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Detection and Identification of *Botrytis* Species Associated with Neck Rot, Scape Blight, and Umbel Blight of Onion

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Primary Economic Host: Onion (*Allium cepa* L.)

Diseases: Neck rot, scape blight, and umbel blight

Pathogens: *Botrytis allii* (Munn) Yohalem, *B. aclada* (Fresen.) Yohalem, *B. byssoidea* J.C. Walker, and *B. squamosa* J.C. Walker

Taxonomy

Seven *Botrytis* spp. have been associated with diseases of *Allium* crops (Table 1) (19,34). Four of these species have known teleomorphs in the genus *Botryotinia*, family Sclerotiniaceae, order Helotiales, and class Discomycetes (21). Three species are considered the primary causal agents of neck rot of onion, namely *B. aclada*, *B. allii*, and *B. byssoidea* (22,55). Although *B. squamosa* and *B. tulipae* have also been associated with neck rot, these species are not typically primary causes of neck rot (34,45,46).

Table 1. Species of *Botrytis* associated with onion crops.

Anamorph	Teleomorph	Onion diseases[x]
B. aclada (Fresen.) Yohalem	Unknown	Neck rot, bulb rot, scape blight, and umbel blight
B. allii (Munn) Yohalem	Unknown	Neck rot, bulb rot, scape blight, and umbel blight
B. byssoidea J.C. Walker	*Botryotinia allii* (Sawada) Yamamoto	Mycelial neck rot
B. cinerea Pers.:Fr.	*Botryotinia fuckeliana* (de Barry) Whetzel	Botrytis brown stain (cosmetic), Botrytis leaf blight, umbel blight
B. porri Buchw.	*Botryotinia porri* (van Beyma Thoe Kingma) Whetzel	Neck rot of leek and garlic, but recently detected in onion seed crops and onion seed[y]
B. squamosa J.C. Walker	*Botryotinia squamosa* Vien.-Bourg.	Botrytis leaf blight, neck rot, umbel blight
B. tulipae (Lib.) Lind	Unknown	Neck rot, bulb rot

[x] Adapted from Mohan and Schwartz (34).

[y] Although primarily a pathogen of garlic and leek, *B. porri* has been isolated from naturally-infected onion seed crops and onion seed (11,12).

Until the recent taxonomic and nomenclatural clarification by Yohalem et al. (55) of the primary onion neck rot species of *Botrytis*, *B. aclada* (Fresen.) Yohalem was considered by many to be synonymous with *B. allii* (Munn) Yohalem (21), while *B. byssoidea* J.C. Walker was regarded by some as conspecific with *B. aclada* (25). The lack of distinction among these species was due, in part, to limitations at differentiating these species using classical morphological and cultural methods (41,42). Nonetheless, Owen et al. (39) demonstrated that *B. byssoidea* and *B. aclada* are valid species. Two subgroups within *B. aclada* (AI and AII) have been distinguished based on chromosome number and conidial dimensions (20,47). More recently, polymerase chain reaction (PCR) alleles and internal transcribed spacer restriction fragment length polymorphisms (ITS-RFLPs) have been used to demonstrate that isolates in subgroups AI and AII are distinct from *B. byssoidea* (36,37,38). Nielsen and Yohalem (37) concluded that the larger-spored subgroup (AII) developed as a result of a hybridization event between a small-spored isolate of *B. aclada* and an isolate of *B. byssoidea*. Yohalem et al. (55) proposed that *B. aclada* be reserved for the small-spored subgroup (AI), and *B. allii* for the larger-spored subgroup (AII) of *B. aclada*. A recent evaluation of the molecular phylogeny of the genus *Botrytis*, based on three nuclear protein-coding genes (*RPB2*, *G3PDH*, and *HSP60*), supports this proposed hybrid status (48). Molecular markers were used in that study to revise the phylogenetic relationships within the whole *Botrytis* genus, and may aid in identifying *Botrytis* species (48).

Symptoms and Signs

Onion bulb crops. Symptoms of neck rot typically develop only after onion bulbs have been harvested and placed in storage, even though infection occurs in the field (28,29). The predominantly latent nature of field infections (12,25) complicates efforts to manage this disease and to predict losses in storage. Under very moist field conditions, *Botrytis* conidiophores and conidia may be visible on senescent plant tissues. However, differentiating *Botrytis* species associated with onion tissues requires culturing the fungi on agar media, and/or measurement of conidial dimensions using a compound microscope. During storage, infected onion bulbs develop a semi-watery decay which usually begins inside the neck with no external symptoms visible (Fig. 1A). Infection may spread through the entire bulb (Fig. 1B). Infection can occur at wound sites on other areas of the bulb, in which case the disease may be referred to as shoulder rot, side rot, or bottom rot (Fig. 1C to 1D). White mycelium develops between bulb scales, producing compact gray masses of conidiophores and conidia (Fig. 1D). Dark brown to black, typically flattened sclerotia may form between scales in the neck of the bulb or at other sites of infection (Fig. 1B) (25). Brown stain, caused by *B. cinerea*, differs from neck rot in that infection is typically limited to the outermost dry scales of the bulb, resulting in a cosmetic brown blemish on the surface of the bulb (Fig. 2) (7). In addition to post-harvest losses from neck rot, *B. aclada* and *B. allii* can reduce plant stands and vigor (8), particularly when plants are injured (e.g., by windblown sand, hail, or chemicals) during cool and moist conditions (Fig. 1E to 1G).

