

CHARACTERIZATION OF COLLETOTRICHUM SPECIES CAUSING BITTER ROT OF APPLES IN KASHMIR ORCHARDS

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ABSTRACT

Morphology-based identification, as well as species-specific primer analyses, revealed that the *Colletotrichum* isolates sampled from bitter rot lesions in symptomatic apple from Kashmir orchards belong to two species complexes: the *C. acutatum* species complex and the *C. gloeosporioides* species complex. Multi-locus gene sequence-based identification using the TUB2 and GAPDH genes identified species within the *C. acutatum* species complex as *C. fioriniae* and *C. nymphaeae*, and species within the *C. gloeosporioides* species complex as *C. siamense*, *C. theobromicola*, and *C. fruticola*. *Colletotrichum fioriniae* is distinguished from the other species by the

production of a red pigment in culture, and this enabled *C. fioriniae* to be recognized as the most abundant bitter rot species in Kentucky (approximately 70% of the total number of isolates). Pathogenicity to apple fruit differs between the two species complexes, as well as among the species within each species complex. The *C. gloeosporioides* species complex was more aggressive than the *C. acutatum* species complex, on average. *Colletotrichum siamense* was the most aggressive species among the five, causing larger and deeper lesions. The sensitivity of *Colletotrichum* species to thiophanate-methyl, myclobutanil, trifloxystrobin and captan was different, on average, between the two Species complexes, as well as among each species within each species complex. The *C. acutatum* species complex was more tolerant to tested fungicides compared to the *C. gloeosporioides* species complex. Among all five species, *C. fioriniae* was the most tolerant to the fungicides used in this study. Fingerprinting

using RAPD analyses suggested that *C. fopromoeae* represented a relatively homogenous population. Relatively diverse RAPD banding patterns were observed within species in the *C. gloeosporioides* species complex, indicating a potentially higher level of diversity.

KEYWORDS: RAPD, *Colletotrichum*, PDB, RFLP, FDA, GAPDH and TUBZ.

INTRODUCTION

The horticulture occupies a very significant position in the agricultural sector of Indian economy. The various fruits grow in India & are exported to different countries in the world. However, apple production is most prominent one in India. In India apples are grown as commercial crop in hilly areas. The apple fruit grows especially in the state of J&K. Apple production is one of the important sources of economy in J&K. Apple cultivation in J&K is fast expanding because apple has a comparative advantage over the other crops that can be grown in hilly regions. Kashmir has a rich heritage of fruit cultivation. Walter. R. Lawrence, who in his book "The Valley of Kashmir"(1895) revealed that "Kashmir is the country of fruits & perhaps no country has greater facilities for horticulture, as the indigenous apples, pear, vine, mulberry, walnut, hazel, cherry, peach, apricot, strawberry, raspberry can be obtained without difficulty in most parts of the valley of Kashmir. J&K is major apple producing state accounting for 67% of the total apple production in the country & the production per hectare is 10 metric tons, which is higher than the national average. The production of apple in state has increased from 1093335 metric tons in 2004-05 to 1367805 metric tons in 2009-10. The production per hectare or average yield in kashmir valley was 10.94 metric tons in 2004-2005, it increased to 11.2 metric tons in 2009-10. The state of J&K is the largest apple producing state in India. The cultivation of apple is done in all districts of kashmir valley. Major contributors to the apple production are Baramullaha, Shopian, Pulwma, Budgam, Anantnag, Kulgam etc. Apples are consumed & cultivated across the world & some part of production are consumed fresh & small part of production is processed in juices, jellies, slices & other jam items.

Challenges for Apple Production:-Kashmir apple growers are faced with several challenges and issues in terms of maintaining maximum levels of production. Apple trees and fruits are attacked by numerous destructive pathogens and insect pests. Poor pest management can result in significant crop losses and damage.

Insects:-The insects that cause the most losses in Kashmir orchards are the codling moth and the Apple Maggot. Potato leafhoppers, wooly apple aphids, mites, and Redmite can also

cause serious problems. Additional pest problems in Kashmir include apple maggots, brown marmorated stinkbugs, codling moths, dogwood borers, eastern tent caterpillars, European red mites, fatheaded apple tree borers, green June beetles, Japanese beetles, oriental fruit moths, plum curculio, ribbed cocoon makers, rosy apple aphids, Leafrollers, speckled green fruit worms, white apple leafhoppers, and wooly apple aphids. Most insect pests in Kashmir apple orchards are controlled by insecticides. A few, including codling moths and oriental fruit moths, are first monitored with pheromone traps and then controlled with insecticides.

Weeds:-Weeds compete with apple trees for water and nutrients. Moreover, if left unchecked, weeds may harbor insects and pathogens and provide habitats for destructive wildlife. The seasonal variations in populations of different weeds in apple orchards on neutral to slightly alkaline soils (pH 7.6 to 8.2) as affected by precipitation & temperature in the Kashmir valley are described. Chamaephytes & hemicryptophytes increased with increase in average temperature during different months up to October & decreased thereafter, October supported the highest number of threophytes under extremely low precipitation, though cynodon dactylon, Medicago dentculata & Agropyron repens were the dominant weeds. C. dactylon showed a consistent dominance except of January.

Wildlife:- Toxic chemicals sprayed in orchards to prevent crop diseases are killing black bears in the mountainous Kashmir valley, worrying the regions wildlife conservationists. Most of the state's orchard area (347223 hectares) is situated in the valley, known for its apples & pears. Every year until harvest season farmers' spray trees with many pesticides & insecticides including Endosulphan, a chemical known to cause serious problem even in humans. Wildlife experts say most of the orchards in Kashmir are situated on hinges of forests while more forest area has been encroached upon by orchadists, affecting the natural habitat of wild animals. An expert on wildlife & the chief vet in Kashmir Mansoor Ahmad Mir says he has seen at least 5 bears who died of high toxic levels in their body. The bears eat in large quantity and when the food is toxic; naturally they will fall sick or die due to poisoning.

Horticultural Practices:- Labour is the largest direct cost associated with apple production in the Kashmir., accounting for 60% of total production costs (48). Most horticultural practices associated with apple cultivation, including pruning, thinning, and harvesting, are performed manually. Thinning and harvesting by removing individual fruit is extremely labor intensive and is cost-prohibitive for some commercial orchards. This has become one of the justifications for some commercial orchards to provide u-pick operations for consumers. Unfortunately, u-pick operations can lead to other problems including plant damage and wasted produce. In addition, fruit left on trees or on the orchard floor can serve as a source of pests and pathogens in the orchard, resulting in significant economic losses for the Kashmir apple industry.

Plant Diseases:-Apple trees and fruits are susceptible to a wide range of diseases. Many of these are caused by endemic fungal and bacterial pathogens that also occur on wild crabapple and other members of the Rosaceae family. Monoculture contributes to buildup of primary inoculum of pathogens. This may be intensified in conditions of poor sanitation such as unpruned trees and unharvested fruit. Plant diseases such as fruit rot, fire blight, frog eye leaf spot, rust, scab, sooty blotch/fly speck, and root/collar rot are commonly found in Kashmir orchards. Some diseases are particularly important in specific areas of the Kashmir. For example apple scab, cedar apple rust, and quince rust are important diseases in the eastern Kashmir, while fruit rots are more significant in the southeastern India including in Kashmir. Bitter rot is the most important of these fruit rots. Bitter rot can cause significant economic losses during seasons with extended periods of warm and wet weather. In temperate growing regions with high rainfall, bitter rot can cause crop losses as great as 50%.

Bitter Rot of Apple:-Bitter rot is one of the most common fruit rot diseases of apple. Without proper disease management, bitter rot can destroy entire fruit crops in just a few weeks during periods of warm, wet weather. Bitter rot can be caused by any of several different *Colletotrichum* species.

