A Diagnostic Guide for Volutella Blight Affecting *Buxaceae*

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Pathogens

Volutella blight affecting plants in the *Buxaceae* family is primarily caused by three distinct fungal species: ***Coccinonectria pachysandricola*** (B.O. Dodge) L. Lombard & Crous, ***Pseudonectria buxi*** (DC.) Seifert, Gräfenhan & Schroers, and ***Pseudonectria foliicola*** L. Lombard & Crous. *Coccinonectria pachysandricola* causes Volutella blight on *Pachysandra* spp. (pachysandra) and *Sarcococca* spp. (sarcococca; sweet box), while *P. buxi* and *P. foliicola* cause Volutella blight primarily on *Buxus* spp.

(boxwood) (Castroagudín et al. 2021; Dodge 1944a, b, c; Lombard et al. 2015; Salgado-Salazar et al. 2019).

Volutella pachysandrae W. G. Hutch. was reported causing leaf blight of *Pachysandra terminalis* (Hutchinson 1929). Hutchinson (1929) described it as a weak pathogen of *Pachysandra terminalis* that is also capable of colonizing dead leaves of *B. sempervirens*. Several authors noted the morphological distinction between *C. pachysandricola* and *V. pachysandrae*: namely that *V. pachysandrae* produces conidia 2 to 6 µm long, versus the conidia of *C. pachysandricola* that measure 14 to 20 µm in length (Dodge 1944b; Pirone 1942; Samuels 1977). However, to the best of our knowledge *V. pachysandrae* has not been reported in the U.S. since the 1970s (Farr & Rossman 2020) and there is no evidence that the fungus has been associated with appreciable disease outbreaks since its original description in the early part of the 20th century (Dodge 1944b; Hutchinson 1929; Pirone 1942). Due to the limited information regarding the pathogen following its original description, the lack of a type specimen, and the absence of molecular validation of its species status and generic placement, *V. pachysandrae* is not discussed further in this guide.

In the past, two of the three primary species responsible for Volutella blight, *C. pachysandricola* and *P. buxi*, were known by different names (synonyms). *Coccinonectria pachysandricola* was previously known as *Volutella pachysandricola*. *Pseudonectria buxi* has gone by 25 different names since its original discovery in 1815, of which readers will be most familiar with the old name *Volutella buxi*. Although these fungi are now designated by the names of their sexual reproductive stage (Gräfenhan et

al. 2011; Lombard et al. 2015), it is their old names for the asexual stage that gave rise to the use of Volutella blight as the common disease name.

Taxonomy

Classification. Fungi causing Volutella blight affecting *Buxaceae*, namely *C. pachysandricola*, *P. buxi*, and *P. foliicola*, belong to the kingdom Fungi, subkingdom Dikarya, phylum Ascomycota, subphylum Pezizomycotina, class Sordariomycetes, subclass Hypocreomycetidae, order Hypocreales, and family *Nectriaceae*.

Electronic Resources. The current taxonomic status of *C. pachysandricola*, *P. buxi*, *P. foliicola*, and *V. pachysandrae* can be retrieved from the United States National Fungus Collections Fungal Database at https://nt.ars-grin.gov/fungaldatabases/ (Farr and Rossman 2020).

Symptoms and Signs

Volutella blight of boxwood. Although the symptoms of Volutella blight have led some to refer to the disease as a leaf blight and/or twig dieback, the disease is more properly categorized as a canker disease. This is because the visually striking canopy symptoms of affected plants are typically the outcome of canker-induced girdling. Cankers are most often located lower down on the same branch as symptomatic leaves (Fig. 1A) (Sinclair et al. 1987; White 1931).

Pseudonectria buxi and *P. foliicola* act as primary invaders of wounded stem tissues (Rivera et al. 2018; Shi and Hsiang 2014b). On mature boxwood, the result is often discoloration of one or more large sections of the canopy (Fig. 1B). This discoloration is often described as “yellowing”, but terms such as “straw-yellow” and “tan” are more descriptive of the dull foliage coloration that occurs as the foliage dries out due to girdling caused by supportive branch cankers (Dodge 1944a; Shi and Hsiang 2014b). Macrophoma leaf spot disease caused by the secondary colonizer *Dothiorella candollei* (formerly known as *Macrophoma candollei*) commonly occurs on weakened or dead boxwood foliage. Although the formation of black pycnidia of *D. candollei* (Fig. 1C) (Hansen 2009; White 1931) is sometimes considered as an indication that boxwood foliage is weakened or killed by *Pseudonectria* spp., particular attention should be paid to not confuse signs and symptoms of Volutella blight with those of Macrophoma leaf spot.

In addition to the presence of cankers, the base of boxwood branches supporting necrotic foliage often contains sections of loosened bark over darkly discolored wood (Fig. 1D) (White 1931). However, it is important to note that split and loosened bark may also be an indication of freeze injury rather than a symptom of Volutella blight (Hansen 2009; Malinoski et al. 2020). Freeze injury is innately harmful and creates wounds that predispose boxwood to infection by *Pseudonectria* spp.

Since branch cankers of Volutella blight often occur following wounding caused by snow load or winter freezing injury, disease symptoms often start to appear in spring. When first- and second-year shoots are affected by Volutella blight (in concert with frost injury, for example), short lengths of stem tissue are affected, and dark, brown to black

discoloration or streaking may be seen on the affected shoots (Fig. 1A) (Hansen 2009; Shi and Hsiang 2014b). Under humid conditions, *Pseudonectria* spp. sporulate conspicuously on host tissue, forming sporodochia on abaxial leaf surfaces that are initially whitish, then becoming pinkish-roseate, salmon-, or coral-colored (Figs. 1E to 1I) (Dodge 1944a, c; Shi and Hsiang 2014b). Perithecia, like sporodochia, are produced from stomata, and range in color from grayish yellow green to straw-colored, to orangey-brown (Figs. 1J to 1L) (Rossman et al. 1993).

