaving at 121°C for 30 min, then filtered through paper (Whatman filter paper; Fisher Scientific, Hampton, NH, U.S.A.), and filled to 1 liter with water. Dipotassium phosphate (K_2HPO_4) (2 g/liter) (Fisher Scientific) agar (15 g/liter) was added, pH was adjusted to 6.2, and the infusion was autoclaved. The plates were incubated at 20°C for 7 days at 12-h intervals of fluorescent light and darkness.

For microscopic studies, sporulating structures from either the PDA plate or from the diseased plant tissue were mounted on a slide with distilled water. Observations were made with a Nikon Eclipse E400 (Nikon Inc., Melville, NY, U.S.A.) compound microscope with a Nikon OS-Fi1 camera (Nikon Inc.). Length and width of 25 macroconidia and 15 macroconidiophores were measured from each isolate. Measurements of conidia, conidiophores, and sclerotia ($n = 20$) were performed using Image J software (<https://imagej.nih.gov/ij/>).

Molecular characterization of *Botrytis* isolates

DNA extraction, PCR conditions, and primers used. Nucleic acids were extracted from isolates by scraping 100 mg of mycelia from a 7-day-old culture into 200 μ l of Prep Man Ultra Sample reagent (Applied Biosystems, Foster City, CA, U.S.A.) in 2-ml screw cap tubes following the manufacturer's protocol, except that 20 to 30 silica beads (400 to 600 μ m) were added to aid homogenization in a Genogrinder (Spex SamplePrep 2010; Thermo Fisher Scientific, Waltham, MA, U.S.A.). Extracted DNA was quantified using a Nanodrop 2000c Spectrophotometer (Thermo Fisher Scientific) and stored at -20°C until further use.

Genomic DNA concentration was adjusted to a 50 ng/ μ l and all *Botrytis* isolates were subjected to molecular identification using the *B. cinerea*-specific primer pair C729+/729- (Rigotti et al. 2002). To detect the cryptic species from *B. cinerea* sensu stricto, the *Bc-hch* gene was amplified from all of the isolates using the primer pair 262/520L (Fournier et al. 2003). The amplicons were purified by the

ethanol precipitation method described by Sambrook and Russel (2000), except that sodium acetate or ammonium acetate solution was not added. Purified products were digested with *Hha*I (Thermo Fisher Scientific) following the manufacturer's protocol. PCR assays were conducted in a thermal cycler (Bio-Rad Laboratories, Inc., Hercules, CA, U.S.A.). Primer pairs boty-SF/boty-SR and F300/F1500 were used to detect the TEs *boty* and *flipper*, respectively (Giraud et al. 1997; Kumari et al. 2014). To determine the mating type, primer pairs BcMAT1-F/BcMAT1-R and BcMAT2-F/BcMAT2-R (Qiao and Li 2015) were used, following the PCR conditions of initial denaturation for 5 min at 94°C; 30 cycles of denaturation for 1 min at 94°C, annealing for 45s at 55°C, and extension for 1.5 min at 72°C; and a final extension at 72°C for 3 min. PCR was conducted on all of the isolates using the primer pairs as described by Staats et al. (2005) to amplify partial sequences of *G3PDH*, *HSP60*, and *RPB2* genes. For the novel species, partial sequences of necrosis, ethylene-inducing protein genes *NEP1* and *NEP2* were amplified using the primer pairs as described by (Staats et al. 2007; Walker 2016).

All PCR amplifications were carried out in a 25- μ l reaction comprising 12.5 μ l of Dream Taq Green PCR Master Mix (Thermo Fisher Scientific), 0.5 μ l each of 10 μ M forward and reverse primers, 9.5 μ l of nuclease-free water, and 2 μ l of DNA template. The thermal cycling conditions were followed as described by Staats et al. (2005): 94°C for 5 min (1 cycle); denaturation at 94°C for 30 s, annealing at 64°C (for *G3PDH*) or 55°C (for *HSP60* and *RPB2*) for 30 s, and 72°C extension for 1.5 min (35 cycles); and a final extension at 72°C for 10 min. The PCR products were electrophoresed in 2% agarose prestained with SYBR Safe in 1 $\times$ Tris-borate EDTA buffer and sized with a DNA Ladder Mix (Invitrogen, Thermo Fisher Scientific). The DNA bands were visualized under UV light in a Gel Doc XR+ Imaging System 1 (Bio-Rad Laboratories, Inc.).

Sequencing and phylogenetic analysis. The PCR products were purified as described above and sequenced with primer pairs used for their amplification in both directions at the Molecular Cloning Laboratories (MCLAB, San Francisco, CA, U.S.A.) or ELIM biopharmaceuticals (Hayward, CA, U.S.A.). Assembly of DNA sequences for each gene was carried out using Sequencher 3.0 (Applied Biosystems).

For the phylogenetic analysis of the *Botrytis* isolates, gene sequences *G3PDH*, *HSP60*, and *RPB2* from this study were aligned with newly described *Botrytis* spp. sequences up until May 2022 and sequences of Garfinkel et al. (2017), using *Sclerotinia sclerotiorum* and *Monilinia fructigena* as an outgroup taxon. Multiple sequence alignments for the gene sequences were performed using ClustalW with default parameters in BioEdit software 7.0. The sequences were concatenated using the sequence matrix software. Maximum-parsimony (MP) analysis was performed in PAUP 4.0a 166 (Swofford 1998) with the heuristic search option using the tree bisection reconnection algorithm with 100 random sequence additions to find the global optimum tree. All sites were treated as unordered and unweighted, with gaps treated as missing data. The strength of the internal branches of the resulting trees was tested with bootstrap analysis using 1,000 replications with the stepwise addition option set as simple (Felsenstein 1985). For the analysis of *NEP1* and *NEP2* genes, sequences from this study were aligned with other *Botrytis* sequences of Garfinkel et al. (2019), including the sequences of *Sclerotinia sclerotiorum* and *Monilinia fructigena* as outgroup taxa; alignment of the sequences and tree construction parameters were the same for *G3PDH*, *HSP60*, and *RPB2* genes.