Symptoms and Signs:-Bitter rot appears initially as small circular lesions on fruit as they begin to mature. Lesions then enlarge, especially in warm and humid conditions. Within these lesions, fungal reproductive structures (acervuli, perithecia, or both) develop. Under moist or rainy conditions, salmon-to-pink conidia appear in circular pattern, while in dry conditions, acervuli are visible in lesions According to Sutton, bitter rot symptoms initiated by spores from a perithecial strain (which produces both ascospores and conidia) appear slightly

different from those that are initiated by conidial strains (which produce only conidia). Lesions formed by conidial strains are described as circular, becoming sunken as they enlarge, while those initiated by perithecial strains are described as being darker brown in color and not sunken. Lesions produced by either type of strain appear as V-shaped in cross section. This V-shape is often used to differentiate bitter rot from white rot, which is characterized by a more cylindrical shape.

Disease Cycle:-Sutton reported that the bitter rot pathogens overwinter as perithecia and acervuli in mummified apples, and as mycelia in colonized dead wood, unpruned branches, cuttings that are left on the ground, and in cankers. In addition, unharvested fruits that fall to the orchard floor, as well as mummified fruits that remain attached to trees, can also be sources of inoculum. Conidia and aeciospores are produced by the acervuli and perithecia respectively and are dispersed by rainfall throughout the growing season. Free water induces conidial and ascospore germination and production of appressoria. *Colletotrichum* spp. can penetrate plants directly and/or through wounds. It is commonly reported that infections occur mid to late season. However, infection can also occur before, during and just after bloom. Infected fruit consequently become a source of secondary inoculum throughout the growing season. Early infections combined with persistent wet weather can potentially result in severe disease epidemics.

Disease Management:-Control of bitter rot is primarily focused on preventative actions such as sanitation and fungicide applications. Minimizing the source of primary inoculum by removing mummified fruit cankers, and pruning remnants will significantly reduce epidemics. As a result, significant economic loss can be avoided. Biggs and SS. Miller in 2001 classified several apple cultivars into four relative susceptibility groups to *C. acutatum*, the prevalent bitter rot pathogen in West Virginia and northern Virginia. The most susceptible cultivars were Ginger Gold, Honeycrisp and Pristin; highly susceptible cultivars included Arlet, Enterprise, Sansa, and Yataka; moderately susceptible cultivars were Creston, Golden Delicious, and GoldRush; and the least susceptible cultivar was Fuji. Shi and CR. Rom in 1995 (63) reported that Jonafree, Jonagold, Melrose, Oregon Spur II, Red Cort, Red Delicious, Spartan, and VPI-9 showed good relative resistance to bitter rot pathogens. Most existing apples cultivars are not sufficiently resistant to bitter rot pathogens for the disease to be managed by sanitation alone. Therefore, a protectant fungicide spray program is one of the most important disease control measures. In Kashmir orchards, growers use several

fungicides representing different chemical groups and/or different modes of action to control bitter rot, as well as other diseases. During different stages of fruit development, different fungicides might be applied. Protective fungicides such as sulfur, captan, and ziram are usually used during the dormant and apple green tip stages, while myclobutanil, trifloxystrobin, and thiophanatemethyl are applied during later stages of development including apple tight cluster, apple pink, or apple bloom.

Colletotrichum Species Associated with Bitter Rot of Apple:- Colletotrichum is a cosmopolitan fungal genus that includes nine major clades (aka species complexes) comprised of 118 species. Some Colletotrichum species have wide host ranges, while others can infect only a single ' host specie. There are three Colletotrichum species that are most commonly reported as causal agents of bitter rot of apple. These are *G. cingulata*, *C. gloeosporioides* (usually considered to be an anamorph of *G. cingulata*), and *C. acutatum*. Several studies have been conducted to examine inter and intraorchard variation of the various Colletotrichum spp. that are associated with bitter rot of apple. Shi et al found that *C. acutatum* was the prevalent species associated with bitter rot in Arkansas, North Carolina, and Virginia. Gonzalez and Sutton reported that *G. cingulata* was more abundant in some orchards in North Carolina.

Colletotrichum Taxonomy and Diagnosis:-The taxonomy of Colletotrichum has changed significantly over the years, in the late 19th and early 20th centuries, species were described primarily on the basis of the host from which they were isolated. In the early 1900S, some of these species were combined based on morphology of spores and other structures, including sexual structures produced in culture. This process was taken to its extreme in 1957, when Von Arx reduced several hundred described species of Colletotrichum to just 11 morphological species (11). Over the next three decades, the numbers of described species slowly increased again, based on subtle differences in conidial shape and size; appressorial shape and size; presence or absence of setae; and colony appearance and growth rate. One of the challenges for identifying species within the Colletotrichum genus is morphological similarity among species. For example, *C. acutatum*, *C. fragariae*, and *C. gloeosporioides* (teleomorph, *G. cingulata*) appear similar in culture. In addition, it has been reported that spore shape and size of Colletotrichum isolates is dependent upon the growth medium used (1; 39). Cultural characteristics such as growth rate and colony pigmentation are also influenced by growth substrate and temperature. Furthermore, in the pathogen-host

interaction, some of these *Colletotrichum* species produce very similar disease symptoms and infect the same hosts. The morphological resemblance among *Colletotrichum* species, as well as the disease symptoms that they cause, can potentially raise another issue in disease management, since different *Colletotrichum* species can have different sensitivities to fungicides. Differential sensitivity among Kashmir isolates of *C. acutatum* and *C. gloeosporioides* to certain fungicides has been observed. Thus, accurate pathogen identification is vital for development of effective disease management recommendations. Molecular approaches began to be widely employed in the late 1990s to address issues related to characterization of *Colletotrichum* species. More recently combined approaches that use both traditional and molecular methods, including species-specific polymerase chain reaction (PCR) amplification, RAPD fingerprinting, and sequence analyses of the internal transcribed spacer (ITS) of ribosomal DNA (rDNA), and simultaneous sequencing of multiple genes have been used to accomplish a more reliable identification of species. The result of these studies has been an increase in the number of named *Colletotrichum* species and a realization that the complexes traditionally known as *C. gloeosporioides* and *C. acutatum* actually consist of a large number of individual species.

The Role of Molecular Taxonomy/Diagnosis in Management of Bitter Rot:-Molecular identification of fungi has provided in-depth knowledge and new insights into the diversity and ecology of many different groups of fungi (3). The ITS region of the nuclear ribosomal repeat unit has become the most popular sequence for fungal taxonomy and species identification. The advantages of the ITS include the ability to amplify this variable region using PCR primers that have been designed based on highly conserved flanking sequences. Amplification is facilitated by the fact that the region consists of many copies (3). Some species that cannot be reliably identified based on morphological traits can be clearly differentiated based on variation in the ITS sequence. Specific primer pairs based on the ITS region can be applied to rapidly identify and distinguish among some species. Since the ITS region has been the most commonly used for fungal identification, it has been chosen for the DNA bar-coding initiative, the goal of which is to provide a reliable database for fungal species identification. Comparison of ITS sequences has commonly been used to identify species and species complexes within the *Colletotrichum* genus. Specific primer pairs have been designed based on the ITS region to differentiate morphologically similar *Colletotrichum* species and species complexes. For example, the specific primer pair, Calnt2ITS4 was used initially by Sreenivasaprasad et al for identification of *C. acutatum*

isolated from strawberry. The primer pair CgInt-ITS4 was utilized initially by Mills et al for detection and differentiation of *C. gloeosporioides* derived from several plants. Since then, these primer pairs have been widely used for identification of *C. acutatum* and *C. gloeosporioides* from different crops such as citrus, anemone and fruits including apples. However, ITS based-sequence analysis has some problems as a tool for taxonomy and diagnosis of *Colletotrichum*. One hindrance is that it has insufficient resolution to separate species within *Colletotrichum* species complexes, including some that are morphologically and/or pathologically distinct. Another major obstacle with ITS species identification is that the species names associated with ITS sequences available in the public databases (eg Genbank) are often inaccurate. Crouch et al revealed a high rate of misidentification (86%) based on ITS sequence similarity comparison within the *C. graminicola* species complex. Cai et al. also reported that ITS sequence data were frequently associated with an incorrect name. Utilization of sequence data from multiple genes provides better resolution of the species within complexes, and during the last decade, this has become the preferred method for *Colletotrichum* taxonomy. The number of new species defined using this approach has increased dramatically, and the challenge for plant pathologists now is to evaluate the potential significance of these sequence-based species definitions for disease diagnosis and management.