Only wounded leaves of *Buxus* are susceptible to infection by *Pseudonectria* spp. (Shi and Hsiang 2014b). Pathogen invasion of susceptible host tissue is quick when cut leaves are inoculated experimentally: sporulation appears only three days after infection (Shi and Hsiang 2014b). Infected leaves become discolored and sometimes abscise. Leaf infection may be facilitated by wounds made by boxwood leafminer or by shearing, a practice that is often carried out multiple times per year on highly maintained boxwood.

Information on possible differences in symptoms on different boxwood species other than *B. sempervirens* is limited. A report from China describes leaf spots on *B. bodinieri* with white centers and bronze boundaries (Wang et al. 2017), whereas such an appearance has not been noted on other species, such as *B. sempervirens* (American boxwood) and its hybrids and *B. sinica* var. *insularis* (Korean boxwood) (Shi and Hsiang 2014b).

Volutella blight can be readily distinguished from the boxwood blight disease caused by *Calonectria* spp. (Castroagudín et al. 2020) based on symptoms (Figure 2). Because of the extent of girdling and cankers associated with Volutella blight, the

overall pattern of injury is also markedly different from that of boxwood blight. With Volutella blight, large sections of the affected foliage, often a foot or more in diameter on larger shrubs, are discolored from the stem-girdling effect of cankers on major branches, while other parts of the plant may appear green and healthy (Fig. 1B). Damage due to boxwood blight, on the other hand, is primarily due to direct leaf and stem invasion by the causal pathogens, giving rise to many scattered areas of leaf and stem infection along the tops or sides of hedges or free-standing shrubs (Castroagudín et al. 2020). Another key symptomatological difference between Volutella blight and boxwood blight is defoliation: in the case of Volutella blight, affected leaves are typically retained on the branch for some time, whereas in the case of boxwood blight, defoliation usually occurs rapidly (Castroagudín et al. 2020).

Although the dieback symptoms of Volutella blight may resemble those of another boxwood disease, boxwood dieback caused by *Colletotrichum theobromicola* (Singh and Doyle 2017; Singh et al. 2015), several diagnostic traits can be used to differentiate these two diseases. First, the stems and branches affected by Volutella blight do not show the bright black discoloration under the bark typical of boxwood dieback (Singh and Doyle 2017). Second, *Pseudonectria* spp. often produce abundant sporodochia on the adaxial side of infected leaves, whereas those of the boxwood dieback fungus are mostly localized upon stems and twigs. Third, *Pseudonectria* spp. and *Colletotrichum theobromicola* are different in setae color. A comparison chart including diagnostic traits of these three major fungal diseases of *Buxus* spp., namely Volutella blight, boxwood blight, and boxwood dieback, is provided in Fig. 2.

Volutella blight of pachysandra. The symptoms of Volutella blight on pachysandra caused by *C. pachysandricola* are a familiar sight in many diagnostic laboratories. Leaf lesions are initially circular (Fig. 3A), then grow to form large, irregular blotches, light to dark brown in color, sometimes with a darker brown margin or yellow halo (Figs. 3B and 3C). A zonate pattern is often seen in the lesions (Figs. 3A to 3C), which may continue to expand until leaves and shoots are blighted (Figs. 3D and 3E) (Bai et al. 2012; Douglas 2008; Sinclair et al. 1987). Cankers initially appear water-soaked and darken from greenish brown (Fig. 3D) to darker brown or black (Fig. 3E), eventually expanding to such an extent that they may girdle stems and stolons (Figs. 3E and 3F) (Bai et al. 2012; Douglas 2008; Šafránková 2007; Sinclair et al. 1987). Circular salmon-colored (brighter pink-orange when moist) sporodochia, sometimes bearing hyaline setae, form on recently killed stems (Figs. 3D and 3E) and the abaxial leaf surface (Fig. 3G). During spring and summer months, orange to carmine-red perithecia with short, thick-walled setae form on diseased tissue from reddish stromatic masses (Fig. 3H) (Dodge 1944b; Douglas 2008; Han et al. 2012; Šafránková 2007; Sinclair et al. 1987). The gardener's greatest concern, however, is stem and stolon infections (Figs. 3D to 3F). These often occur in late winter and result in thinning or death of large groundcover plantings of pachysandra (Fig. 3I) (Pirone 1942). Severity of symptoms in pachysandra from this facultative parasite is correlated with plant stress (Hudler et al. 1990). Wounding facilitates but is not required for infection (Salgado-Salazar et al. 2019).

Volutella blight of sarcococca. *Coccinonectria pachysandricola* causes Volutella leaf spots on *S. hookeriana* and *S. hookeriana* var. *humilis* (Castroagudín et

al. 2021; Salgado-Salazar et al. 2019). Infection on *S. hookeriana* is increased by wounding (Castroagudín et al. 2021; Salgado-Salazar et al. 2019). Brown-spotted leaves and stem cankers were initially noted on sarcococca plants growing near pachysandra (Figs. 4A to 4C) (Salgado-Salazar et al. 2019). Leaf lesions exhibit a zonate banding pattern (Figs. 4A and 4B), similar to those seen on pachysandra leaves infected by *C. pachysandricola* (Fig. 4A). Lesions are irregularly shaped, and often start at the leaf tip (Figs. 4A to 4D). In some cases, infected leaves are chlorotic and become completely blighted (Figs. 4B to 4D).

Host Range

Coccinonectria pachysandricola causes foliar blight on *Pachysandra* spp., *S. hookeriana*, and *S. hookeriana* var. *humilis*, but the fungus has not been reported as a pathogen of *Buxus* spp. (Castroagudín et al. 2021; Dodge 1944b; Lombard et al. 2015; Salgado-Salazar et al. 2019). North American native *Pachysandra procumbens* (Allegheny pachysandra) is reported to be less susceptible to this pathogen than the Asian native *Pachysandra terminalis* (Japanese spurge or Japanese pachysandra) (Douglas 2008). All cultivars of *Pachysandra terminalis* are considered susceptible to *C. pachysandricola* (Douglas 2008); however, explicit reports of infection are only available for the cultivars ‘Green Carpet’ and ‘Variegata’ (Šafránková 2007).