Detached-leaf pathogenicity tests. *Botrytis* isolates were grown on PDA for 48 h at 20°C with a daily cycle of 12 h of light and 12 h of darkness. The isolates were tested for their ability to cause lesions on leaves of the chickpea cultivar Sierra the lentil cultivar Richlea and the dry pea cultivar Lifter from 3-week-old greenhouse-grown plants. Each plant species was tested in a separate experiment. Plugs (5 mm in diameter) were excised from the edge of 48-h-old, actively growing cultures and placed mycelium side down onto the adaxial side of surface-sterilized foliage ($n = 5$). A PDA plug without mycelium was used as a control. The inoculated and control leaves

were placed in plastic containers (15 leaves per container) lined with sterilized, moist filter paper and incubated at 20°C for 3 days in a growth chamber. *B. euroamericana* isolate BP19 (Moparthi et al. 2020) was used as a pathogenic standard. The experiment was laid out in a completely randomized design and performed twice. At 3 days postinoculation, the plugs were removed, lesions were measured in two perpendicular directions, and the average was recorded. The leaves that were inoculated with *B. euroamericana* and other novel species that did not sporulate on PDA were further incubated for 7 to 10 days to promote sporulation on the host tissue. To complete Koch's postulates, *Botrytis* was reisolated from the lesions of the detached leaves and confirmed using morphological features and cultural characteristics.

Whole-plant pathogenicity studies. Greenhouse trials were conducted to determine the level of aggressiveness of *Botrytis* spp. in vivo.

Plant material preparation. Plastic cone-tainers (3.8 cm in diameter by 21 cm in depth; Ray Leach Cone-tainer; Stuewe & Sons Inc., Tangent, OR, U.S.A.) were presoaked in 10% sodium hypochlorite and rinsed thoroughly with tap water. The cone-tainers were filled with a 50:50 mix of Plant Growth Center soil mix and Sunshine mix number 1 (Sun-Gro Horticulture, Agawam, MA, U.S.A.). Sunshine mix consisted of Canadian sphagnum peat moss, perlite, dolomitic limestone, and wetting agent. Plant Growth Center soil mix consisted of equal parts by volume of loam soil, washed concrete sand, and Canadian Sphagnum peat moss. AquaGro 2000 G (Aquatrols Corporation of America, Paulsboro, NJ, U.S.A.) wetting agent is blended in at 0.45 kg/1 cubic meter (1 lb./yard³) of soil mix. The mixture is aerated and steam pasteurized at 70°C for 60 min. A seed was placed on the surface of the soil mix and covered with 2.5 cm of soil. Inoculations were carried out on 14-day-old seedlings; each isolate (one representative isolate for each species) was tested against three cultivars (most commonly grown cultivars of Montana) of each pulse crop cultivar. For dry pea, Lifter, DS Admiral, and Ginny green cultivars were chosen. For lentil, Richlea CDC Impress, and Avondale cultivars were chosen. For chickpea, Orion, Sawyer, and Sierra cultivars were chosen. The experiment was laid out as a randomized complete block design with eight replicates in each trial (a cone-tainer with a single plant was treated as the experimental unit) and the experiment was repeated once. An additional eight plants for each cultivar were included in each trial to serve as control plants.

Preparation of inoculum. One representative isolate each from *B. cinerea* (C34), *B. prunorum* (SM20), and *Botrytis* sp. 1 (isolate BG88a) was selected to conduct pathogenicity tests on the whole plant. These isolates were highly aggressive in the detached-leaf assay. Mycelial plugs (5 mm in diameter) were inoculated on a PDA plate and incubated at room temperature until conidiation (7 to 14 days). The plates were flooded with 10 ml of sterile distilled water containing 0.01% Tween 20, and washings were filtered through multilayered, autoclaved cheesecloth. The tubes were vortexed for 30 s and the concentration of each isolate was estimated using a hemocytometer; the concentration was then adjusted to 1×10^5 conidia ml⁻¹.

Inoculation. Inoculum from each isolate was transferred to 500-ml plastic soda bottles (one bottle for each isolate) and attached to a spray chamber (DeVris Manufacturing Inc., Hollandale, MN, U.S.A.) nozzle. Seedlings were placed on a rack holder and placed on the platform of a single-nozzle track spray chamber. The sprayer speed was adjusted to 1.3 mph, 2 feet of length, and 40 psi. Seedlings were sprayed to the point of complete coverage. Seedlings were transferred to a greenhouse bench, allowed to air dry for few minutes, and kept in a moist chamber (an enclosed area that was built with plastic sheets and pipes on a greenhouse bench). One day after inoculation, each plant was covered with a transparent plastic tube to prevent cross-contamination by the sporulating hosts. A mist generator was placed inside the moist chamber to maintain 90% humidity. Plants were watered as needed and maintained in the moist chamber at 22 and 18°C during the day and night, respectively, with cycles of 16 h of light and 8 h of darkness without any nutritional supplements. Disease severity was determined

after 10 days postinoculation. Percent disease was calculated on each plant using a visual assessment method modified from Horsfall and Barrett (1945), where 0% = no disease symptoms, 20% = lower leaves/leaflets showing necrosis, 40% = lower leaves showing necrosis along with growing tips showing water-soaked symptoms, 60% = majority of the leaves or leaflets showing necrosis, 80% = all leaves with stem showing necrosis symptoms, and 100% = death of seedling (Supplementary Fig. S1). *Botrytis* isolates were reisolated from the leaf lesions (one plant from each treatment) and the identity was confirmed using morphological features and cultural characteristics.

Data analysis. Analysis of variance (ANOVA) on disease severity on detached-leaf assays and pathogenicity tests on whole plants was performed using the 'car' and 'agricolae' packages in R version 3.6. Data from both trials were log transformed to satisfy the assumptions of normality and analyzed with ordinary ANOVA to determine severity level differences among isolates and species. Data were analyzed separately for each of the tested crops.