Problems Addressed in This Study: Bitter rot has emerged as one of the most important disease threats to the apple industry in Kashmir. As in other regions of India, *C. acutatum*, *C. gloeosporioides*, and *G. cingulata* have all been reported to cause bitter rot in Kashmir. The particular bitter rot causing species within these complexes have not been identified in Kashmir, although Kou et al. recently reported *C. fiorinae*, a species within the *C. acutatum* complex, as the cause of fruit decay on Nittany apple. Multiple *Colletotrichum* species often occur within the same orchard or area, but the particular species that dominates in each location seems to vary. The relative prevalence of the species causing bitter rot in Kashmir is unknown. Therefore, the first question addressed in this study was the identity and relative representation of the *Colletotrichum* species causing bitter rot in Kashmir. Molecular fingerprinting was used to investigate genetic diversity within the species in the state. Symptomatic fruit were collected from commercial and some private orchards throughout the state. A total of 475 isolates were recovered and stored in a permanent collection. Twenty-six *Colletotrichum* isolates from several orchards representing all regions of the state were selected for more detailed analysis. A combination of morphological and molecular methods

was used to identify these *Colletotrichum* isolates. A second important objective was to determine which identification method or methods would be most successful and reliable for diagnostic purposes. Fungicides are commonly recommended for management of bitter rot, but sometimes they are not effective in controlling epidemics. Thus, this study also addressed whether the *Colletotrichum* species causing bitter rot in Kashmir differ in sensitivity to common fungicides used in orchards. Several other characteristics were also evaluated, including growth rate in vitro, sporulation in vitro and in planta, and pathogenicity to apple fruits. Pathogenicity tests were performed for each strain using detached apples. Fungicide sensitivity of selected strains representing each species was analyzed using common fungicides in vitro. Differential sensitivity of *Colletotrichum* species to benomyl has been reported. However, benomyl is no longer available on the Kashmir market. Usually growers in the Kashmir spray a combination of different fungicides representing different chemical groups and different modes of action, often with combinations of fungicides for each stage of fruit development. This study tested the sensitivity of representative *Colletotrichum* strains to thiophanate-methyl, myclobutanil, trifloxystrobin, and captan, since these fungicides are commonly used in Kashmir orchards. The overall goal for this study was to provide data that could be used to design more effective disease management strategies in the field. Combining better diagnosis of specific species causing bitter rot with data about their relative pathogenicity and fungicide sensitivity can help growers to make the best management decisions.

Characterization of *Colletotrichum* Species causing Bitter Rot of Apples in Kashmir

Orchards:- Bitter rot is one of the most common and economically important summer fruit rot diseases of apple in Kashmir. It can cause significant economic losses during long periods of warm, wet weather. Failure to manage new infections by a combination of spraying preventative fungicides, removing dead and diseased wood, and removing diseased fruit, can exacerbate losses as disease intensity increases. Bitter rot of apple can be caused by several species within the ascomycete fungal genus *Colletotrichum*. The most commonly reported causal species include *C. acutatum*, *C. gloeosporioides*, and *G. cingulata* (teleomorph of *C. gloeosporioides*) (34; 35; 62; 68). Multiple species are often present within the same orchard or location, but prevalence of specific *Colletotrichum* species seems to differ from one region to another. Thus, location-specific species identification is important for effective disease management. It can be challenging to identify species within the genus *Colletotrichum* because many of them are morphologically similar, with morphology sometimes varying in

response to environmental factors. Furthermore, some *Colletotrichum* species produce similar disease symptoms and infect the same hosts, Molecular approaches have been widely employed since the 1990s to more effectively define and identify species of *Colletotrichum*. Combinations of morphological-based identification and molecular approaches have been demonstrated in some studies to accomplish reliable results for identification of *Colletotrichum* species. Sequencing of the internal transcribed spacer (ITS) quickly became the dominant method for fungal taxonomy and species identification beginning in the 1990s, due to its practicality and convenience. ITS consists of large number of copies per cell, so the region can be sequenced even when the quantity of DNA is low. Universal ITS primers that correspond to highly conserved flanking regions have been developed for fungi. In addition, species-specific primers can be designed based on the ITS region; these can be used to rapidly identify and distinguish among species *in vitro* or *in planta*. ITS also has been commonly used to evaluate species diversity in *Colletotrichum*. However, the ITS method also has some recognized limitations for this purpose. Most importantly, ITS has insufficient resolution to separate some morphologically and/or pathologically distinct species within the *Colletotrichum* species complexes. Thus, multigene sequence-based identification has recently emerged as the preferred method for taxonomy and species identification in *Colletotrichum*. With the application of multigene-based identification, the number of new *Colletotrichum* species has increased dramatically over the past decade. It is important to relate these sequence-based *Colletotrichum* species to characteristics such as pathogenicity and fungicide sensitivity. For example, in their characterization of *Colletotrichum* species associated with bitter rot of apple in Brazil and Uruguay, Velho *et al* reported that some species within the *C. gloeosporioides* complex were more sensitive to benomyl fungicide than other species within *C. acutatum* complex. An ability to relate taxonomic data (also including the development of accurate and rapid diagnostic tools) to pathogenicity and fungicide sensitivity data will assist in management of bitter rot of apple. The objectives of this study were to identify *Colletotrichum* species associated with bitter rot of apple in Kashmir and to determine which species were prevalent across the state; to determine the phylogenetic relatedness among the species and genetic diversity within the species; and to characterize and compare the pathogenicity and fungicide sensitivity of the species.

MATERIALS AND METHODS

Isolation of *Colletotrichum* species from symptomatic apple fruit:-In 2013, 475 *Colletotrichum* isolates were recovered from apples with symptoms of bitter rot collected

from 25 counties across Kashmir. Fruits were sampled from commercial orchards and private residences; these included apples treated with regular fungicide regimes as well as non-sprayed trees. The fruits were stored for up to 7 d at 4°C until they were processed. Fruits were disinfested with 70% ethanol for 30 s and then air-dried in a laminar hood. Three small pieces of fruit skin and pulp were taken from the outer margin of each lesion and placed onto potato dextrose agar (PDA, Difco). Cultures were incubated at 25°C with constant light for 7 to 20 d. Monoconidial isolation was performed according to Du et al, and all isolates were stored in a permanent collection as conidial suspensions on silica at -80°C. Two previously characterized monoconidial strains that had been isolated earlier from Kashmir apples with bitter rot symptoms were used as reference isolates for *C. gloeosporioides* and *C. acutatum*.

Morphological characterization:-Morphological traits that were observed included colony color, conidial shape and size, and the ability to produce perithecia in culture. Colony color was determined after 7 to 15 d on PDA at 23°C in constant light (39). Conidia were harvested from 14-day-old cultures by flooding each culture with 10 ml sterile water, scraping the upper surface of the culture with a sterile pestle, filtering the conidia! suspension through a layer of cheesecloth, and then resuspending conidia in 45 ml of sterile water (24). To determine conidia] shape, conidia were mounted in water and observed at 400x by using an Axioscop equipped with an Axio Cam HRc (Carl Zeiss Microscopy). Lengths and widths of 50 randomly chosen conidia were measured at 400x with phase contrast with the measurement module of the Zeiss AxioVision Rel. 48 computer program. The data were analyzed statistically with the univariate analysis and the Duncan's Multiple Range Test available through the SPSS statistical analysis package (IBM©, SPSS©, Statistic Version).