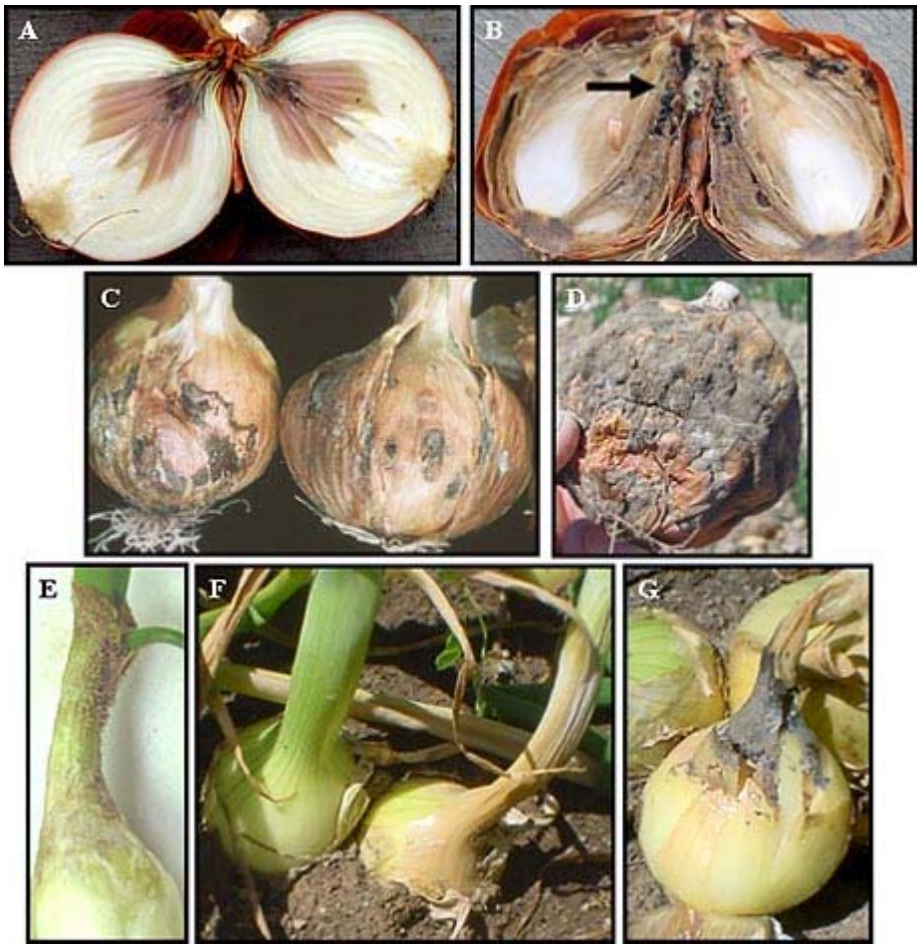

Fig. 1. Common postharvest symptoms (A to D), and less common preharvest (E to G) symptoms, caused by *Botrytis aclada* and *B. allii* on onion bulbs. (A) Internal symptoms of neck rot, including watersoaking and discoloration of the inner scales with white to gray mycelium in the neck. (B) Severe neck rot showing decay of the fleshy scales, shrinkage of the bulb, and black sclerotia (arrow) forming in the neck and shoulders of the bulb. (C) Soil line rot resulting from infection of the bulb at the soil surface (photo courtesy of G.Q. Pelter). (D) Dense sporulation of *B. aclada* or *B. allii* on the dry outer scales of a bulb from a cull pile. (E) Sporulation of *B. aclada* or *B. allii* on the surface of an onion plant under moist field conditions. (F) Neck rot developing on a plant under particularly moist field conditions close to harvest (healthy plant on the left). (G) Sporulation of *B. allii* or *B. aclada* revealed by pulling back the outermost fleshy scale of an infected plant after very moist field conditions.

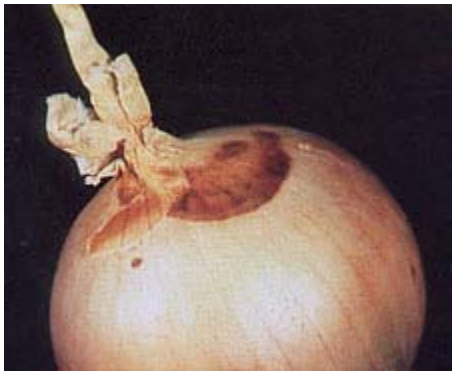

Fig. 2. Brown stain, also known as coffee stain, on the outer dry scales of an onion bulb caused by *Botrytis cinerea* (photo courtesy of Field Fresh Tasmania, Australia).

Onion seed crops. In onion seed crops, blighting and girdling of the scapes (seed stalks), and umbel and flower blight (Fig. 3) have been associated primarily with *B. allii* (usually not differentiated from *B. aclada*), but also with *B. byssoidea*, *B. cinerea*, and *B. squamosa* (1,9,12,13,17,23,31,35, 44,45,46,54,55). Lesions may develop at any point along the scape, typically girdling the scape and causing the scape to lodge, particularly during windy conditions. Scape blight lesions are initially elongated to diamond-shaped and chlorotic, becoming necrotic as they mature. Gray concentric rings may become evident as the fungus produces spores in concentric rings beneath the outer cuticle and wax layers of the scape (Fig. 3B and 3C). Scape and umbel blights reduce seed yield and quality, and may result in infected seed. Seedborne inoculum of *B. allii* and *B. aclada* can be a source of infection resulting in outbreaks of neck rot (28,29,51).

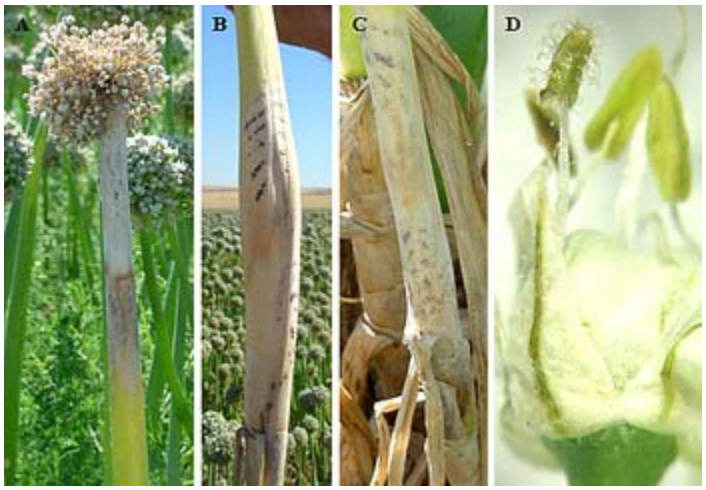

Fig. 3. Symptoms of umbel (A), scape (A to C), and flower blight (D) caused by *Botrytis aclada* and *B. allii*. Lesions can occur at the top (A), middle (B), or lower end (C) of the scape, often resulting in lodging once the lesion has girdled the scape, which affects seed set. Concentric grey rings may form in the girdling lesions as a result of sporulation of the fungus beneath the outer cuticle. The fungi sporulate on senescing tissues, including the spadix (membranous sheath that covers the immature umbel), anthers, and petals (D).

Host Range

The host range of *Botrytis* spp. associated with neck rot, scape blight, and umbel blight appears to be limited to most *Allium* spp., i.e., bulb onion (*A. cepa*), garlic (*A. sativum*), leek (*A. porrum*), shallot (*A. cepa* var. *ascalonicum*), potato or multiplier onions (*A. cepa* var. *aggregatum*), and possibly *Allium* weeds (25). However, Tichelaar (50) observed *B. allii* growing saprophytically on decaying plant material of cereals, flax, lucerne, bean, and pea. Chilvers (3) demonstrated that, in the absence of competition, *B. allii* was capable of colonizing and sporulating profusely on sterilized poppy (*Papaver somniferum*) debris.