Results

Prevalence of *Botrytis* spp. associated with pulse seed

In total, 100 *Botrytis* cultures were recovered (a single colony from each plate was considered as an isolate) from pulse seeds in a 5-year span. The highest numbers of isolates were recovered from chickpea (46 isolates) followed by lentil (36 isolates) and dry pea (18 isolates). *B. cinerea* was the predominant species throughout the years, followed by *B. euroamericana*. *B. prunorum* was the third most frequent species and the remaining species were detected less frequently (Supplementary Table S1). From the phylogenetic analysis, the isolates were grouped to different species. Isolates BG88a and BG89 are hereafter referred to as *Botrytis* sp. 1; isolates BG33, BG45, and BG 80 as *Botrytis* sp. 2; isolates BC28 and BC29 as *Botrytis* sp. 3; isolates BG13, BC31, and BC34 as *Botrytis* sp. 4; and isolate BC33 as *Botrytis* sp. 5. More details on the prevalence of various *Botrytis* spp. from pulse seeds, their host association, and their genetic diversity can be seen in Supplementary Table S1.

Morphological characteristics of *Botrytis* spp.

In the initial days of growth on PDA, all species were morphologically indistinguishable. However, after 10 days, the isolate's morphology differed among the nine species. *B. cinerea* isolates ($n = 54$) were distinct from the other species based on colony morphology on PDA by forming conidia and sclerotia (Fig. 1). The majority of the *B. cinerea* isolates were conidial type (72%), followed by sclerotial type (25%), and very few isolates were mycelial type (3%). Macroconidia were hyaline, single celled, oval to ellipsoidal in shape, and produced in a botryose manner on the conidiophores.

The morphology of *B. prunorum*, *B. mali*, *Botrytis* sp. 1, and *Botrytis* sp. 3 were similar to one another on PDA, with off-white color on the observe and wooly hyphal growth (Fig. 1). Isolates did not produce sclerotia during 30 days of incubation at 20°C. Some isolates eventually produced sclerotia-type structures when the nutrients were completely depleted on PDA (i.e., on dry medium). Both isolates of *Botrytis* sp. 1 sporulated on PDA. The majority of *B. prunorum* isolates produced conidia on PDA after 3 weeks of incubation at 20°C; however, a few isolates did not produce conidia on PDA at 20°C (Supplementary Table S1). The macroconidiophores were typical of *Botrytis*, arising singly or in groups, thick-walled, erect, and septate, with alternate to whorled branches.

B. euroamericana colonies were off white in color, with abundant sclerotia formation in concentric circles within 14 days at 20°C. The morphology of *B. euroamericana* and *Botrytis* sp. 5 was similar on PDA, with white fluffy mycelium composed of dark melanized sclerotia. *B. euroamericana*, *Botrytis* sp. 2, *Botrytis* sp. 3, *Botrytis* sp. 4, and *Botrytis* sp. 5 showed no conidiation on PDA, pea meal agar, or hay agar at 20°C. Some isolates of the *B. euroamericana* and novel species sporulated on host tissue. Microscopic measures of conidia and conidiophores for nine *Botrytis* spp. are shown in Table 1.