Growth rate assay:-Mycelial growth rate of *Colletotrz'chum* isolates was measured by using a race tube assay developed by White and Woodward and modified by another student in the laboratory. Linear mycelial growth was recorded at 3, 5, and 7 d after inoculation for cultures grown on FDA in 27 ml pipet tubes in the dark at 18, 21, 23, 25, or 30°C. Three replications of each isolate at each temperature were evaluated. Growth rate was calculated as the 7-day average of mean daily growth rate (millimeters per day) for each culture and temperature. Growth rate data were analyzed statistically with multivariate analysis and the Duncan's Multiple Range Test available through the SPSS statistical analysis package.

Isolation of fungal DNA:-Conidia from single-spore cultures were loosened by scraping gently with a pestle and were inoculated into 10 ml potato dextrose broth (PDB) in 9 x 50

mm Petri dishes. Cultures were incubated for 7 to 10 d at 23°C. Mycelia were harvested with a spatula. Excess media was removed by blotting the mycelia with sterile paper towels. Mycelia were lyophilized in 15 ml Eppendorf tubes with perforated lids for 24 h. Genomic DNA was extracted as described previously by Panaccione et al. with changes in extraction buffer according to Porebski et al. DNA was dissolved in 0.5 ml 1 x TE buffer (10mMTris-HCl, 1 mm EDTA; pH 8.0) to an approximate concentration of 200 to 500 pg/ml and diluted to a final concentration of 10 ng/μl for PCR.

Molecular characterization:-Species specific PCR was performed by modifying existing protocols of Jelev et al and Du et al using primers CaInt2 (GGGGAAGCCTCTCGCGG) specific for *C. acutatum* and CgInt (GGCCTCCCGCCTCCGGGCGG) specific for *C. gloeosporioides*, each in combination with the conserved primer ITS4. The previously characterized isolates APPR1 and APPY3, both collected earlier from symptomatic apples in Kentucky, were used as reference isolates for *C. acutatum* and *C. gloeosporioides* respectively. Reactions included 200 ng of genomic DNA, 1.5 mM MgCl₂, 1 X PCR buffer, 0.2 mM dNTP, 2.5 u of T aq DNA polymerase (Invitrogen) and 0.4 uM of each primer. The amplification cycle consisted of denaturation at 95°C for 5 m followed by 40 cycles consisting of 30 s at 95°C, 30 s at 50°C and 1.5 m at 72°C. PCR products were separated in agarose gels (2% w/V Methapor Agarose; 15 x 10 cm, W XL) in Tris/Borate/EDTA buffer electrophoresed at 100 V for 1 h. Each gel was stained with ethidium bromide and viewed on a UV transilluminator.

Multigene amplification:-PCR amplifications were performed for variable regions of two nuclear genes: glyceraldehyde-3-phosphate dehydrogenase (GAPDH); and B-tubulin 2 (TUBZ). These two regions have previously been used to differentiate species within the *C. gloeosporioides* and *C. acutatum* complexes and were reported to be the most effective for separating species within these complexes (Ulrike Damm, personal communication). PCR primer pairs used for the experiment included GDF1 (GCCGTCAACGACCCCTTCATTGA) and GDR2 (GGGTGGAGTCGTA CTTG-AGCATGT), for GAPDH amplification; and T1 (AACATGCGTGAG-ATTGTAAGT) and T2 (TAGTGACCCTTGGCCCAGTTG), for TUBZ gene amplification. PCR was performed according to Weir et al. PCR conditions for GAPDH amplification were 4 m at 95°C, and then 35 cycles of 95°C for 30 s, 60°C for 30 s, 72°C for 45 s, and then 7 m at 72°C. The conditions for amplification of TUB2 were similar except that the annealing temperature was 55°C.

RAPD fingerprinting analysis:-RAPD (Random Amplified Polymorphic DNA) fingerprinting analyses were conducted with three different primers: OPA 13 (CAGCACCCAC); OPA 18 (AGGTGACCGT); and UBC 356 (GCGGCCCTCT). All reactions were incubated in a BIO-RAD CIOOOTM Thermal Cycler in a total volume of 26.03 μ l. PCR reactions contained 10.83 μ l sterile water, 2.5 μ l of 50 mM MgCl₂, 2.5 μ l of 1 X PCR buffer, 4 μ l dNTPs (each 0.2mM), 0.22 μ l of 500 U of Taq DNA polymerase (Invitrogen), 3 μ l of 5 pM of primer, and 3 μ l of 10 ng/ μ l genomic DNA. Amplification consisted of 3 cycles of 60 s at 95°C, 60 s at 35°C, 1.5 m at 72°C, followed by 40 cycles consisting of 10 s at 93°C, 20 s at 40°C and 1.5 m at 72°C. PCR products were separated in agarose gels (1% w/v Agarose GPG/LE (American Bioanalytical); 15 x 10 cm, W X L) in Tris/acetate/EDTA buffer electrophoresed at 90 V for 1 h. Each gel was stained with ethidium bromide and Viewed on a UV transdluminator.

Pathogenicity tests:-Pathogenicity tests were performed in the laboratory with two apple cultivars: Golden Delicious, reported to be susceptible to bitter rot and Red Stayman Winesap, reported to be moderately resistant. Apples used in this study had not been sprayed with fungicides and were obtained from the Horticulture Research Farm, Department of Horticulture, SKAUST located in Srinagar, KY. Apples were harvested in August 2014 and stored in the dark at 4°C for up to 2 months before use. Detached fruits were surface-disinfested in 10% bleach for 5 m, washed in sterile distilled water, and then air-dried on sterile paper towels at room temperature. Fruits were wounded with a dissecting needle that had been modified to produce a puncture of 0.5 mm depth. The wounded fruits were then inoculated by placing 6 μ l of conidial suspension (1x10⁵ spores/ml) on the surface of the wound. The conidia used to make suspensions were harvested from 10-day old cultures grown on PDA. Inoculated fruits were secured in plastic containers lined with wet paper towels and incubated for 14 d at 21°C in constant light. Containers were opened once a day for measurements of developing lesions. Lesion diameters were measured using a digital caliper. At 14 days post inoculation (dpi) fruits were cut through the centers of lesions for measurements of lesion depth. The entire surface area of each lesion was then removed with a scalpel and submerged in 10 ml of sterile water with one drop of Tween 80 (Fisher Scientific, Fair Lawn, NJ) in a 50 ml tube (USA Scientific). Spores were dislodged from the tissue samples by using a vortex for 10 s, and then conidia were filtered through a layer of cheesecloth. Spores were counted with a hemocytometer. The experiment was performed twice. The factorial experiment for experiment, replication, cultivar, species complex, and

species, isolate was evaluated using an analysis of variance and means were separated using Least Square Means with Bonferroni correction provided in the statistical algorithms of SAS version 9.3.