Pseudonectria buxi is reliably reported only from hosts in the genus *Buxus* (Farr and Rossman 2020; Salgado-Salazar et al. 2019). Susceptible hosts of *P. buxi* include but are not limited to *B. microphylla*, *B. microphylla* var. *japonica*, *B. sempervirens*

cultivars ‘Arborescens’ and ‘Suffruticosa’, and *B. sinica* var. *insularis* (Bezerra 1963; Farr and Rossman 2020; Rivera et al. 2018). Infection was also noted on hybrid cultivars. For example, *P. buxi* was recovered from cultivar ‘Glencoe’ (‘Chicagoland Green’) in British Columbia, Canada (Shi and Hsiang 2014b). Unusually severe and widespread infections (>50% loss) during nursery propagation in Ontario, Canada were reported on plants belonging to the Sheridan hybrids (*B. sinica* var. *insularis* × *B. sempervirens*) (Shi and Hsiang 2014b). Specifically, cultivars ‘Green Gem’, ‘Green Velvet’, ‘Green Mound’, ‘Green Mountain’, and ‘Pincushion’ were reported to show different levels of susceptibility to *P. buxi* under experimental conditions (Shi and Hsiang 2014b). Shi and Hsiang (2014b) reported that cultivar ‘Pincushion’ with low susceptibility to insects also exhibited low susceptibility to *P. buxi* inoculation.

Pseudonectria foliicola was first described as a boxwood pathogen, but it has been occasionally recovered from sarcococca (Lombard et al. 2015; Salgado-Salazar et al. 2019). The ability of *P. buxi* and *P. foliicola* to colonize plants outside the *Buxaceae* as endophytes is not known, but they are so frequently isolated from surfaced-sterilized boxwood tissues in diagnostic laboratories that they appear to be endophytes or latent pathogens of *Buxus* spp.

Coccinonectria pachysandricola, *P. buxi*, and *P. foliicola* are shown to exhibit variability among isolates in pathogenicity and in their dependence on the presence of wounds; however, differences in aggressiveness are poorly understood (Castroagudín et al. 2021; Dodge 1944b; Hudler et al. 1990; Salgado-Salazar et al. 2019). As of yet, no extensive, methodical research has been conducted on the susceptibility of different species and varieties of *Buxus*, *Pachysandra*, and *Sarcococca* and their companion

plants, such as begonia, geranium, iris, liriope, and thyme, to Volutella blight. Several *Pseudonectria* spp. have been described as endophytes, saprophytes, and secondary invaders (Collado et al. 1999; Lombard et al. 2015; Salgado-Salazar et al. 2019). It is not unusual to recover the Volutella blight pathogens from diagnostic clinic samples of boxwood, pachysandra, and sarcococca that have been killed or weakened by other aggressive pathogens, such as the boxwood blight fungi (Castroagudín et al. 2021; Castroagudín et al. 2020) and *Phytophthora* spp. (Erwin and Ribeiro 1996; Hansen 2009; Reeser et al. 2015), or by environmental stresses, particularly winter injury.

Geographic Distribution

Coccinonectria pachysandricola has been documented from reports and herbarium specimens (www.MycoPortal.org) from China, the Czech Republic, England, Germany, Japan, Korea, the Netherlands, and United Kingdom (Bai et al. 2012; Cannon et al. 1985; Han et al. 2012; Kobayashi 2007; Lombard et al. 2015; Šafránková 2005). In North America, it has been formally documented through reports and herbarium specimens (www.MycoPortal.org) in the U.S. states of Connecticut, Delaware, Florida, Kansas, Maryland, New Jersey, New York, North Carolina, Ohio, Pennsylvania, Virginia, and West Virginia, and District of Columbia (Anonymous 1960; Gräfenhan et al. 2011; Rogerson 1953; Salgado-Salazar et al. 2019).

Following its original description when recovered from dead and dying leaves of *Buxus* sp. in France in 1815, *P. buxi* has been documented from at least 25 countries. In Europe and Asia, reports and herbarium specimens (www.MycoPortal.org) show that

P. buxi colonizes boxwood in Armenia, Austria, Belgium, Bulgaria, China, the Czech Republic, Denmark, England, France, Germany, Greece, Ireland, Italy, the Netherlands, Norway, Poland, Portugal, Russia, Scotland, Spain, Slovakia, Sweden, Turkey, and Ukraine (Bobev 2009; Cannon et al. 1985; de Sousa Dias et al. 1987; Eriksson 1992; Farr and Rossman 2020; Garibaldi et al. 2016; Lombard et al. 2015; Mulenko et al. 2008; Munk 1957; Pantidou 1973; Salgado-Salazar et al. 2019; Shi and Hsiang 2014a; Simonyan 1981; Simsek et al. 2019; Spetik et al. 2019; Unamuno 1941; Wang et al. 2017). In South America, *P. buxi* has been reported in Brazil (Andrade et al. 2017). In North America, formal reports and specimens of *P. buxi* come from Canada (Shi and Hsiang 2014b) and from sixteen U.S. states: Alabama, California, Connecticut, Florida, Illinois, Kansas, Maryland, Mississippi, Ohio, New Jersey, New York, North Carolina, Oregon, Pennsylvania, Rhode Island, and Virginia (Alfieri et al. 1984; Farr and Rossman 2020; French 1989; Grand 1985; Lambe and Wills 1975; Rogerson 1957; Salgado-Salazar et al. 2019).

The recently described species, *P. foliicola*, has been documented from boxwood in three countries. These reports were made from the Czech Republic, New Zealand, and within North America from six U.S. states, namely Illinois, Maryland, Massachusetts, North Carolina, Tennessee, and Washington (Baysal-Gurel et al. 2021; Lombard et al. 2015; Salgado-Salazar et al. 2019; Spetik et al. 2020).

Any discussion of the geographic distribution of these two *Pseudonectria* spp. is not complete without reminding the reader that more than 200 years of taxonomic changes and multiple new names makes it difficult to specifically connect disease reports prior to 2015 to either *P. buxi* or *P. foliicola*. Since *P. foliicola* was identified for

the first time in 2015, it is possible that many of the pre-2015 morphology-based identifications of *P. buxi* might represent misdiagnosed instances of *P. foliicola* (Lombard et al. 2015).