Geographic Distribution

Onions are an important food crop worldwide. In 2005, approximately 65,000 hectares of storage and non-storage onions were harvested in the US, representing a farmgate value of $922 million (USDA National Agricultural Statistics Service). Losses to the onion industry from neck rot occur worldwide, but are variable and sporadic with some seasons bringing greater than 50% losses to individual crops (10,28,29,31). Neck rot species of *Botrytis* are distributed in all areas of the world where onions are produced, but greatest losses have been reported from temperate regions (4,5,10,12,28,29,31,36,38). Although *B. allii* and *B. aclada* are the predominant species reported to cause neck rot of onion, these species are difficult to distinguish morphologically because of similar growth patterns on agar media, and overlapping spore sizes

(55). Although *B. byssoidea* is only occasionally reported as causing neck rot (42,53), the distribution and significance of *B. byssoidea* as a neck rot pathogen may be underestimated compared to *B. allii* and *B. aclada* because of the difficulty of identifying *B. byssoidea*, which sporulates sparsely on most media used to isolate fungi from plant tissues (41,42).

Previous reports on neck rot typically have not differentiated between *B. allii* and *B. aclada*, making it difficult to discern the relative distribution and significance of these two species as neck rot pathogens. However, since description of a PCR-RFLP assay by Nielsen et al. (38) for differentiation of the primary neck rot *Botrytis* spp., *B. allii* and *B. aclada* can be differentiated more readily. Nielsen et al. (36) reported that of 29 isolates collected from Belgium, Canada, Denmark, England, Germany, Japan, the Netherlands, Norway, Scotland, and the US, and identified morphologically as *B. allii* or *B. aclada*, 14 and 15 of the isolates were identified as *B. aclada* and *B. allii*, respectively, using the PCR-RFLP assay. An additional seven isolates were identified morphologically as *B. byssoidea*, but PCR-RFLP analysis determined one isolate to be *B. allii* and three to be *B. squamosa*, highlighting the difficulty of morphological differentiation of these species (36). Using the PCR-RFLP procedure, Chilvers et al. (5) examined 23 isolates from onion crops in Australia that had been identified morphologically as *B. allii* or *B. aclada*. The results differentiated 22 of the isolates as *B. allii* and 1 as *B. aclada*. du Toit et al. (12) collected 469 isolates identified morphologically as *B. allii* (undifferentiated from *B. aclada*) from onion seed crops and onion seed in central Washington. Using PCR-RFLP analysis, 60 and 40% of the isolates were identified as *B. aclada* and *B. allii*, respectively (Chilvers and du Toit, *unpublished*). These studies demonstrate that both *B. allii* and *B. aclada* appear to be distributed worldwide. Further research is needed to evaluate *B. aclada*, *B. allii*, and *B. byssoidea* for differences in epidemiological significance as neck rot pathogens.

B. squamosa is primarily a leaf blight pathogen of onion in temperate regions of bulb or seed production during seasons with high relative humidity and rainfall (44,45,46). Although *B. squamosa* is occasionally associated with neck rot, scape blight, or umbel blight in these regions, this species does not appear to be associated with neck rot, scape blight, or umbel blight in the semi-arid regions of onion bulb and seed production in the western US (12,17,24).

Pathogen Isolation

Plants. An effective method of inducing sporulation of *Botrytis* spp. on onion plants with latent infections by these fungi was described by du Toit et al. (12). Whole plant samples were placed in plastic bags in a cool (5 to 10°C) dark environment for 4 to 7 weeks. The plants were then removed from cold storage, cut longitudinally, and placed into clear plastic containers lined with moist paper towel to create moist chambers. The plants were incubated in the moist chambers at ambient temperature (24 to 28°C) on approximately a 12-h/12-h day/night cycle for 5 to 7 days, and then examined macroscopically and microscopically for development of *Botrytis* conidiophores and conidia (Fig. 4). Surface-sterilizing the plants with 0.5% NaOCl for 60 s, then rinsing the plants with tap water and incubating them in a moist chamber, did not affect the efficacy of this protocol for triggering latent infections to become active and promote sporulation of *Botrytis* spp. (12). The duration of cold storage needed to trigger latent infections has not been determined, but Kritzman (23) demonstrated that storing onion bulbs for 2 days at 4°C, followed by 16 days at 15°C, activated latent infections by *B. allii* (undifferentiated from *B. aclada*). This, in turn, altered the pH of the infected bulb tissue. The change in pH was then visualized by cutting the bulbs longitudinally and staining them with an alcoholic solution of bromocresol green and methyl red. Healthy bulbs stained green but infected bulbs stained red. Alternatively, detached onion leaves can be placed directly into plastic containers lined with moist paper towel, and incubated at room temperature for 7 days. *Botrytis* conidiophores and conidia develop as infected tissues senesce (4,28).

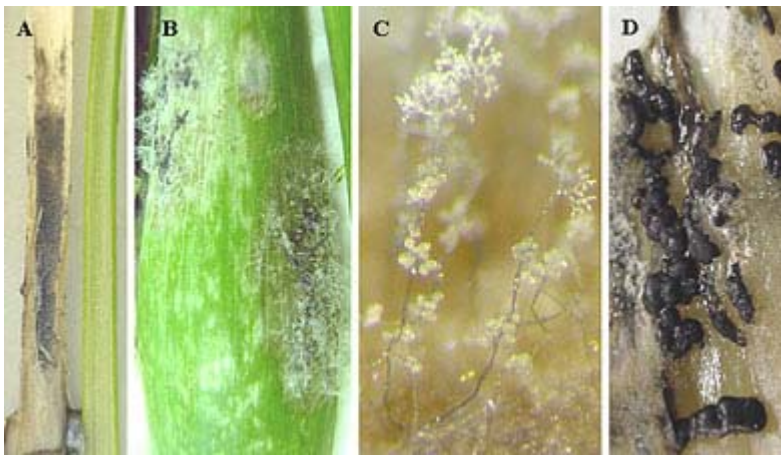

Fig. 4. Detection of latent infection by *Botrytis aclada* and *B. allii* on asymptomatic onion plants sampled from an onion seed crop, placed in cold storage (5 to 10°C) for four weeks, and then incubated in a moist chamber for 5 to 7 days. (A) Dense sporulation on an onion scape (noninfected scape on the right). (B) White mycelium and gray sporulation on a scape. (C) Conidiophores and conidia (48× magnification) of *B. aclada* or *B. allii*. (D) Sclerotia and sporulation of *B. aclada* or *B. allii* on an infected scape.

Pure cultures of *Botrytis* spp. can readily be established by transferring conidia onto agar media from the host material on which the fungi are sporulating. Potato dextrose agar (PDA) or similar general media support growth of all *Botrytis* spp. associated with *Allium* crops, although media differ in the propensity to promote sporulation. Agar media may be amended with antibiotics (e.g., streptomycin at 100 ppm) to reduce bacterial contamination. Single-spore isolates can be obtained by streaking conidia, or by dilution plating (18).