Fungicide sensitivity assays:—Effect of fungicides on mycelial growth Fungicide sensitivity of isolates was tested on PDA amended with fungicides in 25 ml race tubes as described above. Fungicides included thiophanate-methyl (Topsin M WSB 70 WP, Nippon Soda Company Ltd.; FRAC 1), myclobutanil (Rally 40 WSP, Dow AgroSciences LLC; FRAC 3), trifloxystrobin (Flint, Bayer Crop Science; FRAC 11), and captan (Captan 80 WDG, Arysta LifeScience North America CO.; FRAC M). Race tube assays were performed according to Buiate (9), as discussed previously. Fungicide concentrations in the amended media were 0.01; 0.1; 1; 10; and 100 µg/ml for Topsin, Rally and Flint, and 0.1; 1; 10; 100; and 1,000 µg/ml for Captan. The linear grth of each isolate was measured at 10 d after inoculation. Percent inhibition was calculated by [(growth of the control growth of the experimental)/growth of the control]*100. Relative grth was calculated by [100 percent inhibition]. Half maximal effective concentration (EC₅₀) was determined based on the relative growth data. Each treatment was replicated three times within the experiment, and the experiment was . repeated once. The factorial experiment for experiment, replication, fungicide, species complex, species, isolate was evaluated using an analysis of variance and means of EC₅₀ were separated using Least Square Means with Bonferroni correction provided in the statistical algorithms of SAS version 9.3.

Effect of fungicides on conidial germination:—Trifloxystrobin (a systemic fungicide) and captan (a protectant fungicide) were tested for their ability to inhibit spore germination. Fungicide solutions and fresh conidial suspensions were mixed with sterile water to a total of 15 ml. The final concentrations of fungicide were 0.001; 0.01; 0.1; 1; and 10 µg/ml, and the final concentration of conidia was 1×10^4 conidia per ml. Three 100-µl droplets of each suspension was placed into an empty 100-mm polystyrene Petri dish and then placed in humidity chambers and incubated for 20 to 24 h. After incubation, each droplet was covered with a cover slip and conidia were scored at 100x as either non-germinated or germinated according to Chaky et al and Vincelli and E. Dixon.

RESULTS

Morphological characterization:—The 475 Colletotrichum bitter-rot isolates that were collected from 25 counties including Kashmir were divided into four morphological types

(morphotypes) based on colony color and Spore shape. These morphotypes corresponded to the descriptions of the three *Colletotrichum* species most commonly associated with bitter rot of apple: *C. acutatum*; *C. gloeosporioides*; and *G. cingulata*. Isolates belonging to Morphotype 1 were characterized by the production of a pink to red coloration in the media. Orange conidial masses were submerged in the mycelia. Conidia were predominantly iilsiform with pointed ends. Perithecia were not observed. Based on these morphological characteristics, all of the isolates in Morphotype 1 corresponded to *C. acutatum*. Morphotype 2 isolates did not produce pigment in the growth medium. Isolates formed white-togray mycelia, occasionally with a light yellow tint, and an abundance of orange conidial masses. Conidia of the isolates in Morphotype 2 were mostly thsiform, with ends that were somewhat more rounded than spores of Morphotype 1. No perithecia were observed in cultures of this morphotype. The conidial shape of the isolates in Morphotype 2 corresponded to the description of *C. acutatum*. Isolates of Morphotype 3 were differentiated from other morphotypes by the presence of gray mycelia, with no coloration in the growth medium. Some isolates produced abundant aerial mycelia that were white-to-gray. Sectors were observed in the colony mycelia of some isolates of Morphotype 3 such as KY254; KY301; KY152; and KY153 (Appendix 1). Conidia of all isolates were cylindrical with rounded ends. Conidia were produced in small orange masses over the entire colony in most isolates. A few isolates produced larger orange scattered masses of conidia. No perithecia were observed in culture. The characteristics of the isolates in Morphotype 3 were consistent with descriptions of *C. gloeosporioides*. The mycelia of the isolates in Morphotype 4 were dark gray, with abundant mycelia. Isolates of Morphotype 4 produced no pigmentation in the media. Conidia were cylindrical with rounded ends, similar to those produced by isolates in Morphotype 3. Perithecia were produced in culture, and asci and ascospores were observed under the microscope. Based on these morphological characteristics, the isolates in Morphotype 4 belonged to *G. cingulatas*. Among the total of 475 isolates, 335 (70.53%) belonged to Morphotype 1; 15 (3.16%) belonged to Morphotype 2; 106 (22.32%) belonged to Morphotype 3; and 19 isolates (4%) belonged to Morphotype 4. Sizes of conidia produced by each of the 26 selected isolates and two reference isolates were measured and compared. Average conidial length and width were significantly different among the four morphotypes ($P < 0.05$). Isolates of Morphotype 3 had the longest conidia, averaging 17.89 μm , while isolates of Morphotype 1 had the shortest, averaging 12.43 μm . Conidia of Morphotype 4 were the widest, with an average of 6.39 μm , while conidia of Morphotype 1 isolates were the narrowest, averaging 4.61 μm . Length-width ratios of isolates of Morphotype 1 and

Morphotype 4 were not significantly different from one another ($P = 0.375$). Likewise, conidial length width ratios of Morphotype 2 and Morphotype 3 did not differ from one another ($P = 0.323$). Mycelial growth rate The independent variables morphotype and temperature both had a significant effect on mycelial growth rate ($P < 0.05$). The growth rate of each morphotype was significantly different from the others ($P < 0.05$). The maximum growth rate occurred at 25°C for all isolates. Isolates of Morphotype 4 grew the fastest (average growth 4.99 mm/day), and isolates of Morphotype 2 were the slowest (average growth 3.43 mm/d).

Molecular identification and phylogenetic analysis (Species-specific primer analyses) :-

DNA amplification with the species-specific primers for *C. acutatum* (CaInt2-ITS4) yielded a single, relatively intense 490-bp product for isolates of Morphotype 1 and Morphotype 2, as well as from the *C. acutatum* reference isolate, APPY3. Multiple amplification products of lower intensity, including one of 490-bp, were generated with this primer for most of the isolates of Morphotype 3 and Morphotype 4, but not for the APPRI *C. gloeosporioides* reference isolates. Primers specific for *C. gloeosporioides* (CgInt-ITS4) yielded an intense 450-bp DNA product from all isolates in Morphotype 3 and Morphotype 4, as well as from the reference isolate of *C. gloeosporioides*, APPRI. No amplification products were produced with CgInt-ITS4 for isolates of Morphotype 1 and Morphotype 2.

Multigene analyses:-GAPDH and TUBZ DNA fragments were successfully amplified and sequenced from all test isolates. The PCR products from the GAPDH and TUBZ amplification were approximately 250 and 700 bp, respectively. Homology searches with each individual sequence suggested that all tested isolates corresponded to the genus *Colletotrichum*. Based on the sequence comparison of 26 tested isolates with published sequences in GenBank, all isolates in Morphotype 1 and Morphotype 2 belonged to the *C. acutatum* species complex. All isolates in Morphotype 1 (12 isolates) matched *C. fioriniae*, and all isolates in Morphotype 2 (3 isolates) could be identified as *C. nymphaeae*. Isolates within Morphotype 3 and Morphotype 4 belonged to the *C. gloeosporioides* species complex. Seven isolates in Morphotype 3 matched *C. siamense*, and 2 isolates were identified as *C. theobromicola*. All isolates in Morphotype 4 corresponded to *C. fructicola*. Homology searches to confirm species identity for the GAPDH and TUBZ sequences against the Qbank fungal data base was consistent with the homology searches by using megablast against the GenBank nr database. Molecular phylogenetic analyses based on GAPDH and TUBZ

regions in a separate analysis by PhyML grouped the *Colletotrichum* isolates into two major clades, corresponding to the *C. acutatum* species complex and *C. gloeosporioides* species complex, with bootstrap support of 100%. The *C. acutatum* clade consisted of two separate sub-clades, which corresponded to *C. fioriniae* and *C. nymphaeae*. The *C. gloeosporioides* clade consisted of three sub-clades, corresponding to *C. siamense*, *C. theobromicola*, and *C. fructicola*.