Pathogen Isolation

Coccinonectria pachysandricola, *P. buxi*, and *P. foliicola* can be isolated by directly transferring conidia, which are produced on the sporodochia that are usually present on dying leaves and stems (Figs. 1E to 1I, and 3D, 3E, 3G), to a growth medium. Specifically, transfers can be made from these fruiting bodies by dipping a sterile loop or pick into sterile water, lightly touching a sporodochium with the tool, then streaking the conidia onto full- or half-strength potato dextrose agar (PDA) amended with streptomycin (50 to 100 mg/liter) or tetracycline (100 mg/liter) to inhibit bacterial growth (Castroagudín et al. 2021; Shi and Hsiang 2014b). When sporodochia are absent, their production can be facilitated by incubating infected leaves and stems in a moist chamber at 20 to 25ºC in the dark or under a 12-h photoperiod (Salgado-Salazar et al. 2019). Surface disinfestation of plant tissues is optional but may help reduce contamination by bacteria and other fungi. Surface sterilization can be performed by placing tissues into 1% NaOCl for 30 s to 1 min, rinsing in 70% EtOH, then allowing the leaves to dry briefly in a laminar flow hood before incubating in a moist chamber. White, immature sporodochia develop within 2 to 7 days (Fig. 5A). Once the sporodochia have turned salmon pink color, the conidia are mature enough to be transferred to new plates as described above (Castroagudín et al. 2021; Shi and Hsiang 2014b). Conidia

germinate within 1 to 2 days and at this point single-spore isolates can be transferred to new PDA plates. Cultures develop a salmon pink center with a white edge within a few days after they have been placed at 20 to 25ºC. Care must be taken not to confuse cultures of *Coccinonectria* and *Pseudonectria* with those of *Fusarium* spp. Species of *Fusarium* commonly inhabit healthy and dying boxwood tissues, and in common with *P. buxi* and *P. foliicola*, may produce visually similar pink sporodochia and colonies. However, mature *Fusarium* cultures usually develop a magenta pink to red colony center (Fig. 6D), while those of *Coccinonectria* and *Pseudonectria* spp. are more salmon pink to orange in color (Figs. 6A to 6C) (Lombard et al. 2015; Salgado-Salazar et al. 2019). Furthermore, *Fusarium* spp. can be differentiated from the fungi causing Volutella blight based on conidial morphology. *Fusarium* spp. commonly develop two spore types: (i) microconidia, aseptate or septate, short and ellipsoidal, may potentially be confused with the conidia of *Coccinonectria* and *Pseudonectria* spp.; (ii) macroconidia, septate, with or without a foot cell, long and curved to crescent or sickle-shaped and very distinct from the morphological characters of *C. pachysandricola*, *P. buxi*, and *P. foliicola*, which are detailed in the next section.

Pathogen Identification by Morphology

***Pseudonectria buxi*.** Bezerra (1963) examined boxwood tissues infected by *P. buxi* and described light reddish to pink hemispherical sporodochia (Figs. 1E to 1G) 60 to 110 µm in diameter (Fig. 5B), with setae up to 190 µm long (Fig. 5A). Rossman et al. (1993) described the sporodochia as nonstromatic, easily detached from substrate, 50

to 240 µm in diameter, with red-tipped, hyaline setae 80 to 180 µm long. Conidiophores bearing phialides (Fig. 5C) were 45 to 75 µm long (Bezerra 1963), or 19 to 43 µm long and weakly verticillate (Rossman et al. 1993). Conidia were fusoid, hyaline, smooth, one-celled (Fig. 5D), 8 to 12 × 2.5 to 3 µm (Bezerra 1963); 4.4 to 5.8 × 2.1 to 2.9 µm on plants and 2.8 to 8.7 × 1.9 to 4.3 µm in culture (Rossman et al. 1993); 6 to 9 × 2 to 2.5 µm on Korean boxwood (Shi and Hsiang 2014a); 8.7 to 14.0 × 1.9 to 3.5 µm on leaves and 3.6 to 6.5 × 1.8 to 3.5 µm on PDA (Garibaldi et al. 2016); 3.8 to 8.6 × 1.9 to 5.2 µm (Wang et al. 2017). When Bezerra (1963) grew *P. buxi* in oatmeal agar, mycelia grew immersed in the agar and produced sporodochia similar to the ones observed on host tissue. Aerial mycelia developed at the margins of older cultures, which contained chlamydospores that were elliptic to globose, one-celled, hyaline, terminal, sometimes intercalary and 13 to 25 × 13 to 22 µm. Bezerra (1963) reported abundant growth of *P. buxi* both immersed and aerially on PDA, but no sporulation. Rossman et al. (1993) observed *P. buxi* colonies 2.2 to 2.4 cm in diameter after 5 days on PDA, with sparse, white, aerial mycelia turning pale salmon due to production of sporodochia (Fig. 6A), eventually producing diffuse sporodochia with sparse, red-tipped setae.

From diseased leaves, Bezerra (1963) described perithecia as pear shaped, light orange to greenish, setose, 135 to 190 µm in diameter and 180 to 250 µm high; hyaline setae were 50 to 110 µm long (Figs. 1J to 1L). The unitunicate asci measuring 50 to 70 × 8 to 10 µm were clavate and contained eight ascospores (Fig. 5E). Ascospores were fusoid, rounded at both ends, hyaline, thin-walled, one-celled, 11 to 17 µm long and 3 to 5 µm broad, greyish yellow to green (Fig. 5E). Rossman et al. (1993) described perithecia as 190 to 204 µm high × 168 to 175 µm wide, luteous, straw-colored or with

hyaline setae (25 to) 56 to 160 µm long with finely granular encrustations on surface (Figs. 1J to 1L). Asci measured 43 to 52 × 7.4 to 11.2 µm. Ascospores measured 11.7 to 15.7 (to 17.6) × 3.7 to 4.3 (to 5) µm (Fig. 5E).