Seed. Various procedures can be used to isolate *Botrytis* spp. from onion seed, including plating seed onto agar media or incubating seed on damp blotters. However, there is currently no certified, internationally-accepted protocol for assaying onion seed for neck rot fungi. This compounds the controversy over the relative significance of seedborne inoculum vs. alternative sources of inoculum (infested onion residues, culled onions, infected volunteers, and soilborne sclerotia) of *B. allii* or *B. aclada* in development of neck rot in storage (12,25,26,28,29,31,51). The controversy is not addressed by this paper. The most common difficulty when assaying onion seed for *Botrytis* spp. is the presence of fast-growing fungi that outgrow *Botrytis* spp. present in or on the seed. In particular, species of *Rhizopus* and *Aspergillus* may prevent, or significantly limit, detection of *Botrytis* on some onion seed lots, unless selective agar media are used (Table 2). Two versions of an agar media assay for detecting *Botrytis* spp. associated with onion seed were described by du Toit et al. (12):

(i) *Surface (non-sterilized) seed assay:* To assay onion seed for infestation by *Botrytis* spp., onion seed was rinsed under running deionized water for 60 min to remove spores of fast-growing contaminant fungi present on the seed surface, and to assist with removal of fungicides on the surface of treated seed. The rinsed seed was then dried in a laminar flow hood on sterile paper towel, and plated onto Kritzman's agar (22) with 20 seeds spaced uniformly in each 10-cm-diameter Petri plate (Fig. 5). The plates were then sealed with Parafilm and incubated at 20°C with a 12-h/12-h day/night cycle (cool white fluorescent light and near ultraviolet light by day) for 14 days. The seed were examined microscopically (up to 100× magnification) at approximately 4-day intervals for up to 14 days after plating, for development of *Botrytis* spp. (12).

(ii) *Internal (surface-sterilized) seed assay:* To differentiate surface-contamination (infestation) from infection of onion seed by *Botrytis* spp., onion seed can be surface-sterilized using a disinfectant, rinsed thoroughly, and assayed for *Botrytis* spp. du Toit et al. (12) rinsed onion

seed in 0.5% NaOCl with agitation for 60 s, triple-rinsed the seed in sterile deionized water, and then air-dried, plated, and examined the seed as described above for the non-sterilized seed assay. The particular disinfectant used, the concentration of the disinfectant, and the duration of surface-sterilization all affect the results of such seed assays (12,22,28,31,32,51).

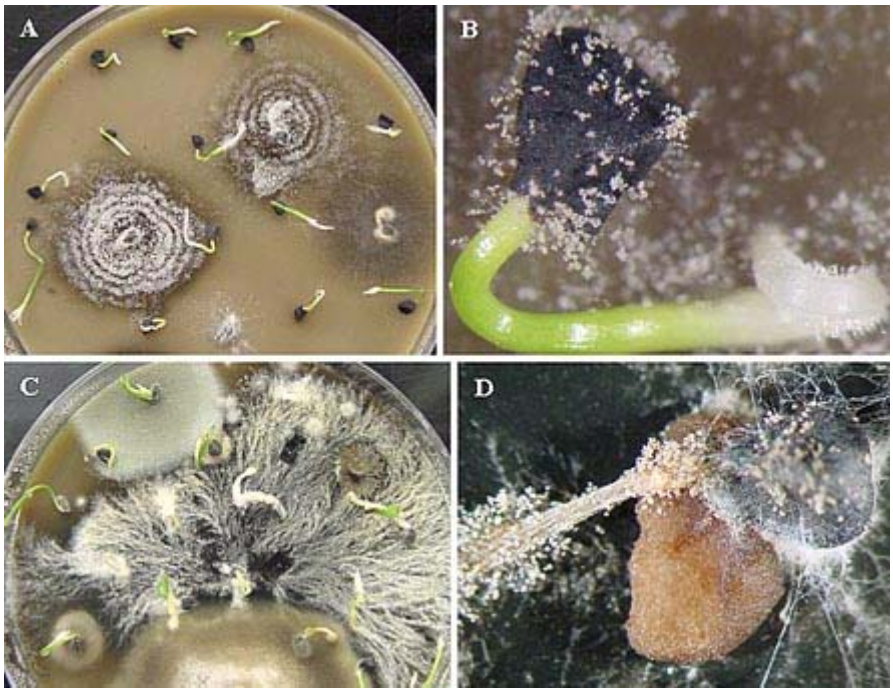

Fig. 5. Onion seed assayed for *Botrytis* spp. on Kritzman's agar (22). (A) Concentric rings of sporulation with darkening of the agar medium resulting from polyphenoloxidase (PPO) activity of *B. allii* or *B. aclada*. (B) *B. aclada* or *B. allii* sporulating on an onion seed. (C) Fast-growing, flat, white, fan-shaped or 'ropy' mycelium of *B. porri* that sporulates sparsely and also darkens Kritzman's agar as a result of PPO activity. (D) Sporulation and a brown sclerotium of *B. porri* developing from an infected onion seed.

Table 2. Efficacy of four agar media for determining the percentage of seeds in an onion seed lot that were infected with *Botrytis* spp. [adapted from Metcalf (32)].

	Agar medium[x]			
Characteristic assessed	**PDA**	**PLA**	**Kritzman**	**Pectin**
Mean percentage of seed with *Botrytis*[y]	5.3	5.4	4.4	5.2
Repeatability of nutrient consistency	+[z]	+	+++	+++
Freedom from contaminant fungi	+	+	+++	++
Internationally accepted for *Botrytis* spp.	+	+++	+++	+
Ease of preparation	+++	+	+	++
Ease of identifying *Botrytis*	++	++	+++	++

[x] PDA = potato dextrose agar, PLA = prune lactose agar, Kritzman = agar medium developed by Kritzman and Netzer (22), Pectin = pectin agar (33). Refer to the appendix for details of the media.

[y] Three replications of 550 seeds were assayed on each medium (standard deviation = 1.5%).

[z] + = low, ++ = good, +++ = very good.

Soil. To isolate *Botrytis* spp. from soil, aliquots of a dilution series of soil samples suspended in water or dilute water agar (approximately 2% to help keep the soil in suspension) can be plated onto selective media such as those described by Kritzman and Netzer (22) or Lorbeer and Tichelaar (27). For the Kritzman and Netzer (22) medium, polyphenoloxidase (PPO) activity of *Botrytis* spp. converts the tannin in the medium to a dark brown pigment (Fig. 5). This, in combination with the antibiotic and fungicide amendments that inhibit or slow development of other fungi, makes the medium very selective for identification of *Botrytis* spp. (22). Kritzman and Netzer (22) found a positive correlation between inoculum concentration in soil and the number of brown-pigmented colonies observed on the medium. They were able to identify brown-pigmented colonies within 48 h of incubation at 24°C, and each of the colonies was verified as *B. allii* (not differentiated from *B. aclada*) upon sporulation after an additional 4 days of incubation. Kritzman's agar has been used by others to isolate *Botrytis* spp. from soil (3,30).