RAPD fingerprinting analyses:- Amplification products were obtained from all 26 test isolates with the random primers OPA 13, OPA 18, and UBC 356. Similar banding patterns were observed among isolates of *C. Fioriniae* (Morphotype 1), and also among isolates of *C. nymphaeae* (Morphotype 2), suggesting that these populations are relatively homogeneous. Banding patterns within *C. siamense*, *C. fructicola*, and *C. theobromicola* isolates appeared to be more variable, indicating a higher level of diversity within those populations.

Pathogenicity tests:- All test isolates of *Colletotrichum* produced typical bitter rot symptoms on Golden Delicious, Ambri, & American Trel fruits. These symptoms included sunken lesions, and conical zones of necrotic tissue extending beneath the lesions. Apple cultivar had no significant effect on lesion diameter or on lesion depth ($P > 0.05$). A significant difference was observed between the two replicate experiments that were performed for each cultivar ($P < 0.05$). The variable of species complex had a significant effect on lesion development ($P < 0.0001$). Isolates belonging to the *C. gloeosporioides* species complex, on average, produced larger and deeper lesions (34.22 mm and 22.03 mm, respectively) compared with the *C. acutatum* species complex isolates (20.11 mm and 12.12 mm, respectively). The interaction of species complex and apple cultivar had no effect on lesion diameter or on lesion depth ($P > 0.05$). The variable of species ($P < 0.0001$), as well as the interaction of species and apple cultivar ($P = 0.0135$), had significant effects on lesion development. Isolates of *C. siamense* produced the largest and deepest lesions (average of 40.18 mm in diameter and 25.17 mm deep). Even though *C. fioriniae* and *C. nymphaeae* belong to the same species complex, *C. fioriniae* caused significantly larger lesions (average diameter 21.15 mm) than *C. nymphaeae* (average diameter 15.93 mm). Isolates of *C. fioriniae* also produced significantly deeper lesions than *C. nymphaeae* ($P = 0.0002$). However, the depth of lesions caused by *C. fioriniae* and by *C. theobromicola* was not significantly different ($P > 0.05$).

Apple cultivar had a significant effect on the sporulation of *Colletotrichum* spp. ($P = 0.0097$). The average number of spores produced by *Colletotrichum* lesions on Red Delicious apples

was 0.56×10^5 spores/mm² while the mean number of spores produced by lesions on Golden Delicious was 0.37×10^5 spores/mm². The variable of species complex also had significant effect on sporulation ($P < 0.0001$). The mean number of spores produced by lesions caused by members of the *C. acutatum* complex was 0.88×10^5 spores/mm², while the average produced by lesions caused by isolates of the *C. gloeosporioides* complex was 0.06×10^5 spores/mm². Lesions caused by *C. fioriniae* produced more spores (average concentration 1.01×10^5 spores/mm²) than any of the other species. The number of spores produced by *C. nymphaeae*, *C. siamense*, *C. theobromicola*, and *C. fructicola* was not significantly different ($P > 0.05$).

Sensitivity of Colletotrichum isolates to fungicides :- The independent variables fungicides (thiophanate-methyl, myclobutanil, trifloxystrobin, and captan), species complex, species, interaction of fungicide and species complex, and interaction of fungicide and species all had significant effects on the ECSOs of selected fungicides against mycelial growth of *Colletotrichum* ($P < 0.0001$). No differences were observed between the two replicate experiments ($P > 0.05$). The EC₅₀ of captan against the mycelial growth of *Colletotrichum* spp. was higher than that of any of the other fungicides in the study (mean EC₅₀ = 299.21 mg/L). The ECSOs of thiophanate-methyl, myclobutanil, and trifloxystrobin were not significantly different from one another ($P > 0.05$). Based on mycelial growth, isolates belonging to the *C. gloeosporioides* complex were more sensitive, on average, to the four fungicides than isolates in the *C. acutatum* species complex. Isolates of *C. fioriniae*, *C. nymphaeae* and *C. theobromicola* were significantly less sensitive to the four fungicides than isolates of *C. siamense* and *C. fructicola* ($P < 0.05$). The sensitivities of *C. fioriniae*, *C. nymphaeae*, and *C. theobromicola* were not significantly different from one other ($P > 0.05$). Similarly, *C. siamense* and *C. fructicola* did not differ in fungicide sensitivity ($P > 0.05$). The independent variables fungicide (trifloxystrobin and captan), Species complex, species, fungicide-species complex interaction, and fungicide-species interaction all had significant effects on the ECSOs of selected fungicides to the spore germination of each *colletotrichum* species evaluated ($P < 0.05$). The EC₅₀ of trifloxystrobin (mean EC₅₀ = 0.02 mg/L) for conidial germination of *Colletotrichum* spp. was significantly lower than the EC₅₀ of captan (mean EC₅₀ = 0.33 mg/L). The fungicide sensitivity of spores among members of the *C. gloeosporioides* species complex was, on average, significantly higher than the sensitivity of spores of isolates within the *C. acutatum* species complex. The sensitivity of spores of *C.*

theobromicola to the two selected fungicides was significantly higher than the sensitivity of spores of the other species ($P < 0.05$).

CONCLUSION

The major finding of this work suggested that *Colletotrichum* isolates causing bitter rot of apple in Kashmir orchards belong to five different species: *C. fioriniae*; *C. nymphaeae*; *C. siamense*; *C. theobromicola*; and *C. fruticola*. Among these, only *C. fioriniae* has been reported previously to cause bitter rot of apple in India. However, all five species have been found associated with bitter rot in other countries including Croatia (43), Brazil, and Uruguay (70). Furthermore, the publications that established and named these five new species included several isolates of *C. fioriniae*, *C. siamense*, and *C. fruticola* that had been recovered from apple in the Kashmir. To my knowledge, this is the first study that has compared fungicide sensitivity and pathogenicity of these species on apple. Diagnosis of species associated with bitter rot has traditionally been based on morphological features, particularly spore shape and type. This has allowed the recognition of three species of *Colletotrichum* as causal agents: *C. acutatum*, *C. gloeosporioides*, and *G. cingulata*. The 475 isolates recovered from diseased apples in Kashmir could be divided into two groups on the basis of spore shape, one with the spindle-shaped spores of *C. acutatum*, and the other with the ovoid spores of *C. gloeosporioides*. Among the latter group, a small percentage formed perithecia containing asci and ascospores in culture. These were grouped as Morphotype 4, differentiating them from the non-perithecial members of *C. gloeosporioides* which were named Morphotype 3. Isolates of *C. gloeosporioides* that form perithecia have traditionally been referred to as *G. cingulata*. However, there has recently been a move to eliminate the binary naming system for ascomycete fungi, and thus the teleomorph name *G. cingulata* has been rejected and both the sexual and asexual forms should now be referred to as *C. gloeosporioides*. The assumption has generally been made that perithecial and non-perithecial isolates of *C. gloeosporioides* causing bitter rot represent the same strains undergoing different phases of development. However, this work clearly showed that isolates of *C. gloeosporioides* that formed perithecia were not the same as those that did not develop perithecia; perithecial isolates belonged to a different species within the *C. gloeosporioides* complex, *C. fruticola*. Isolates of *C. gloeosporioides* that did not form perithecia included two other species within the complex, *C. siamense* and *C. theobromicola*, which could not be distinguished by morphological features alone. Some of the Morphotype 4 isolates that had initially formed perithecia lost this ability in subsequent cultures. The loss of fertility in