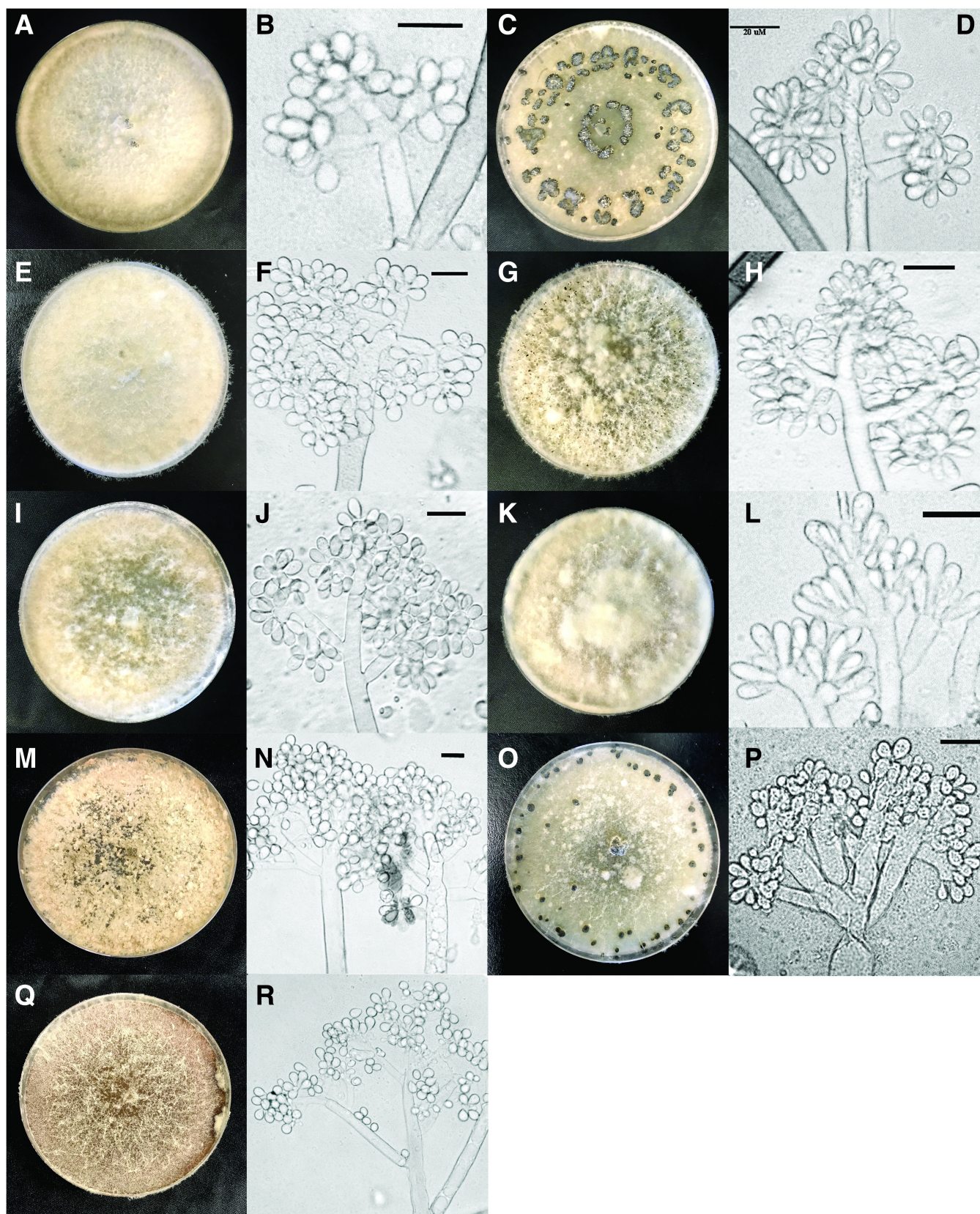


Fig. 1. Morphological characteristics of *Botrytis* spp. in culture. Bar = 20 μ M. **A**, *Botrytis cinerea* on potato dextrose agar (PDA) and **B**, macroconidiophore; **C**, *B. euroamericana* on PDA and **D**, macroconidiophore; **E**, *B. prunorum* on PDA and **F**, macroconidiophore; **G**, *B. mali* on PDA and **H**, macroconidiophore; **I**, *Botrytis* sp. 1 on PDA and **J**, macroconidiophore; **K**, *Botrytis* sp. 2 on PDA and **L**, macroconidiophore; **M**, *Botrytis* sp. 3 on PDA and **N**, macroconidiophore; **O**, *Botrytis* sp. 4 on PDA and **P**, macroconidiophore; and **Q**, *Botrytis* sp. 5 on PDA and **R**, macroconidiophore.

PCR amplifications

PCR amplification was attempted on all of the *Botrytis* isolates using the *B. cinerea*-specific primer pair C729+/729– (Rigotti et al. 2002). All *B. cinerea* isolates were amplified and an amplicon of 750 bp was observed (Supplementary Fig. S2). A few isolates (BG18, BG33, BG37, and BG73) that were not *B. cinerea* were also amplified with these primers (the taxonomic categorization of these isolates can be found in Supplementary Table S1). To detect the cryptic species from *B. cinerea* sensu stricto, the *Bc-hch* gene was amplified from 57 of the 100 isolates using the primer pair 262/520L and an expected amplicon of 1,150 bp was observed (Supplementary Fig. S3). After *Hha*I digestion of the *Bc-hch* amplified fragments, 54 isolates showed a restriction profile consistent with group II haplotype with a band size of 517 bp, indicating that all of these isolates are *B. cinerea* sensu stricto, whereas three isolates of *Botrytis* sp. 4 showed a restriction profile consistent with a group I haplotype with a band size of 600 bp (Supplementary Fig. S4).

TEs of *Botrytis* spp.

All isolates were analyzed to detect the TEs *boty* and *flipper*. The majority of the isolates were *boty* type (46%), followed by *transposa* (27%), *vacuma* (16%), and *flipper* (6%). The frequencies of TEs with respect to *Botrytis* spp. indicated that *boty* type was predominant in *B. cinerea* and *B. prunorum* whereas the *vacuma* type predominated for *B. euroamericana*. The undescribed *Botrytis* spp. varied in terms of the TEs. Detailed information of TEs from various *Botrytis* isolates with respect to their host is shown in Supplementary Table S1.

Mating type distribution

Amplification of the *MAT* locus for mating type determination using specific primer pairs revealed heterothallic isolates. In all, 35 isolates were of *MAT* 1-1 type and 36 isolates were of *MAT* 1-2. In addition, 32 isolates were of *MAT* 1-1 type and 21 isolates were *MAT* 1-2 in *B. cinerea* (amplification was not obtained for 2 isolates). Amplification was not observed for *B. euroamericana* isolates except for BG-48, which was of *MAT* 1-1 type. In the species *B. prunorum*, seven isolates were of *MAT* 1-1 type and four isolates were of *MAT* 1-2 type. All *Botrytis* sp. 2 and *Botrytis* sp. 4 isolates were of *MAT* 1-1 type; amplification was not observed in *Botrytis* sp. 1 (BG-88a and BG89), *Botrytis* sp. 3 (BC28 and BC29) and *Botrytis* sp. 5 (BC33).

Phylogenetic analysis

Sequences were submitted to GenBank and accession numbers were obtained (Supplementary Table S2). Phylogenetic analysis of individual genes showed consistent branching patterns among the *G3PDH*, *HSP60*, and *RPB2* genes and the *NEP1* and *NEP2* genes. Therefore, concatenated sequences were used to further consider relationships among species. GenBank accession numbers of additional sequences, apart from those of Garfinkel et al. (2017), used in the phylogenetic analysis for this study are shown in Supplementary Table S3. MP analyses of *G3PDH*, *HSP60*, and *RPB2* sequence data consisted of 2,961 characters. Among those, 2,145 were constant, 277 were variable but parsimony uninformative, and 539 were parsimony informative. In total, 67,267 equally parsimonious trees with 1,768 steps were constructed. All of the topologies were consistent among the trees, except for branching orders of the terminal branches and lengths. Only the representative MP tree constructed by *G3PDH*, *HSP60*, and *RPB2* data sets is presented (Fig. 2).

Partial sequences of necrosis, ethylene-inducing protein genes *NEP1* and *NEP2* were amplified for novel species only. MP analyses of *NEP1* and *NEP2* sequence data consisted of 1,525 characters, in which 837 were consistent, 189 were variable but parsimony uninformative, and 499 were parsimony informative. In total, 16 equally parsimonious trees with 1,594 steps were constructed. All of the topologies were consistent among the trees, except for branching orders of the terminal branches and lengths. Thus, only the representative MP tree constructed by *NEP1* and *NEP2* data sets is included (Fig. 3). Phylogenetic analysis also confirmed the identities of various *Botrytis* isolates recovered from pulse seed in Montana.

Based on phylogenetic analysis of *G3PDH*, *HSP60*, *RPB2*, *NEP1*, and *NEP2* partial gene sequences, *Botrytis* sp. 2 isolates were closely related to *B. prunorum*, *Botrytis* sp. 3 isolates were closely related to *B. byssoides*, and *Botrytis* sp. 4 isolates were closely related to *B. medusae* and other *B. cinerea* cryptic species *B. pseudocinerea*, *B. californica*, and *B. sinoviticola*. More in-depth studies are underway to determine the taxonomic placement of these novel species.