Differentiation of *Botrytis* spp. Isolated from Onion Plants

Macroscopic identification. *Botrytis* spp. are readily cultured on various agar media. Differentiation among the species associated with onion based on morphology is possible for most species, except to differentiate *B. allii* from *B. aclada* (Table 3, Fig. 6) (41,42). However, accurate species identification based on macroscopic morphology is difficult for non-sporulating cultures. Nielsen et al. (36) found that four of seven non-sporulating isolates initially identified using morphological characteristics as *B. byssoidea*, were later classified by DNA fingerprinting techniques as *B. allii* or *B. squamosa*. Spore production by these species is enhanced by near-ultraviolet light and by adding onion tissue to the medium (42).

Table 3. Morphological characteristics of *Botrytis* spp. isolated from onion crops when cultured on potato dextrose agar.

***Botrytis* species**	**Morphological characteristics**[w]				
	Optimum temp. (°C) **for sporulation**	**Production of conidiophores & conidia**	**Production of sclerotia**	**Colony color & morphology**	**Relative rate of growth**[x]
B. aclada	20	+ (abundant short conidiophores, about 1 mm long)	—	Tan (dense mass of conidiophores)	Intermediate
B. allii	20	+ (abundant short conidiophores, about 1 mm long)	—	Tan (dense mass of conidiophores)	Intermediate
B. byssoidea	15	—[y]	—	White to off-white mycelium	Intermediate
B. cinerea	15	+[z] (long, frequently 2 mm or more, dematiaceous, branched conidiophores)	+ (medium to large, smooth-surfaced, light to dark brown)	White mycelium, gray to brown colony as conidiophores mature	Fast
B. porri	—	+	+ (medium to large, brown, often cerebriform)	White, flat, 'ropy' mycelium	Fast
B. squamosa	5	—[y]	+ (abundant, small, black)	White mycelium that collapses onto agar surface	Intermediate

[w] Adapted from Presly (41,42), Ellis (14), and from observation of cultures on potato dextrose agar (PDA) (Fig. 6).

[x] Intermediate vs. fast growth rates = 6 to 10 vs. 3 to 6 days, respectively, for the fungus to cover the surface of a 10-cm diameter plate of PDA from a colonized 5 mm-diameter plug placed in the center of the plate (du Toit, *unpublished*).

[y] *B. byssoidea* and *B. squamosa* produce spores sparsely when exposed to near-ultraviolet light, but do not produce spores when cultured in the dark. *B. squamosa* produces a very marked concertina-like collapse of conidiophores.

[z] *B. cinerea* produces abundant spores when exposed to near-ultraviolet light, but very few spores in the dark. Sealing plates with Parafilm also may inhibit sporulation of *B. cinerea*.

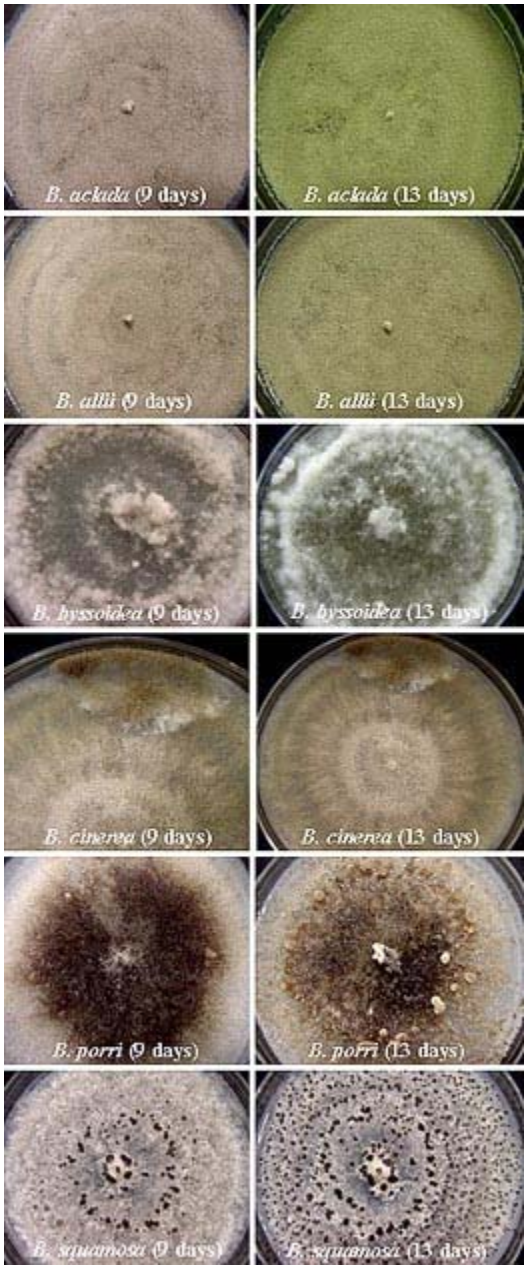

Fig. 6. Appearance of isolates of *Botrytis aclada*, *B. allii*, *B. byssoidea*, *B. cinerea*, *B. porri*, and *B. squamosa* on potato dextrose agar (PDA) after 9 or 13 days of growth at ambient temperature (25 ± 3°C).

Microscopic identification. *Botrytis* spp. associated with onion plants can be differentiated to some degree based on conidial dimensions. However, spore dimensions of isolates of the three predominant neck rot species overlap, complicating efforts to differentiate these species morphologically (Table 4, Fig. 7).

Table 4. Dimensions of conidia, average number of nuclei/conidium, and number of chromosomes/nucleus for *Botrytis* spp. isolated from onion or leek crops.

***Botrytis* species**	**Length of conidia** (µm)	**Width of conidia** (µm)	**Average number of nuclei/ conidium**[w]	**Number of chromo-somes/ nucleus**[w]
B. aclada	8.4 - 12.1[u] 6.5 - (8.6) - 11.0[v]	4.6 - 5.6[u] 4.0 - (4.6) - 6.0[v]	1.4	16
B. allii	9.7 - 14.1[u] 9.0 - (10.2) -15.0[v]	5.3 - 7.3[u] 5.0 - (5.7) - 6.5[v]	1.5	32
B. byssoidea	12.0 - 14.0[w] 12.0 - 15.0[x]	8.0 - 9.0[w] 6.5 - 8.5[x]	5.1	16
B. cinerea	9.0 - 11.0[w] 6.0 - 18.0[y]	6.0 - 8.0[w] 4.0 - 11.0[y]	4.6	16
B. porri	14.5 - 16.5[x]	8.5 - 11.0[x]	—[z]	—
B. squamosa	21.0 - 22.5[x]	16.5 - 17.0[x]	18.4	16

[u] Nielsen et al. (36).
[v] Yohalem et al. (55), with the mean dimension reported in parentheses.
[w] Shirane et al. (47).
[x] Presly (41).
[y] Ellis and Waller (15).
[z] "—" = unknown.