culture is not uncommon in *Colletotrichum*. Thus, presence of perithecia in culture may be a useful trait for diagnosis of *C. fructicola*, but their absence is not informative for diagnosis of bitter rot isolates within the *C. gloeosporioides* complex. Isolates with the spindle-shaped conidia of *C. acutatum* occurred as two different color morphs in culture. A pink to red pigmentation was observed in some isolates (categorized as Morphotype 1). Other isolates with *C. acutatum*-type spores did not produce pigment and were categorized as Morphotype 2. Gonzalez et al also reported the presence of two color morphs in the *C. acutatum* species. Isolates that produced red pigment in culture were identified as chromogenic *C. acutatum* and isolates that did not produce red pigmentation were identified as non-chromogenic *C. acutatum*. A similar observation was made in Du et al. (24) including some isolates from apple. Isolates of Morphotype 1 corresponded to chromogenic *C. acutatum*, and this work showed that all isolates of Morphotype 1 belonged to the species *C. fioriniae*. All the non-chromogenic isolates with *C. acutatum*-type spores belonged to *C. nymphaeae*. Thus, a combination of spore shape and color is useful for diagnosing species causing bitter rot within the *C. acutatum* complex in Kashmir. *Colletotrichum fioriniae* was the most common causal agent of the bitter rot disease throughout the state. The dominance of *C. fioriniae* is consistent with previously published research by Shi et al., who determined that most of the *Colletotrichum* isolates derived from apple bitter rot in the Southeastern India were chromogenic *C. acutatum*. Although the authors did not perform molecular characterization of their isolates, results of my study suggest that those isolates were probably *C. fioriniae*. RAPD fingerprinting has been widely used for characterization of diversity within *Colletotrichum* populations. RAPD fingerprinting suggested relatively low levels of genetic diversity within the *C. fioriniae* population in Kentucky, and likewise among *C. nymphaeae* isolates. Results indicated a somewhat higher level of diversity within the *C. gloeosporioides* complex species *C. siamense* and *C. fructicola*. Greater relative diversity among isolates of *C. gloeosporioides* compared with *C. acutatum* has been frequently reported. Reasons for this difference are unknown, but it has been suggested that it may be related to the presence of a sexual phase (*G. cingulata*) in *C. gloeosporioides*. The teleomorph of *C. acutatum* (*G. acutata*) has been observed only in the laboratory in the US. (37), although it has been reported in the field associated with some fruit rots in Asia (42). A sexual phase would be expected to increase population diversity due to marker recombination in the absence of selection. However, measuring genetic diversity of a population by using RAPD has several limitations. RAPD fingerprinting only determines amplification or lack of amplification (two alleles) for each amplicon locus; it does not provide measures of genetic diversity affected by

the number of alleles at a locus. In addition, some technical limitations such as the size and specificity of RAPD primers, sensitivity to reaction condition, and the possibility of co-migration, may cause non-reproducible amplification products. Spore shape was generally useful for differentiating *C. gloeosporioides* and *C. acutatum*. However, spores of the non-Chromogenic *C. nymphaeae* were more rounded than the spindle shaped spores of the chromogenic *C. fioriniae*, and thus they were more difficult to classify as fusiform with complete confidence. Species-specific primers developed to identify *C. acutatum* and *C. gloeosporioides* gave consistent and reliable results in this study and may be a useful additional diagnostic technique in cases where spore shape is ambiguous. Random PCR products were often generated with *C. acutatum*-specific primers when they were used with *C. gloeosporioides*, and some of these amplicons matched the expected size for the specific *C. acutatum* product. However, the bands produced with *C. gloeosporioides* isolates were less intense. Additionally, if both primer pairs were used, results were unambiguous. These species-specific primers were designed from the ITS 1 region of the ribosomal DNA (rDNA). ITS sequences have been widely used for *Colletotrichum* species identification, but they do not allow separation of species within species complexes. GAPDH and TUBZ sequences have been used more recently to identify and differentiate *Colletotrichum* species within the *C. acutatum* and *C. gloeosporioides* complexes (19; 77). This study used these sequences to reveal the presence of five species associated with bitter rot of apple in Kentucky. In the future, these sequences could be used to design new species-specific primers that will allow identification and detection of each of these five species. The presence of five different species causing bitter rot disease raised the possibility that they may differ in traits relevant to disease epidemiology and management. To my knowledge, my study is the first to thoroughly test this hypothesis. Isolates within the *C. acutatum* species complex had a slower average growth rate than isolates in the *C. gloeosporioides* species complex. This result agrees with a previously published report. All test isolates had an optimal growth rate at 25°C, and that growth of all isolates was significantly inhibited at 30°C, in agreement with a previous report by Velho et al. In this study, both species within the *C. acutatum* complex (*C. nymphaeae* and *C. fioriniae*) grew at a similar rate. For isolates within the *C. gloeosporioides* complex, *C. theobromicola* grew more slowly than the other two species, and its growth rate was not significantly different from the two *C. acutatum* isolates. These results are in contrast with Velho et al., who reported that *C. nymphaeae* grew more slowly than *C. theobromicola*. All tested isolates from all five *Colletotrichum* species were pathogenic to wounded apple fruits. On average, isolates within the *C. acutatum* species complex produced smaller lesions than

isolates in the *C. gloeosporioides* species complex. However, this study found significant differences in aggressiveness among the species within each complex. Thus, *C. siamense* in the *C. gloeosporioides* complex was the most aggressive species overall and was significantly more aggressive than the other two species within that complex. Similarly, within the *C. acutatum* species complex, *C. fiorinae* was more aggressive than *C. nymphaeae*. Isolates of *C. theobromicola* and *C. fuctz'cola* within the *C. gloeosporioides* complex were not significantly different in aggressiveness from the two *C. acutatum* species. Thus, a diagnosis to species complex alone would be insufficient for a prediction of relative aggressiveness. Lesion sizes did not differ on the two tested apple cultivars, Golden Delicious and Red Stayman Winesap. Red Stayman Winesap is reported to have partial resistance to bitter rot, whereas Golden Delicious is reported to be susceptible to the disease. This partial resistance may relate to factors other than resistance of the pulp to colonization once the skin has been broken. Most existing apples cultivars are not sufficiently resistant to manage the disease effectively in the absence of chemical treatments. Isolates of *C. fiorinae* produced significantly more spores per unit lesion area than the other four species. Perhaps its relative fecundity is related to its dominance across the state of Kashmir. According to Shi et al one of the variables influencing the frequency of a fruit rot pathogen is the abundance of initial inoculum. On average, significantly more spores were produced on the Red Stayman Winesap apples than on the Golden Delicious apples. One possible explanation for this result could be differences in nutrient content, e.g. sugars or amino acids, between the two cultivars. Nour et al. reported considerable variation in biochemical characteristics and mineral content between various apple cultivars, and nutritional factors are known to influence the sporulation of fungi in vitro. Differences due to variable storage periods between Golden Delicious and Red Stayman Winesap apple cannot be discounted as a contributing factor to this observation, as well as the lack of variation in susceptibility of the two cultivars. This study used the Golden Delicious apples in the first and second experiments, and during that time, the Red Stayman Winesap apples were kept in cold storage for 4 weeks. There was a significant difference in lesion size between the first and second experiments with each cultivar, with apples exhibiting greater susceptibility in the second experiment in each case. Apples used in the second experiments had been stored for about two weeks longer than apples used in the first experiments in each case. However, there was no difference in sporulation between the first and second experiments with each cultivar, so storage effects do not explain that difference. Kashmir growers typically manage bitter rot and other fungal diseases on apples with timed applications of fungicide mixes that include different modes of