Pathogenicity tests on detached leaves

A test for homogeneity of variance for the two pathogenicity trials was conducted, and there was no significant difference between the

Table 1. Dimensions of sclerotia, macroconidiophores, and macroconidia formed (either in vitro or in vivo) by various *Botrytis* spp. isolates

Species	Isolate	Dimensions (μM) ^x		
		Sclerotia	Macroconidiophores	Macroconidia
<i>Botrytis cinerea</i> ^y	BG8	NA	1,000–1,519 × 6.5–14.8	9.6–(12.4)–15.8 × 4.8–(6.8)–8.2
	C36	0.8–1.5 × 1.45–2.1	962–1,672 × 7.15–12.52	7.0–(9.1)–12.1 × 5.1–(7.5)–9.7
	C47	0.5–1.8 × 0.6–1.4	1,041–1,556 × 5.5–11.9	6.2–(8.2)–10.5 × 6.1–(7.3)–9.4
<i>B. euroamericana</i> ^z	SM20	1.2–3.48 × 0.6–2.4	510–1,581 × 7.6–13.3	8.8–(13.3)–16.6 × 5.7–(7.5)–9.3
	BE2	0.5–3.22 × 0.7–2.6	388–596 × 4.7–8.3	4.4–(6.0)–7.8 × 2.5–(3.4)–4.1
	BG18a	1.6–3.5 × 0.6–1.59	306–826 × 3.6–9.4	5.8–(5.1)–4.4 × 3.8–(4.2)–4.8
<i>B. prunorum</i> ^y	BG75	NA	475–1,872 × 8.6–19.8	8.3–(12.4)–17.7 × 4.6–(6.3)–8.3
	SM 20	1.3–2.9 × 1.0–2.7	559–1,400 × 6.6–15.5	8.3–(10.59)–12.7 × 6.7–(8.9)–12.0
	BG–18b	NA	601–1,356 × 7.4–10.8	12.1–(14.3)–16.8 × 5.8–(7.3)–9.3
<i>Botrytis</i> sp. 1 ^y	BG88a	0.8–1.4 × 0.6–1.4	384–769 × 7.2–6.6	9.4–(12.5)–17.6 × 6.6–(8.2)–10.9
	BG89	NA	448–1,178 × 5.1–13.03	8.9–(10.7)–12.1 × 5.3–(6.8)–7.7
<i>Botrytis</i> sp. 2 ^z	BC28	NA	494–700 × 9.0–12.9	7.5–(10.3)–12.3 × 4.2–(5.6)–7.1
	BC29	NA	174–439 × 9–21	7.0–(10.5)–15 × 5.0–(5.8)–8.0
<i>Botrytis</i> sp. 3 ^z	BG45	1.1–4.4 × 1.0–2.7	353–1,229 × 7.5–14.7	6.0–(8.9)–13.1 × 3.3–(4.8)–6.0
	BG80	NA	403–1,232 × 9.9–13.4	6.8–(11.5)–14.9 × 4.7–(6.3)–7.6
<i>Botrytis</i> sp. 4 ^z	BG13	0.8–6.0 × 0.75–4.3	NA	NA
	BC31	1.5–3.45 × 1.5–3.1	731–1,291 × 9–23	5.2–(8.8)–12.6 × 3.2–(4.2)–5.6
	BC34	0.8–1.47 × 0.5–0.98	NA	NA
<i>B. mali</i> ^z	BM7	1.4–17 × 0.8–1.6	585–1,362 × 7.4–17	10.1–(12.8)–16.4 × 5.3–(6.9)–10.2
	BM37	1.1–2.5 × 1.1–1.8	460–1,606 × 7.3–16.4	10.8–(13.8)–19.7 × 5.1–(7.4)–9.3
<i>Botrytis</i> sp. 5 ^z	BC33	1.4–2.5 × 1.6–3.48	378–680 × 4.3–6.9	4.7–(6.4)–7.9 × 2.3–(3.3)–4.7

^x NA = no formation of sclerotia and macroconidia.

^y Macroconidia formed in vitro.

^z Macroconidia formed in vivo.



Fig. 2. Maximum-parsimony tree of combined *G3PDH*, *HSP60*, and *RPB2* gene sequences (Staats et al. 2005) describing the phylogenetic relationship between isolates used in this study and voucher sequences of named *Botrytis* spp. obtained from GenBank using *Sclerotinia sclerotiorum* and *Monilinia fructigena* as outgroups.

two ($P > 0.05$); therefore, data from the two trials were combined for analysis. Under the trial conditions, all isolates from nine species were able to cause lesions on the detached leaves of at least one host, except *B. mali* isolates. Pathogenic isolates produced >1-mm lesions within 3 days postinoculation on detached leaves without wounding. The pathogens were successfully reisolated from symptomatic

tissues but not from control leaves. There was a significant difference in the lesion diameter ($P < 0.05$) caused by various *Botrytis* isolates on the tested host. Pathogenic standard isolate *B. euroamericana* isolate BP19 produced lesions on dry pea (10 mm), chickpea (16 mm), and lentil leaves (9 mm). *B. cinerea* and *B. euroamericana* produced larger lesions on dry pea, chickpea and lentil. Both isolates