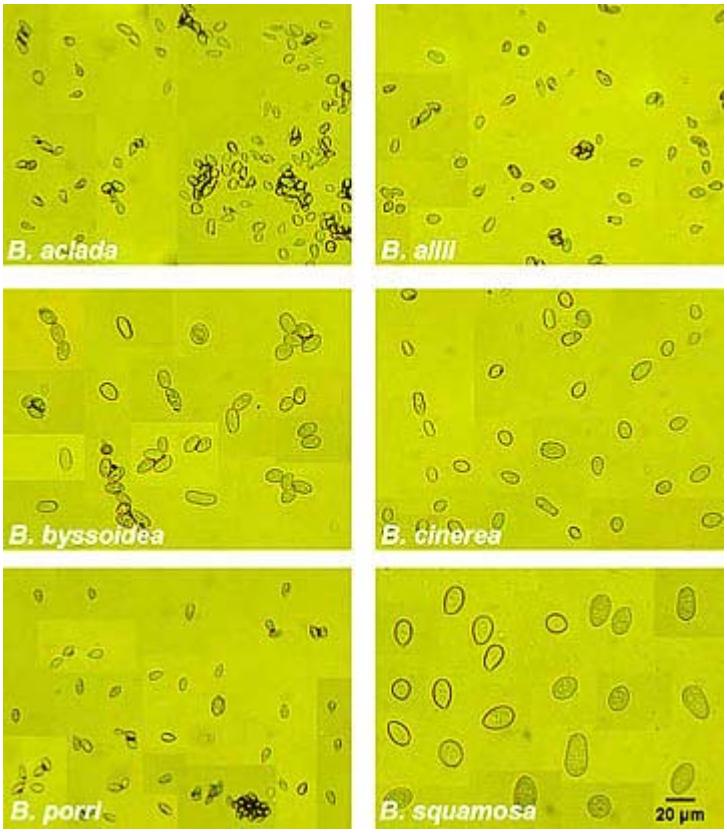

Fig. 7. Microscope photos (200x magnification) of conidia of each of six *Botrytis* species isolated from onion plants and grown on potato dextrose agar (PDA). Cultures were maintained for 7 to 14 days at ambient temperature (25 ± 3°C) with a 12 h/12 h day/night cycle (cool white fluorescent light), except for *B. byssoidea* and *B. squamosa* which were maintained at 15 ± 2°C under constant near-ultraviolet light for 7 days.

Key to *Botrytis* species on *Allium* based on macroscopic and microscopic characteristics. Use of this key, together with matching the *Botrytis* characteristics[x] observed with those shown in Table 3, Table 4, Fig. 6 and Fig. 7 will enable identification of most isolates:

1. Sclerotia readily produced on PDA . . . . (*B. cinerea*, *B. porri*, *B. squamosa*) 2

1'. Sclerotia not produced on PDA . . . . (*B. aclada*, *B. allii*, *B. byssoidea*) 3

2. Conidia longer than 20 µm and wider than 16 µm; sclerotia black and 1 to 3 mm in diameter . . . . . *B. squamosa*[y]

2'. Conidia shorter than 20 µm and narrower than 16 µm; sclerotia medium to dark brown and variable in size . . . . (*B. cinerea*, *B. porri*) 4

3. Conidia have a minimum length of 12 µm; very sparse sporulation on PDA; mycelium white to off-white on PDA . . . . . *B. byssoidea*

3'. Most conidia shorter than 12 µm; dense gray mass of conidiophores and conidia on PDA; sparse mycelium on PDA . . . . (*B. aclada*, *B. allii*) 5

4. Conidia have a minimum length of 14 µm and a minimum width of 8 µm; conidiophores about 1 mm long; sclerotia smooth to cerebriform . . . . . *B. porri*

4'. Conidia often shorter than 14 µm and often narrower than 8 µm; conidiophores typically 2 mm or longer; sclerotia smooth-surfaced
. . . . . *B. cinerea*

5. Conidia have a maximum length of 14 to 15 µm, and mean dimensions of 10.2 × 5.7 µm
. . . . . *B. allii*

5'. Conidia have a maximum length of 11 to 12 µm, and mean dimensions of 8.6 × 4.6 µm
. . . . . *B. aclada*

[x] Excluding *B. globosa*, which has spherical to sub-spherical conidia, commonly 12 to 18 µm long, and which occurs on wild garlic, *Allium ursinum*; excluding *B. sphaerosperma*, which has spherical conidia commonly 20 to 26 µm long, and which occurs on three-cornered leek, *Allium triquetrum*; and excluding *B. tulipae* (records on *Allium* are extremely infrequent).

[y] In Ellis (14), conidia of *B. squamosa* are more highly variable in length than indicated in Table 4, but are up to 26 µm long; and also highly variable in width, but up to 18 µm wide, i.e., in spite of variability, the maximum length and maximum width of conidia exceed those of other species.

Molecular Identification and *Botrytis* Detection

PCR detection and RFLP differentiation of *Botrytis* spp. causing neck rot. Nielsen et al. (38) developed the following DNA primers for amplification and subsequent differentiation of five *Botrytis* spp. associated with neck rot, using sequence characterized amplified regions (SCARS):

BA2f: 5'-GTGGGGGTAGGATGAGATGATG-3'
BA1r: 5'-TGAGTGCTGGCGGAAACAAA-3'

Using a polymerase chain reaction (PCR) assay, the primers amplify a 413 bp DNA product from all five neck rot species. Restriction fragment length polymorphism (RFLP) analysis of the PCR amplicon using the restriction enzyme *Apo*I distinguishes the following diagnostic DNA bands (Fig. 8) (38):

B. aclada AI = 413 bp,
B. allii (*B. aclada* AII) = 413 + 298 bp,
B. byssoidea = 298 bp,
B. cinerea = 250 bp,
B. squamosa = 269 bp.

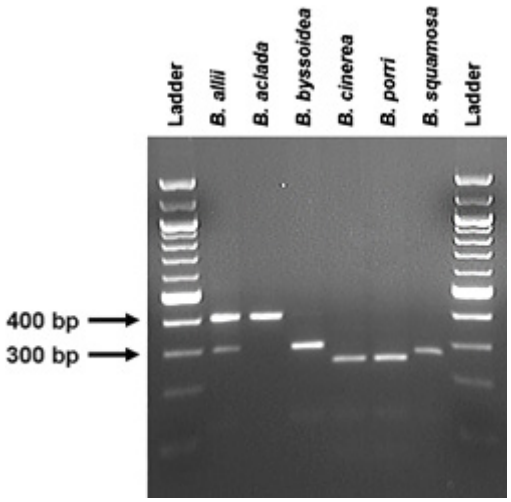

Fig. 8. PCR-RFLP analysis using the protocol described by Nielsen et al. (38) for six *Botrytis* spp. isolated from onion plants or seed. 100 bp ladder (New England Biolabs, Beverly, MA). Arrows indicate 300 and 400 bp DNA ladder fragments.