action. This study tested the sensitivity of the *Colletotrichum* bitter rot species to some common fungicides used in Kashmir orchards. On average, species within the *C. acutatum* complex were more tolerant to thiophanate-methyl, myclobutanil, trifloxystrobin, and captan, than members of the *C. gloeosporioides* species complex. Similar results have been reported previously. However, this study suggests that the story is more complex than this. The *C. acutatum* species *C. fioriniae* and *C. nymphaeae* were both more tolerant to fungicides used in this study compared with the *C. gloeosporioides* species *C. siamense* and *C. fruticola*. However, *C. theobromicola*, also within the *C. gloeosporioides* complex, did not differ significantly from the two *C. acutatum* isolates in its sensitivity to fungicides; it was significantly more tolerant than the other two *C. gloeosporioides* species. Thus, a diagnosis to species complex may not be sufficient to predict fungicide sensitivity among Kashmir bitter rot isolates. Mycelial growth of *Colletotrichum* species was significantly less inhibited by captan than thiophanate-methyl, myclobutanil, or trifloxystrobin. Captan is a protectant fungicide with a multi-site mode of action; it is normally applied in higher doses compared with single-site mode of action fungicides. As a protectant fungicide, captan functions as a barrier to prevent infection from occurring by preventing spore germination. The mean EC50 of captan on conidial germination of *Colletotrichum* spp. is higher than the mean EC50 of trifloxystrobin. It confirms that *Colletotrichum* species in general are more sensitive to trifloxystrobin than to captan. Trifloxystrobin is a single-site fungicide from the group of quinone outside inhibitors (QoI) that inhibit respiration by targeting cytochrome *b_L* at the (QoI) site (cyt *b* gene), while captan is a multi-site fungicide. On average, spores of members of the *C. acutatum* species complex were more tolerant to captan and trifloxystrobin than members of the *C. gloeosporioides* species complex. However, when individual species were considered, only *C. fioriniae* and *C. theobromicola* differed significantly from one another, with *C. fioriniae* being more tolerant.

In conclusion, these findings demonstrate the importance of accurate pathogen identification for bitter rot management. It also shows the necessity to identify species beyond the species complex, because species within the species complex sometimes differed significantly in traits relevant to disease management, eg. pathogenicity and fungicide sensitivity. Further study on comparing biological behavior of species within the species complex is necessary, especially for applied purposes such as disease management.

Table 2.4. Average growth rate of Colletotrichum isolates at five selected temperatures

Temperature (°C)	Mean growth rate (mm/day) ^x
18	2.67 a
21	3.25 b
23	4.69 c
25	6.12 d
30	3.90 e

*Values followed by the same letter were not significantly different based on Duncan's multiple range test ($P < 0.05$).

Significance of the study:- Morphology-based identification, as well as species-specific primer analyses, revealed that the *Colletotrichum* isolates sampled from bitter rot lesions in symptomatic apple from Kashmir orchards belong to two species complexes: the *C. acutatum* species complex and the *C. gloeosporioides* species complex. Multi-locus gene sequence-based identification using the TUB2 and GAPDH genes identified species within the *C. acutatum* species complex as *C. fioriniae* and *C. nymphaeae*, and species within the *C. gloeosporioides* species complex as *C. siamense*, *C. theobromicola*, and *C. fruticola*. *Colletotrichum fioriniae* is distinguished from the other species by the production of a red pigment in culture, and this enabled *C. fioriniae* to be recognized as the most abundant bitter rot species in Kentucky (approximately 70% of the total number of isolates). Pathogenicity to apple fruit differs between the two species complexes, as well as among the species within each species complex. The *C. gloeosporioides* species complex was more aggressive than the *C. acutatum* species complex, on average. *Colletotrichum siamense* was the most aggressive species among the five, causing larger and deeper lesions. The sensitivity of *Colletotrichum* species to thiophanate-methyl, myclobutanil, trifloxystrobin and captan was different, on average, between the two Species complexes, as well as among each species within each species complex. The *C. acutatum* species complex was more tolerant to tested fungicides compared to the *C. gloeosporioides* species complex. Among all five species, *C. fioriniae* was the most tolerant to the fungicides used in this study. Fingerprinting using RAPD analyses suggested that *C. fopromoe* represented a relatively homogenous population. Relatively diverse RAPD banding patterns were observed within species in the *C. gloeosporioides* species complex, indicating a potentially higher level of diversity. The overall differences observed between the two species complexes causing bitter rot were consistent with previous reports in the literature. However, this study is the first, to my knowledge, to thoroughly examine differences among species within species complexes in

the India and one of the first worldwide. It revealed that there are differences in pathogenicity and fungicide sensitivity among these species that could have significant implications for disease management. A diagnosis to species complex is not sufficient for prediction of behavior for these traits. Accurate pathogen identification is the first step in the process of developing an effective disease management program. Colletotrichum species identification can be challenging due to morphological similarity among species and morphological plasticity in culture. In addition, the wide range of hosts and the ability of some Colletotrichum species to infect the same host make species boundaries ambiguous and confusing. This study included cases in which several Colletotrichum species were found infecting the same tree in the same orchard. Moreover, two different species were sometimes found in the same lesion. Different Colletotrichum species cause very similar bitter rot symptoms, which makes disease diagnosis in the field even more challenging. In the meantime, errors in disease diagnoses may result in ineffective disease control. Each pathogen identification method used to address the complexity of Colletotrichum species identification has advantages and disadvantages. The traditional identification technique, which is based only on pathogen morphology, seems to be straightforward. However, it is time-consuming and this study clearly exhibited the inadequacy of morphologically-based identification to differentiate Colletotrichum species. Identification by sequencing or species-specific amplification provided rapid and reliable identification to species complex by using PCR. However, the disadvantages of this method include the possibility for DNA contamination or inaccurate identifications of standards in Genbank, as well as insufficient resolution to differentiate species within species complexes of Colletotrichum. Multi-locus sequence based identification is currently the preferred method for discrimination of species within the species complexes, and it was applied successfully for this study. However, this technique required more advanced methods including sequencing technology and cost more compared to other techniques. My research findings demonstrated the importance of identifying and studying the behavior of species within the species complexes of Colletotrichum. Findings suggest that species within each species complex differed in pathogenicity and in fungicide sensitivities. Thus it is important to be able to apply multi-locus sequence-based identification. Future work should include development of specific primer pairs based on the variable multi-locus sequences. For the apple industry in Kashmir, these research findings provide some important information about the causal agent of bitter rot. Most importantly, *C. fiorim'ae* appears to be the most common species causing bitter rot in Kashmir orchards; it also appears to be relatively tolerant to common fungicides that are used in Kashmir orchards. However, the ECSO of some common

fungicides to *C. fioriniae* showed in this study are still far below the lowest label rates; thus thiophanate-methyl, myclobutanil, trifloxystrobin and captan should still provide control for *C. fioriniae* in Kashmir orchards, provided optimal conditions and coverages with fungicides are achieved. However, optimal condition and optimal coverage of fungicide in fields is often difficult to achieve, and this may lead to variation in efficacy. Applications of protectant fungicides and/or other fungicides based on local recommendations is critical. Growers should also incorporate cultural practices such as maintaining orchard sanitation and monitoring disease incidence and disease spread. Preliminary fingerprinting analyses using the RAPD technique suggested that the dominant *C. fioriniae* population within the state was relatively homogenous. However, further studies on the genetic diversity of isolates using more stringent fingerprinting techniques such as RFLP are necessary to evaluate the genetic diversity of species. Genetic diversity data will be useful to describe how the population disperses in nature. A genetic diversity study might also be expanded to include these same species that infect different crops surrounding orchards, such as blueberry and strawberry, in order to study cross-infection potential, diseases cycle and potential cultural practices to control diseases. Caused by these pathogens. Cross infection potential of species could also be evaluated by conducting infectivity tests (Koch's postulates) to different fruits. Differences in sporulation of *Colletotrichum* spp. on two different apple cultivars suggest that planting more resistant or tolerant cultivars might be able to reduce the rate of disease spread in Kashmir orchards. However, field research would be needed to test this hypothesis, since my work was done in the laboratory with a limited numbers of isolates. Field studies on the pathogenicity of species-within-species complex is needed to illustrate the ability of each species in causing disease in orchards and to show whether or not some species are experiencing fitness penalty in field.

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