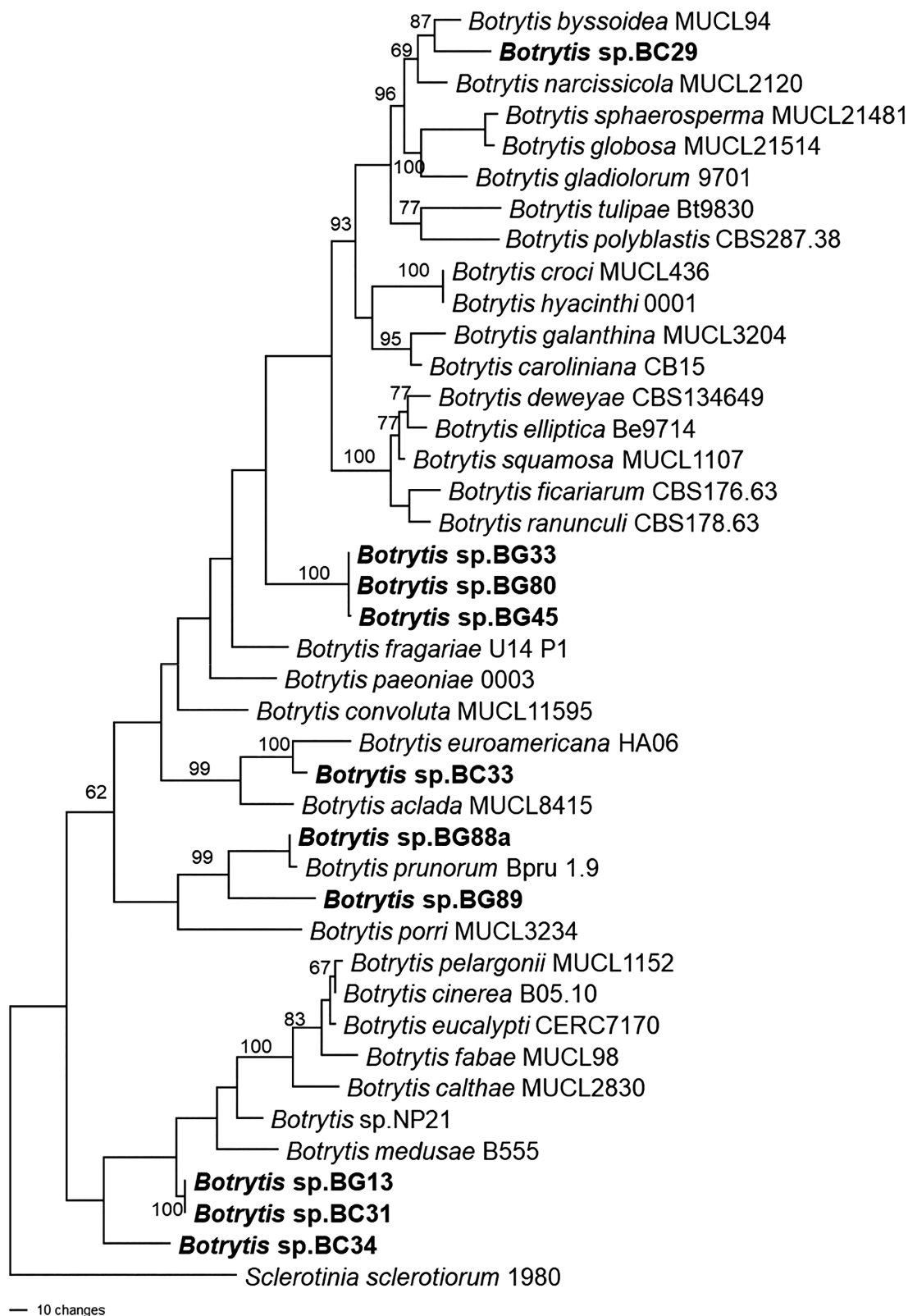


Fig. 3. Maximum-parsimony tree of combined *NEP1* and *NEP2* gene sequences (Staats et al. 2007) describing the phylogenetic relationship between isolates used in this study and voucher sequences of named *Botrytis* spp. obtained from GenBank.

from *Botrytis* sp. 3 were aggressive on all host plants. A majority of *B. prunorum* isolates caused either small lesions or no lesions on the dry pea and chickpea (Supplementary Table S4; Supplementary Fig. S4).

Greenhouse studies

Of the nine species evaluated, only three species (*B. cinerea*, *B. prunorum*, and *Botrytis* sp. 1) sporulated on PDA. One isolate of each species that produced larger lesions (C36, SM20, and BG88a) on the detached leaves was chosen for the greenhouse pathogenicity trials. Five days after inoculation, all of the inoculated plants showed water-soaked lesions on leaves followed by necrosis when the disease progressed, the control plants remained healthy. None of the plants, except two chickpea plants in trial one, showed sporulation 10 days after inoculation. Disease severity was significantly ($P < 0.05$) higher in all of the hosts that were inoculated with *B. cinerea*, followed by *Botrytis* sp. 1. Disease severity was low on all chickpea, dry pea, and lentil cultivars inoculated with *B. prunorum*. Chickpea cultivar Sierra, lentil cultivar Richlea, and dry pea cultivar DS Admiral showed more symptoms with *B. cinerea* and *Botrytis* sp. 1 compared with other two cultivars. Further details on aggressiveness of *Botrytis* spp. on the tested hosts is shown in Table 2 (with transformed data) and Supplementary Table S5 (original values).

Discussion

The current study revealed that nine phylogenetically distinct species of *Botrytis* are associated with three types of pulse seeds from 15 different counties in Montana. Frequency of recovered *Botrytis* spp. was high from chickpea and lentil seed samples compared with dry pea samples. This could be because more chickpea samples are submitted to the RPCDL or due to the relative susceptibility of the host to the pathogen. Species were identified by phylogenetic analysis of *G3PDH*, *HSP60*, and *RPB2* sequences. Cultural differences were noted among the identified species. *Botrytis* spp. diversity on pulse seed included *B. cinerea*, *B. euroamericana*, *B. prunorum*, *B. mali*, and five novel species. *B. euroamericana* was first reported as a pathogen of peony and grape in the United States and Europe (Garfinkel et al. 2017), and it is a newly documented pathogen of chickpea from Montana in 2020 (Moparthi et al. 2020). However, this is a first report on lentil and dry pea in the United States and world as well.

B. prunorum was reported on various hosts such as Japanese plum, kiwi fruit, and grape from Chile (Elfar et al. 2017; Esterio et al. 2020; Ferrada et al. 2016). This species was not reported from pulse crops previously. The remaining species are novel to pulses, including three cryptic isolates resembling *B. cinerea*. It is fascinating to recover diverse *Botrytis* spp. from pulse seeds, and this can be credited to comprehensive phylogenetic analysis and availability of sequences in GenBank by various researchers. Currently, 38 species

are recognized within the *Botrytis* genus and the number of described species is constantly increasing as new species are reported (Garfinkel 2021). The high diversity of *Botrytis* spp. associated with pulse seeds could be due to the cooler climate of the state of Montana. A previous study by Garfinkel et al. (2019) also showed numerous *Botrytis* spp. associated with peony from Alaska.