Although Nielsen et al. (38) apparently did not amplify DNA of *B. porri* using these primers, we detected an amplicon of similar size which showed the same restriction pattern as *B. cinerea* for isolates of *B. porri* obtained from onion plants and seed in Washington (Fig. 8) (Chilvers and du Toit, *unpublished*).

Magnetic capture hybridization (MCH)-PCR. Although the PCR-RFLP assay described by Nielsen et al. (38) facilitates more rapid detection and differentiation of neck rot species of *Botrytis* compared to traditional methods of isolating the fungi onto selective media, onion seeds contain compounds that inhibit the PCR and can yield false-negative results (52). To overcome this problem, Walcott et al. (52) developed a magnetic capture hybridization (MCH)-PCR protocol for seedborne *Botrytis* spp. In summary, magnetic beads were coated with DNA probes based on the SCAR described by Nielsen et al. (38). The probes captured the fungal DNA from crude seed extracts, and PCR inhibitors were washed off the captured DNA, which was then amplified by PCR using the primers described by Nielsen et al. (38). The MCH-PCR assay reduced the time required to assay onion seeds from 10 to 14 days when using selective agar media, to less than 24 h. However, the agar assay yielded higher detection frequencies of *B. aclada* for seed lots with low infection levels (2.1%) compared to the MCH-PCR assay, and the MCH-PCR assay lacked adequate reproducibility or the ability to quantify the incidence of infection in individual seed lots. With further refinement, and in combination with quantitative real-time PCR, the MCH-PCR assay has potential as a rapid commercial method of screening onion seed lots for infection by *Botrytis* spp. (52).

Real-time PCR assay. A real-time fluorescent PCR assay based on SYBR green chemistry has been developed to quantify the amount of neck rot *Botrytis* spp. (*B. allii*, *B. aclada*, and *B. byssoidea*) present in onion seed (6). The nuclear ribosomal intergenic spacer (IGS) region of target and non-target *Botrytis* spp. was sequenced, aligned, and used to design primers specific to *B. allii*, *B. aclada*, and *B. byssoidea*. Primers and amplification parameters were optimized to avoid amplifying the related species *B. cinerea*, *B. porri*, *B. squamosa*, and *Sclerotinia sclerotiorum*, as well as 15 other fungal species commonly found on onion seed. The primers were capable of detecting 10 fg genomic DNA extracted from pure cultures of *B. allii* and *B. aclada* (6). The real-time PCR assay appears to be more sensitive than plating onion seed onto selective agar media, as the PCR assay enabled DNA of *Botrytis* neck rot species to be detected from seed lots that tested negative using conventional seed plating assays (6).

ELISA assays. Linfield et al. (26) developed polyclonal antisera for an indirect ELISA assay to detect latent *B. allii* (undifferentiated from *B. aclada*) infection in stored onion bulbs. The ELISA enabled earlier detection of *B. allii* in bulb tissue compared with conventional isolations onto agar media. The antisera did not cross-react with *B. byssoidea*, *B. porri*, or *B. squamosa*, but showed some cross-reaction with *B. cinerea*. Antiserum developed to cell wall extracts of *B. allii* displayed less cross-reaction with *B. cinerea* than antiserum developed from surface washings of *B. allii* cultures, as the latter contained whole spores and mycelial fragments. Linfield et al. (26) also demonstrated that non-uniform infection of onion neck tissue by *B. allii* necessitates sampling whole slices across the neck of each bulb for more accurate detection of the incidence of bulbs with latent infection. Neogen Europe Ltd. (Auchincruive, UK) offers a commercial, non-quantitative ELISA IDENTIKIT for *B. allii* (undifferentiated from *B. aclada*). To our knowledge, this is the only commercial ELISA kit available for onion neck rot fungi.

Storage of isolates. Isolates of *Botrytis* spp. can readily be stored using a number of methods, e.g., on colonized PDA slants held at 4°C (16), as spore suspensions in 15% glycerol stored at −80°C (36), or on colonized and dried filter paper stored at −20°C (40) (du Toit and Chilvers, *unpublished*).

Pathogenicity Tests

Inoculum production. Spores of *B. allii* and *B. aclada* can be produced readily on a variety of media, including PDA and onion leaf extract agar (37), or on infected onion tissue (senescing leaves or infected bulbs) maintained under

high relative humidity and at moderate temperatures (15 to 25°C). A conidial suspension can then be prepared in water. Adding a small amount of surfactant (e.g., Tween 80 at 0.01% v/v) counteracts the hydrophobic nature of *Botrytis* spores in suspension. Alternatively, naturally infected, decaying onion bulbs with symptoms of neck rot can be used as inoculum, particularly as a source of sclerotia. Production of sclerotia in culture can be achieved by autoclaving 10 g unprocessed natural bran and 250 g sand (<500-µm-diameter particles) in a flask, and inoculating the contents of the flask with a conidial suspension of the pathogen. The inoculated bran-sand mixture is incubated for 6 weeks in the dark at approximately 20°C, and then the sclerotia are separated by wet-sieving the mix through a 500-µm mesh sieve (3).

Inoculum application. To inoculate onion bulbs with *B. allii* or *B. aclada*, a spore suspension of the pathogen can be injected into the neck of each bulb using a hypodermic needle and syringe, or the necks can be cut horizontally and a spore suspension or a colonized disk of agar medium applied to the freshly-cut surface of each bulb (29,42). Inoculated bulbs should be stored at ambient temperature (15 to 25°C) for 2 to 4 weeks, and then cut longitudinally and incubated at high relative humidity for about 7 days to promote sporulation of the pathogen.

To inoculate onion plants with neck rot species of *Botrytis*, a spore suspension of the pathogen can be sprayed onto plants. Inoculated plants are maintained under high humidity for 2 to 3 days at moderate temperatures (15 to 25°C), where possible, to promote spore germination and establishment of infection. In field trials, plants should be inoculated during cool and moist conditions that promote extended periods of leaf wetness (3,33). Alternatively, neck rot-infected bulbs can be placed within the field as a source of inoculum (3,33).

Pathogenicity tests. Yohalem et al. (55) tested the virulence of *Botrytis* isolates by placing an agar disk with mycelium of the appropriate isolate onto the cut neck of each onion bulb. The depth of maceration of each inoculated bulb was assessed after incubating the bulbs in a dark moist chamber at 18°C for 14 days. Stewart and Mansfield (49) placed drops of a conidial suspension of each *Botrytis* isolate on detached onion leaves or onion scales to test for pathogenicity.