Cautious interpretation of sexual structures for diagnostic purposes is recommended. In particular, our ability to uniquely connect the sexual structures described for the boxwood-infecting *Pseudonectria* with either *P. buxi* or *P. foliicola* is incomplete and additional research on the topic is needed. In the past, two distinct sexual morphs were described for boxwood-infecting *Pseudonectria* under the dual-name system of fungal nomenclature: *P. rousseliana*, which is a synonym of *P. buxi*; and *P. coronata*, which is a homotypic synonym of *Nectriella coronata* (Rossman et al. 1993). Both of these sexual morphs can coexist on a single boxwood leaf, as was documented from the type specimen of *P. rousseliana* (Rossman et al. 1993). However, studies of the sexual morphs took place several decades before mycologists were aware of the existence of *P. foliicola*, and sexual fruiting bodies were not identified when *P. foliicola* was described (Bezerra 1963; Lombard et al. 2015; Rossman et al. 1993). The sexual structures detailed above for *P. buxi* are based on descriptions of *P. rousseliana*; for a complete description of *P. coronata*, consult Rossman et al. (1993).

Gräfenhan et al. (2011) correlated perithecial color with *Pseudonectria* spp., distinct from species belonging to the *Volutella sensu stricto* clade, noting that *Pseudonectria* spp. produced green perithecia and *Volutella* spp. produced red perithecia. While preparing images for this guide, we observed a range of perithecial colors associated with the setose sporodochia of *P. buxi* on boxwood leaves, ranging from orangey-brown to orange to greenish to grayish yellow green to straw colored

(Figs. 1J to 1L). It is worth noting that perithecia of additional nectriaceous fungi have also been observed from dying or dead boxwood leaves, including *Hyponectria buxi* and *Sesquicillium buxi* (Rossman et al. 1993).

***Pseudonectria foliicola*.** *Pseudonectria foliicola* was described as a fungus similar to *P. buxi*, but without a known perithecial state, whose sporodochia on plant tissue lacked setae and whose conidia could form both on single conidiophores on plant tissue and multiple conidiophores on sporodochia (Fig. 5F) (Lombard et al. 2015). On diseased leaves, the single conidiophores are often visible first, appearing as a pale pink lawn of erect hyphae with irregularly verticillate spore droplets that persist when sporodochia arise. Conidia are hyaline, aseptate, fusiform to ellipsoidal (Fig. 5G), (5 to) 6.5 to 7.5 (to 8) × 2 to 3 µm (average 7 × 3 µm) (Lombard et al. 2015); or 4.5 ± 0.9 × 2.9 ± 0.4 µm (Spetik et al. 2020). Lombard et al. (2015) reported fast growing colonies on malt extract agar (MEA), reaching 90 mm in 10 d at 24°C with hyaline chlamydospores, globose to subglobose, 35 to 60 µm in diameter, intercalary in chains or solitary. Spetik et al. (2020) reported colonies on PDA with white aerial mycelia and scattered pink to salmon, slimy masses of conidia on sporodochia at the margins (Fig. 6B).

Lombard et al. (2015) indicated that *P. foliicola* can be differentiated from *P. buxi* by its simple conidiophores, something not reported for *P. buxi* (Bezerra 1963; Rossman et al. 1993). Additionally, conidia of *P. foliicola* (Fig. 5G) are smaller than those of *P. buxi* (Fig. 5D), which are 8 to 12 × 2.5 to 3 µm (Bezerra 1963). Also, the sporodochia of *P. foliicola* are not surrounded by setae, while formation of setae is a characteristic of *P. buxi* (Fig. 5A) (Bezerra 1963; Rossman et al. 1993). However, in our own experience, we have not always observed setae from *P. buxi* grown in culture, but

these features are regularly observed from natural infections of host tissue by *P. buxi* (C. Salgado-Salazar, *personal communication*). In general, we have also observed that *P. foliicola* sporulates profusely, whereas *P. buxi* does not sporulate as much, but we do not know how widespread this trait might be across all populations of these two fungal species.

***Coccinonectria pachysandricola*.** Signs of *C. pachysandricola* on the plant are mucoid, salmon-colored sporodochia with hyaline or faintly colored setae (Figs. 3D, 3E, and 3G). The tapered setae (Fig. 5H) measure 100 to 200 µm long (Dodge 1944b) or 60 to 100 µm long (Han et al. 2012). A dissecting needle touched to the spore-laden surface of a sporodochium, then touched to a drop of water on a microscope slide, releases spores which, under magnification, are aseptate, smooth, hyaline, and ellipsoidal to fusiform (Fig. 5I). Conidial dimensions of *C. pachysandricola* from pachysandra were given by Dodge (1944b) as 14 to 24 × 2 to 4 µm (more variable in size and shape in culture on PDA); by Rossman et al. (1993) as (6.8 to) 12 to 21 × 3.1 to 4.3 µm; by Han et al. (2012) as 11 to 26 × 2.5 to 4.0 µm; and from sarcococca by Salgado-Salazar et al. (2019) as 11 to 23 × 2 to 5 µm (average. 15.4 × 3.1 µm). According to Salgado-Salazar et al. (2019), isolates of *C. pachysandricola* (Fig. 5I) could be readily distinguished from *P. buxi* and *P. foliicola* based on the size of their conidia (*P. buxi*: 8 to 12 × 2.5 to 3 µm; *P. foliicola:* 6.5 to 7.5 × 2 to 3 µm).

Rossman et al. (1993) described colonies of *C. pachysandricola* from pachysandra grown on PDA as 2 cm in diameter with sparse aerial mycelia near the margins, with solitary conidiophores that are unbranched, irregularly verticillate with monophialidic conidiogenous cells 9.3 to 19 µm × 2.5 to 3.7 µm at the base and 1.9 µm

at the apex (Fig. 5J). Salgado-Salazar et al. (2019) described colonies of isolates from sarcococca as effuse and salmon-white on PDA (Fig. 6C), reaching 26 to 35 mm in diameter after 10 days at 25ºC. Pale, salmon-colored sporodochia produced in culture lacked setae (Salgado-Salazar et al. 2019), whereas setae surrounding sporodochia were observed on infected pachysandra leaves (Fig. 5H). With the aid of a scanning electron microscope, we were also able to observe a previously unreported sporodochia-forming pattern of *C. pachysandricola* on naturally infected pachysandra, which was distinct from that of *Pseudonectria* spp. on boxwood. Namely, we observed that hyphae of *Pseudonectria* spp. first exited the stomata, then sporodochia began to form on the surface of plant tissues (Figs. 5B and 5F). In contrast, *C. pachysandricola* formed spore-bearing conidiophores within stomata. The conidiophores of *C. pachysandricola* then erupted through stomata to develop into larger mature sporodochia (Fig. 5H) (C. Salgado-Salazar, *personal communication*). This finding is supported by a closer observation of diagnostic plant samples, as the sporodochia of *C. pachysandricola* are usually restricted to the sites of stomata, while limited mycelia are seen on the surface of plant tissues (Fig. 3G). Whether the sporodochia-forming pattern is consistent across isolates of each species warrants further investigations.