In the pulse seed, *Botrytis* spp. might be surviving in the form of either conidia or mycelium because no sclerotia were observed on the pulse seed. Further studies are needed to know the actual location of these pathogens in the pulse seeds. Conidiation was observed in some of the old cultures of *B. prunorum* isolates on PDA, whereas other *B. prunorum* isolates showed no conidiation on either PDA or pea agar medium at 20°C. Ferrada et al. (2016) observed the highest conidial production on pea agar medium. Some newly reported *Botrytis* spp. (Garfinkel et al. 2017; Li et al. 2012b; Zhong et al. 2019) lacked conidiation on PDA; some produced conidia on host tissue. It is perplexing why some isolates from the novel species did not produce conidia on host tissue; for instance, only one isolate (BC31) in *Botrytis* sp. 4 sporulated on host tissue. Similarly, BG33 (*Botrytis* sp. 2) did not sporulate on the host tissue even after several trials of inoculations on pulse crops along with other leguminous crops such as faba bean, pole bean, red clover, and alfalfa (data not shown). Cohrs et al. (2016) reported that lack of conidiation is common in field strains and hypothesized that numerous photoreceptive pathways might be responsible for this phenomenon. Park and Yu (2012) also reported that conidiation in filamentous fungi is associated with many factors, including spatial and temporal regulation of gene expression, specialized cellular differentiation, intra- or intercellular communications, and response to environmental factors.

Conidial measurements are important in differentiating the *Botrytis* spp.; however, the size and shape of the conidia among different species in this study are similar and are not useful for species differentiation. Earlier studies also showed that morphology and conidial measurements were identical, and differentiation of the species based on this criterion was not feasible (Walker et al. 2011). In the genus *Botrytis*, prominent morphological diversity exists among the various isolates of the same species, which is commonly referred to as intraspecific variation and makes morphological identification questionable (Garfinkel 2021).

In this study, the overall sampled population showed a 1:1 distribution of mating loci. However, there was no 1:1 ratio within each species and none of the isolates showed both mating genes, indicating that all of the isolates in the study were heterothallic. According to Qiao and Li (2015), mating type ratio can be affected by the number of samples and sampling method. Further studies are required to understand why amplification was not achieved in the case of some novel species (*Botrytis* sp. 1, 3, and 5) and *B. euroamericana*.

B. cinerea-specific primers C729+/729– amplified all isolates of *B. cinerea* in this study, except for one. These primers also amplified

Table 2. Pathogenicity of *Botrytis cinerea* (C36), *B. prunorum* (SM20), and *Botrytis* sp. 1 (BG88a) on 2-week-old host plants^z

<i>Botrytis</i> spp.	Chickpea		Lentil		Dry pea	
	Cultivar	DS (%)	Cultivar	DS (%)	Cultivar	DS (%)
Control	Sierra	0 a	Richlea	0 a	Lifter	0 a
Control	Orion	0 a	CDC Impress	0 a	DS Admiral	0 a
Control	Sawyer	0 a	Avondale	0 a	Ginny green	0 a
<i>Botrytis cinerea</i>	Sierra	1.72 e	Richlea	1.36 e	Lifter	1.03 c
<i>B. cinerea</i>	Orion	1.56 de	CDC Impress	1.17 de	DS Admiral	1.31 d
<i>B. cinerea</i>	Sawyer	1.53 d	Avondale	1.33 e	Ginny green	1.06 cd
<i>B. prunorum</i>	Sierra	0.82 b	Richlea	0.80 bc	Lifter	0.50 b
<i>B. prunorum</i>	Orion	0.99 b	CDC Impress	0.65 b	DS Admiral	0.67 b
<i>B. prunorum</i>	Sawyer	0.93 b	Avondale	0.96 cd	Ginny green	0.57 b
<i>Botrytis</i> sp. 1	Sierra	1.58 de	Richlea	1.23 e	Lifter	0.75 b
<i>Botrytis</i> sp. 1	Orion	1.28 c	CDC Impress	0.87 bc	DS Admiral	1.28 cd
<i>Botrytis</i> sp. 1	Sawyer	0.89 b	Avondale	0.91 c	Ginny green	0.68 b

^z Data were log-transformed and analyzed separately for each host tested. DS (%) = percent disease severity. Means followed by the same letter within each column are not significantly different according to Tukey's honestly significant difference.

two isolates of *B. prunorum*, one isolate of *B. euroamericana*, and a single isolate of *Botrytis* sp. 1, which makes the specificity of the primers questionable. Based on this work, it is not suggested to solely rely on C729+/729– primers to discriminate *B. cinerea* from other *Botrytis* spp., because these primers were developed (Rigotti et al. 2002) before the discovery of *B. euroamericana*, *B. prunorum*, and other novel *Botrytis* spp. The primers that amplify the Bc-*hch* region were found to be reliable in discriminating *B. cinerea* and the cryptic species of *B. cinerea*. Amplification data of the Bc-*hch* region corresponded with the sequence data for *G3PDH*, *HSP60*, and *RPB2* regions in the identification of *B. cinerea*.

Based on PCR-amplified Bc-*hch* gene restriction enzyme polymorphism, we found that the majority of *B. cinerea* isolates in the study were of group II type. Previous studies have also shown that *B. cinerea* group II isolates were dominant on infected plants (Fournier et al. 2005; Isenegger et al. 2008; Karchani-Balma et al. 2008; Váczy et al. 2008); The *vacuma* transposon was the only genotype associated with the group I, whereas all four TEs were detected in group II (Fournier et al. 2005; Isenegger et al. 2008; Karchani-Balma et al. 2008; Ma and Michailides 2005; Martinez et al. 2008; Rajaguru and Shaw 2010; Váczy et al. 2008). However, in the present study, the isolates that belong to the group I haplotype showed *boty* elements, indicating that no correlation exists between transposon type and group-specific haplotypes. The majority of *B. cinerea* populations from green pea were *vacuma* type at the end of the vegetative period (Isenegger et al. 2008). However, in the current study, *boty* isolates were dominant from dry pea seed. The *flipper* type was dominant among the *B. cinerea* populations from chickpea (Kumari et al. 2014), whereas the data from this study shows that *boty* isolates were dominant from chickpea seed. Studies by Ferrada et al. (2016) showed only *boty* elements in *B. prunorum* from Japanese plum; however, in the current study, along with *boty* isolates, *transposa* isolates were also detected. Studies have shown that the frequency and type of TE elements differ not only on the host plant but also with the phenological stage of the host (Giraud et al. 1997; Martinez et al. 2005). In this study, the relationship between TE elements and pathogenicity was observed because the isolates that caused larger lesions on the detached leaves were of *transposa* and *boty* type. Studies conducted by Kumari et al. (2014) showed that *transposa* isolates were highly pathogenic compared with *vacuma* isolates.