Detecting Latent *Botrytis* Infection in Onion Bulbs

Latent *Botrytis* infections in onion bulbs are typically detected by storing harvested bulbs for 8 to 16 weeks, and then cutting the bulbs vertically to assess for internal symptoms of neck rot (4). However, the need exists for a rapid predictive test at, or prior to, harvest to determine the incidence of bulbs with latent infection. This would facilitate decisions regarding storage, processing, and sale of the bulbs. As described above, Kritzman (23) developed a method of detecting latent bulb infections by *B. allii* that was based on triggering latent infections to become active by changing the temperature during incubation of the bulbs. The pH in infected bulb tissues dropped to 4.4 when infection by *B. allii* was activated, compared to pH 5.2 for healthy tissue. The pH change was visualized using bromocresol green and methyl red, which stained infected tissue red and healthy tissue green. Kritzman (23) stated that, although culturing bulb tissue onto selective agar media provided more accurate diagnosis than the staining method, the latter enables large numbers of bulbs to be tested at very low cost without the need for laboratory facilities. However, other factors can cause a change in the pH of bulb tissues, which could result in false positives when using this staining procedure (33).

Chilvers et al. (2) attempted to predict the incidence of neck rot in onion bulbs by removing a core of neck tissue from each bulb using an apple corer, and incubating the core for 7 days in humid conditions. They were unable to detect a consistent relationship between the number of bulbs from which the sampled cores developed *Botrytis* spp. and the number of bulbs that developed neck rot in storage (2,3). In contrast, the indirect ELISA developed by Linfield et al. (26) gave a "good indication" at harvest of the probable incidence of neck rot in storage, for both non-inoculated plants and plants inoculated in the field with *B.*

allii. Isolations from bulbs onto agar media consistently showed a lower incidence of latent infection detected than the ELISA, until the bulbs had been stored for several months, by which time the ELISA and conventional isolation methods gave similar results. The researchers noted the necessity of sampling whole slices across the neck of each bulb for accurate detection of latent infection, because of non-uniform infection of onion neck tissue by *B. allii*. This might explain the poor results for the core sampling method evaluated by Chilvers et al. (2,3).

Prithiviraj et al. (43) demonstrated that detecting volatile metabolites can be used to discriminate among onion bulbs infected by *Erwinia carotovora* subsp. *carotovora*, *Fusarium oxysporum*, and *B. allii*, and to differentiate healthy bulbs from infected bulbs. Onions inoculated with *B. allii* released 1-ethenyl-4-ethyl-benzene, whereas healthy bulbs and bulbs inoculated with the other two pathogens did not release this metabolite (43). Although sampling for volatile metabolites may facilitate determination of the incidence of bulbs infected with *Botrytis* in a bulb lot, the procedure may be limited to detection after an incubation period, combined with a protocol that triggers latent infections to become active, as described by Kritzman (23).

With any method of assessing the incidence of latent infection in onion bulbs for predicting the incidence of neck rot in storage, research is needed to determine the optimum sampling time and sample size for accurate prediction of the incidence of neck rot in storage, as noted by Linfield et al. (26). Although morphological and molecular tools are available for accurately differentiating among *Botrytis* spp. that infect onion plants, the predominantly latent nature of these infections remains a hurdle to managing the pathogens effectively.

Conclusions

Although traditional methods of isolating fungi onto standard agar media, followed by classic morphological and microscopic techniques, provide relatively easy and accessible methods to differentiate *B. porri*, *B. cinerea*, and *B. squamosa* from *B. aclada* and *B. allii* in association with onion tissues, accurate differentiation of *B. allii* and *B. aclada* is difficult using these techniques because of very similar morphological characteristics of the latter two species. Furthermore, identification of *B. byssoidea* is complicated by the fact that this species typically does not sporulate or form sclerotia on agar media commonly used to isolate fungi from plant tissues. Therefore, combining classic morphological and microscropic examination of cultures with newer molecular techniques, such as PCR-RFLP (38) or real-time PCR (6), will enable accurate differentiation of these species. Real-time PCR assays may prove valuable as a rapid means of detecting latent *Botrytis* infections in onion bulbs or in plant samples. The choice of which method(s) to use for detecting and identifying *Botrytis* species associated with onion samples essentially depends upon the resources available and the requirements for identification, i.e., for extension or diagnostic purposes, identifying the causal organism as one of the "neck rot species" (*B. allii*, *B. aclada*, or *B. byssoidea*) using isolations onto standard agar media, followed by microscopic examination of the cultures, is probably sufficient. However, confident identification to the species level may be required for detailed epidemiological or etiological research, which is possible with the combined use of morphological and molecular techniques.

Complicating this issue further is the lack of epidemiological research that demonstrates the relative significance of *B. byssoidea* vs. *B. aclada* vs. *B. allii* as neck rot pathogens under the diversity of environmental conditions in which onion bulb crops are grown. Until such information is generated, the three species probably should be considered of equal importance as causal agents of neck rot, and differentiation among the three species may not be as important for applied research purposes as differentiating these species from *B. porri*, *B. cinerea*, and *B. squamosa*. Even with the ability to differentiate accurately among all *Botrytis* spp. associated with onion plants, a suitable sampling strategy remains perhaps the biggest challenge to applying these techniques to estimate the incidence of onion plants or bulbs with latent infection in the field or at harvest as a predictor of the incidence of neck rot in storage.

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Appendix

Onion leaf extract agar

(i) Place the following ingredients into a 2 liter flask:

Agar	16 g
Dried onion leaves, cut or crushed	15 g
Distilled or deionized water to	1000 ml

(ii) Autoclave and dispense the medium into Petri plates.

Kritzman's (modified) agar [adapted from Kritzman and Netzer (22)]

(i) Place the following ingredients into a suitable flask:

$NaNO_3$	1.0 g
K_2HPO_4	0.9 g
$MgSO_4 \cdot 7H_2O$	0.2 g
KCl	0.15 g
Glucose	20.0 g
Agar	25.0 g
Distilled or deionized water to	1000 ml

(ii) Autoclave the suspension.
(iii) Add the following ingredients to the autoclaved suspension, starting with the tannic acid and ensuring it is dissolved completely before adding the copper sulfate:

Tannic acid	5.0 g
$CuSO_4$.hydrated	1.7 g

(iv) Adjust the pH of the medium to 6.0 using 1 N NaOH.
(v) Autoclave, cool the medium to 50°C, then add the following ingredients just prior to pouring:

PCNB (99%)	0.005 g
Dithane (8%)	0.005 g
Chloramphenicol	0.025 g

(vi) Dispense the medium into Petri plates.

Pectin Agar

(i) Mix the following ingredients in a blender for 1 to 2 minutes, until the pectin dissolves:

$NH_4H_2PO_4$	0.9 g
$(NH_4)_2HPO_4$	2.0 g
$MgSO_4 \cdot 7H_2O$	0.1 g
KCl	0.5 g
Citrus Pectin	10 g
Agar	30 g
Distilled or deionized water to	1000 ml

(ii) Adjust the suspension to pH 4.0 using HCl.
(iii) Autoclave and dispense the medium into Petri plates.