On plant tissue but not in culture, setose perithecia of *C. pachysandricola* develop from old sporodochia or on stromatic tissue erupting from plant tissue (Dodge 1944b), with one or more perithecia developing from the stroma. Mature perithecia on pachysandra are orange-red to carmine-red (Fig. 3H), measuring 240 to 280 × 200 to 225 µm (Dodge 1944b) or 235 to 240 × 180 to 195 µm (Rossman et al. 1993). Setae are yellowish, 135 µm long, interspersed with shorter hairs (Rossman et al. 1993). The

asci are unitunicate, slightly clavate with a broad apex, measuring 46 to 58 × 7 to 8 µm (Rossman et al. 1993) or 60 to 80 × 7 to 10 µm (Dodge 1944b). Asci contain eight ascospores measuring 9.3 to 13.2 × 3.1 to 3.7 µm (Rossman et al. 1993) or 10 to 15 × 3 to 4.5 µm (Dodge 1944b). To date, perithecia of *C. pachysandricola* have not been observed on sarcococca.

Molecular Methods for Pathogen Identification and Detection

Identification of fungi causing Volutella blight using molecular tools relies on sequencing of one or more DNA markers, such as the rDNA internal transcribed spacer region (ITS), the universal barcode marker for fungi (Schoch et al. 2012), and the second largest subunit of the RNA polymerase I (*rpb1*) and II (*rpb2*) and larger subunit of the ATP citrate lyase (*acl1*) for species discrimination in the *Nectriaceae* (Gräfenhan et al. 2011; Lombard et al. 2015). The DNA regions can be used for sequence analysis by finding regions of similarity between the query and reference sequences using nucleotide blast programs such as BLASTn provided by NCBI (Johnson et al. 2008). Phylogenetic reconstructions can be carried out to pinpoint the phylogenetic positions of the query sequences in relation to reference sequences of *C. pachysandricola*, *P. buxi*, and *P. foliicola*, which are publicly accessible in GenBank (Sayers et al. 2020). It is recommended that users rely on sequences obtained from types and/or phylogenetically and morphologically authenticated isolates for sequence comparison and phylogenetic analysis, such as those summarized in Table 1 by Salgado-Salazar et al. (2019).

Coccinonectria pachysandricola can be readily identified based on the ITS sequence as it shares less than 97% similarity with the most closely related species, *C. rusci* (Lombard et al. 2015). To discriminate between the morphologically similar *P. buxi* and *P. foliicola*, two species which also share more than 98.7% similarity in the ITS sequence, alternative DNA markers such as *acl1*, *rpb1*, or *rpb2* can be used, either alone or in combination (Gräfenhan et al. 2011; Lombard et al. 2015; Salgado-Salazar et al. 2019).

With the advent of whole-genome sequencing, genomes of many boxwood-infecting fungi, including *C. pachysandricola*, *P. buxi*, and *P. foliicola* (Castroagudín et al. 2021; Rivera et al. 2018), have been sequenced. Whole-genome sequences of *C. pachysandricola*, *P. buxi*, and *P. foliicola* have the potential to aid the diagnosis of Volutella blight (Malapi-Wight et al. 2016b). Furthermore, although rapid diagnostic assays for Volutella blight are currently unavailable, a genomics approach can be used to identify informative DNA markers for rapid detection and subsequently facilitate the development of these assays, such as loop-mediated isothermal amplification (Malapi-Wight et al. 2016a).

Pathogen Storage

Active cultures of *C. pachysandricola*, *P. buxi*, and *P. foliicola* are usually maintained in PDA (Bezerra 1963; Gräfenhan et al. 2011; Rivera et al. 2018; Rossman et al. 1993; Shi and Hsiang 2014a, b). Other media types for these, or related species, include corn meal agar + 2% glucose, MEA (Rossman et al. 1993), and oatmeal agar

(Bezerra 1963). For short term storage, isolates without contamination can be maintained for one to two years at 4ºC in PDA in Petri dishes or glass slants sealed with Parafilm (Nakasone et al. 2004). Routine subculturing at least once a year should take place to maintain pure, live isolates (Nakasone et al. 2004). However, it is important to note that routine subculturing in a metabolically active state may result in phenotypic and genetic changes, including changes in virulence of fungal species (Crous 2002). Utilizing a combination of the long- and short-term storage methods can preserve the integrity of the collection, reducing the likelihood of these changes.

Several options exist for long term storage of *C. pachysandricola*, *P. buxi* and *P. foliicola*, including preservation with sterile mineral oil (Johnson and Martin 1992; Nakasone et al. 2004; Smith and Onions 1983; Stebbins and Robbins 1949), immersion in sterile water (Johnson and Martin 1992; Nakasone et al. 2004), and immersion in sterile glycerol (Nakasone et al. 2004). Mineral oil overlay of slant cultures is a traditional technique for preserving fungal isolates that is both economical and efficient, yielding several years of viability (Nakasone et al. 2004; Smith and Onions 1983). To preserve isolates using the mineral oil overlay technique, colonies of pure isolates grown on PDA slants are fully covered one centimeter above the top of the slant with twice sterilized (121ºC for 15 minutes) high-quality mineral oil (Smith and Onions 1983; Stebbins and Robbins 1949). Smith and Onions (1983) revived a culture of *Pseudonectria* sp. stored with this technique 32 years after preparation. Sterile water storage is a low-cost and effective method for preserving filamentous fungi for years (Johnson and Martin 1992; Nakasone et al. 2004). To apply this method, fungal isolates are grown on PDA for 7 to 10 days at 25ºC. Five to ten plugs (5 mm^3) are excised from