Phylogenetic studies play a crucial role in *Botrytis* classification; *G3PDH*, *HSP60*, *RPB2*, *NEP1*, and *NEP2* loci are most favored for the genetic analysis in differentiating the species (Garfinkel 2021). All five novel *Botrytis* spp. in this study were distinguishable from related *Botrytis* spp. through phylogenetic analysis of five partial gene sequences. Analysis of *G3PDH* + *HSP60* + *RPB2* and *NEP1* + *NEP2* partial gene sequences provided clear evidence of novel species because they share unique clades. *Botrytis* sp. 1 (BG88a and BG89) BLAST queries of five genes resulted as *B. prunorum* with the highest identity. Similarly, *Botrytis* sp. 5 (BC33) BLAST queries of five genes resulted as *B. euroamericana* with the highest percent identity. However, in the phylogenetic analysis, these two species had their unique clade, indicating that the phylogenetic analysis of five genes is crucial in identifying a novel species.

This current study showed that morphologically and genetically diverse *Botrytis* spp. not only share the same ecological niche but also share common hosts. Previous studies have shown several *Botrytis* spp. infecting a single host. Studies conducted by Garfinkel et al. (2019) showed that 16 *Botrytis* spp. were found in association with *Paeonia lactiflora* in Alaska and the Pacific Northwest of the United States. Similarly, in onion, seven *Botrytis* spp. were found to be associated with neck rot (Chilvers et al. 2007). *B. prunorum* isolates from this study are phylogenetically similar to *Botrytis* spp. from peony (Garfinkel et al. 2019) and plum (Ferrada et al. 2016), indicating the polyphagous nature of this species. Previous studies have shown *Botrytis* spp. that are polyphagous (Mercier et al. 2019); *B. mali* was reported from an herbarium specimen in apple by O’Gorman et al. (2008) and, recently, this species was also reported from strawberry (Dowling and Schnabel 2017). In this study, even

though *B. mali* did not cause any lesions on leaves, it may deserve additional research efforts because it has been reported as a major postharvest pathogen in apple (Mikani et al. 2008, O’Gorman et al. 2005), and it may affect seed quality. *Botrytis* sp. 3 isolates are closely related to *B. allii* and *B. byssoides*. *Botrytis* sp. 5 isolates are closely related to *B. aclada*, the seedborne pathogen of onion (du Toit et al. 2004; Özer and Köycü 2004). Although the frequency of the isolates in the current study is very low, more in-depth studies are needed to characterize these seedborne pathogens and their effect on seed quality.

A majority of the species were able to cause lesions on the detached leaves of at least one crop. According to Garfinkel (2021), it is not easy to differentiate *Botrytis* spp. based on symptoms alone. In this study, the isolates that produced lesions on the detached leaves were able to cause disease on whole plants in the greenhouse. The aggressiveness of the tested isolates was lower on whole plants compared with the detached-leaf assay because the plants might have resisted these pathogens. Dry pea is more resistant to gray mold pathogens than the other two tested hosts, as demonstrated in the greenhouse trials. Earlier studies by Liu et al. (2007) showed a significant difference in symptom development on the attached- and detached-leaf assays. Differences in defense responses were found when the leaves intact with the plant and the detached leaves were inoculated with the same pathogen and, in detached leaves, symptom development was associated with senescence (Liu et al. 2007). Pathogenicity assays revealed differences among the *Botrytis* spp. in terms of aggressiveness. Detached-leaf assays and greenhouse studies confirmed that *B. cinerea* is more aggressive compared with other *Botrytis* spp. *B. prunorum* causes less disease on whole plants compared with the detached-leaf assay on lentil. *B. prunorum* either caused no lesions or small lesions in chickpea, indicating that it is less aggressive than *B. cinerea*.

Some *Botrytis* spp. such as *B. narcissicola* have special epidemiology where the ascospores act as primary inoculum and infect flowers only (Garfinkel 2021). *B. pseudocinerea* has shown insensitivity to the fungicide fenhexamid (Fournier et al. 2005; Walker et al. 2011); thus, identifying the *Botrytis* isolates at the species level would be useful to manage the disease in the field. Studies are limited on fungicide response by the newly reported species such as *B. prunorum* and *B. euroamericana*. A recent study conducted by Dowling et al. (2018) has shown that *B. fragariae* isolates showed reduced sensitivity to the fungicide Polyoxin-D compared with *B. cinerea* isolates. Future studies on fungicide sensitivity of these newly described species would be of importance in establishing the most effective chemical control programs. *Botrytis* spp. can affect the world’s agricultural systems regarding agricultural exports (Garfinkel 2021). The association of numerous *Botrytis* spp. with pulse seeds is a major concern because these pathogens would serve as initial inoculum for disease. Field surveys of major pulse-growing regions would generate applied knowledge on the prevalence of *Botrytis* spp.

In conclusion, this study revealed numerous species of *Botrytis* that are associated with pulse seed in Montana. These species could be aggressive on multiple crops, which may have implications for disease management. Our findings from this study emphasize the need for effective seed treatment practices to prevent the initial infection of seedlings. Future research in determining the pathogen’s location within the seed tissues would help in making effective seed treatment practices. In addition, studying *Botrytis* spp. transmission rate to the seedling and their sensitivity to fungicides would help in determining management strategies and fungicide choices.

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