the leading edge with a cork borer or scalpel and submerged in sterile water in a glass vial with screw-cap lid or a polypropylene microcentrifuge tube sealed with Parafilm. Isolates in sterile water can be stored at room temperature (*c.* 22ºC) or at 4ºC for several years. It is important to note that the effectiveness of sterile water is yet to be determined for storing *C. pachysandricola*, *P. buxi*, and *P. foliicola*, despite the fact that this method has been successful for other fungal species including *Pseudonectria* (Babu et al. 2015; Johnson and Martin 1992). Lastly, isolates of *C. pachysandricola*, *P. buxi*, and *P. foliicola* can be maintained in long-term storage in a metabolically inactive state at -20 or -80ºC in 10 to 15% sterile glycerol solution by immersing 5 to 10 plugs (5 mm^3) excised from the leading edge of an active colony grown in PDA (Abd-Elsalam et al. 2010; Nakasone et al. 2004). This method has reliably worked for other pathogenic fungi and resulted in multiple years of viability with limited phenotypic changes (Abd-Elsalam et al. 2010).

While other methods of storage may also work for *C. pachysandricola*, *P. buxi*, and *P. foliicola*, the discussed methods are simple and cost effective and do not require specialized equipment to preserve cultures. In general, it is our practice and recommendation to store cultures of *C. pachysandricola*, *P. buxi*, and *P. foliicola* in duplicate using multiple methods to ensure long-term viability and to safeguard against losses due to unexpected equipment failures or other issues.

Pathogenicity Tests

Pathogenicity of *C. pachysandricola*, *P. buxi*, and *P. foliicola* has been determined using detached leaves (Salgado-Salazar et al. 2019; Shi and Hsiang 2014a), potted plants (Garibaldi et al. 2016; Shi and Hsiang 2014a, b; Wang et al. 2017), or whole plants in the field (Spetik et al. 2020), with some variations in the inoculation process and incubation duration. It is common to create wounds on detached leaves during pathogenicity trials. This can be done with light scratches using sterile dissecting needles on the adaxial surfaces of leaves (Shi and Hsiang 2014a) or at either side of the leaf midrib (Salgado-Salazar et al. 2019). When stems and leaves of whole plants are inoculated, they can be wounded with a sterile 5-mm cork borer (Spetik et al. 2019). The number of wounded leaves can vary from 3 to 10 per plant (Garibaldi et al. 2016; Shi and Hsiang 2014a).

Wounded leaves are inoculated by spraying a conidial suspension that consists of 1×10^5, 10^6, or 10^8 conidia/ml until runoff (Salgado-Salazar et al. 2019; Shi and Hsiang 2014a). Conidial suspensions are prepared by rinsing 7 to 21-day-old PDA cultures of *C. pachysandricola*, *P. buxi*, or *P. foliicola* with sterile distilled water. Control plants are sprayed with sterile water. For whole plants, a 5-mm mycelial plug taken from a 10-day-old PDA culture is placed onto each wound and secured with Parafilm or aluminum foil (Spetik et al. 2020).

Age of boxwood plants used in pathogenicity tests for *P. buxi* and *P. foliicola* can vary from 2 to 3-month-old rooted cuttings (Salgado-Salazar et al. 2019; Shi and Hsiang 2014b) to 2 to 3-year-old potted plants (Garibaldi et al. 2016; Shi and Hsiang 2014a; Spetik et al. 2020; Spetik et al. 2019; Wang et al. 2017). The number of replications has also varied with the inoculation method. Each trial using whole plants typically includes

3 to 8 inoculated replications plus at least one non-inoculated control plant (Garibaldi et al. 2016; Shi and Hsiang 2014a; Spetik et al. 2020; Spetik et al. 2019; Wang et al. 2017). For inoculating detached leaves, at least 10 replications are recommended to be used in each trial (Salgado-Salazar et al. 2019).

After inoculation, potted plants are covered with plastic bags (Garibaldi et al. 2016; Shi and Hsiang 2014a, b), while detached leaves are placed in closed moist chambers at 20 to 25ºC with a 14-h photoperiod for approximately three days to facilitate infection (Salgado-Salazar et al. 2019). Lesions with sporodochia on wounded leaves are usually observed three days after inoculation, followed by yellowing and browning of tissues (Bai et al. 2012; Garibaldi et al. 2016; Shi and Hsiang 2014a, b), while the development of lesions under the bark of whole plants in the field can take up to 2 to 3 months (Spetik et al. 2020; Spetik et al. 2019).

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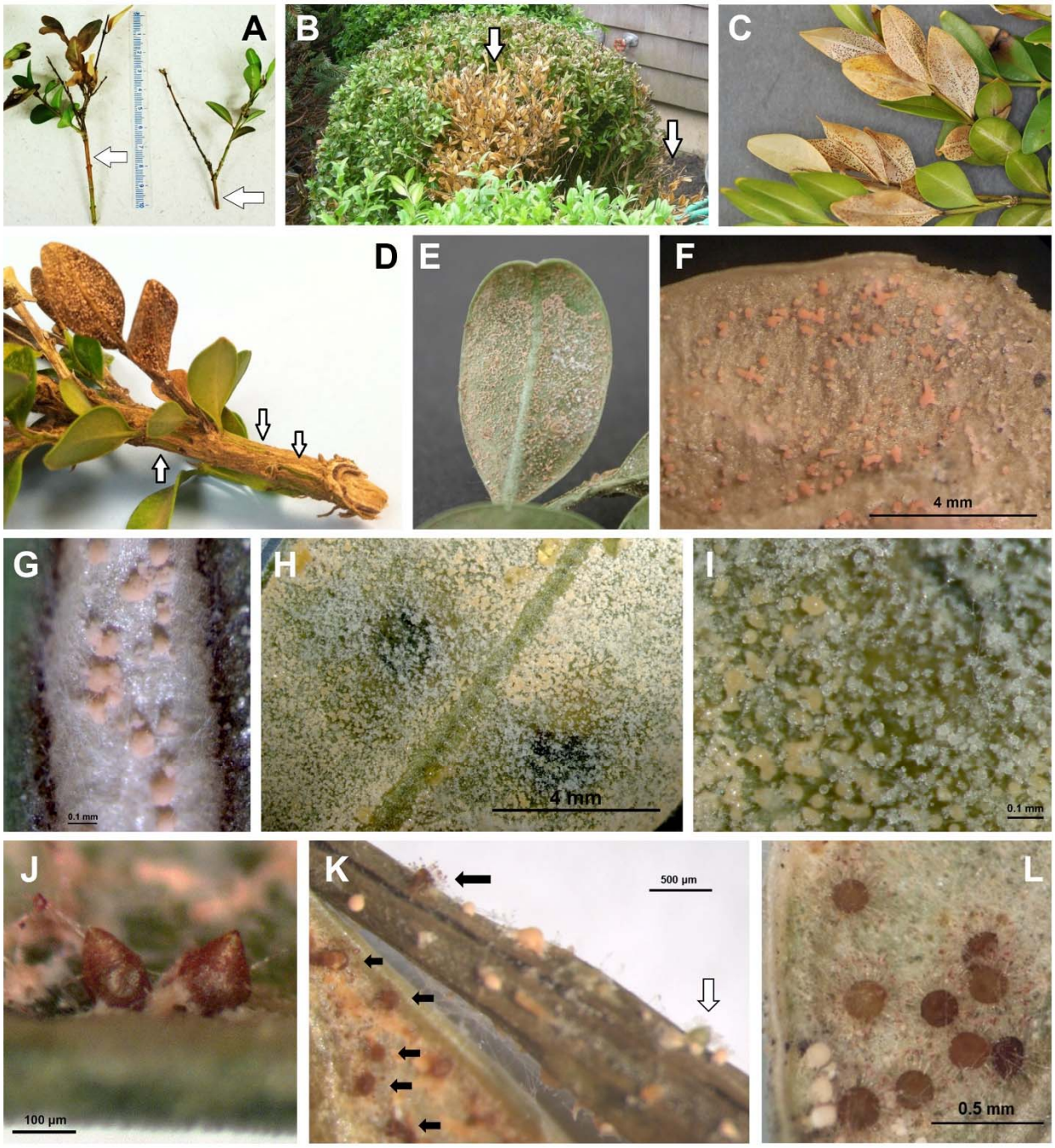

Figure 1. Symptoms and signs of Volutella blight on boxwood. **(A)** Darkly discolored canker on lower branches (arrows) and subsequent development of leaf and twig blight. **(B)** “Straw-yellow” discolored canopy sections (arrows) of a diseased plant at a landscape site. **(C)** Foliage weakened by Volutella canker, then colonized by black

pycnidia of a secondary invader, *Dothiorella candollei*. **(D)** Split and loosened bark (arrows) at the base of a branch supporting blighted foliage. **(E, F, G)** Pinkish-roseate or coral sporodochia of *Pseudonectria buxi* on abaxial surface. **(H, I)** Pink to salmon-colored slimy masses of conidia on sporodochia of *P. foliicola* on abaxial surfaces of infected leaves. **(J)** Two orangey-brown perithecia of *P. buxi*. **(K)** Orange to orangey-brown perithecia (black arrows) and a greenish perithecia (white arrow) of *P. buxi*. **(L)** Nine perithecia of *P. buxi* range in color from grayish yellow green to straw-colored, to orangey-brown. Panel C courtesy of Lorraine Graney (Bartlett Tree Experts Company).

Diagnostic traits		Volutella blight	Boxwood blight[a]	Boxwood dieback[b]
Symptoms on boxwood	**Blighted canopy pattern**	Relatively large sections of the canopy due to infection on supporting branch bases	Starts with scattered, relatively small areas due to leaf infection, then spreads to the entire foliage	Relatively large sections of the canopy due to infection on supporting branch bases and individual twigs
	Leaf spots	None	Extensive at the early stage following infection of *Calonectria henricotiae* and *Ca. pseudonaviculata*	*Colletotrichum theobromicola* occasionally causes leaf spots (R. Singh, personal communication).
	Defoliation	Minimum, blighted leaves tend to remain attached	Rapid and extensive, occurs within days after infection under favorable conditions	Minimum, tan-colored leaves tend to remain attached
	Stem cankers	Brown to black streaks, girdling, but can be vertical when associated with loosened bark caused by winter damage; some discoloration of wood associated with loosened and peeling bark	Extensive vertical black streaks on susceptible hosts; discoloration is shallow, limited to the bark, rarely extends to the xylem.	Stems with dieback show bright black discoloration under the bark; discoloration can extend into the xylem and pith.
Pathogen morphology	**Color of sporodochia**	Initially white, quickly turning pinkish-roseate, salmon-, or coral-colored	White	Salmon-colored
	Location of sporodochia	Abundant on the adaxial surface of leaves; sometimes observed on the stems	Abundant on the leaf adaxial surface and stems, associated with leaf spots and stem cankers	Sometimes developed on infected stems and twigs
	Conidial shape	Fusoid (*Pseudonectria buxi*); Fusiform to ellipsoidal (*P. foliicola*)	Two-celled, elongated cylindrical	Single-celled, short cylindrical
	Setae color	Hyaline to whitish	Not observed	Black
	Perithecia	Dark brown, straw-colored, or grayish yellow green, pear shaped, appear at the end of the season	Not observed on host tissue; Rounded, orange to red perithecia produced by crosses of two boxwood blight fungi on carrot agar	Not observed on host tissue or artificial media

[a] Additional information including diagnostic images of boxwood blight can be found in Castroagudín et al. (2020).

[b] Additional information including diagnostic images of boxwood dieback can be found in Singh and Doyle (2017).

Figure 2. Comparative diagnostic characters of three major fungal diseases on *Buxus* spp. including Volutella blight caused by *Pseudonectria buxi* and *P. foliicola*, boxwood blight caused by *Calonectria henricotiae* and *Ca. pseudonaviculata*, and boxwood dieback caused by *Colletotrichum theobromicola*.

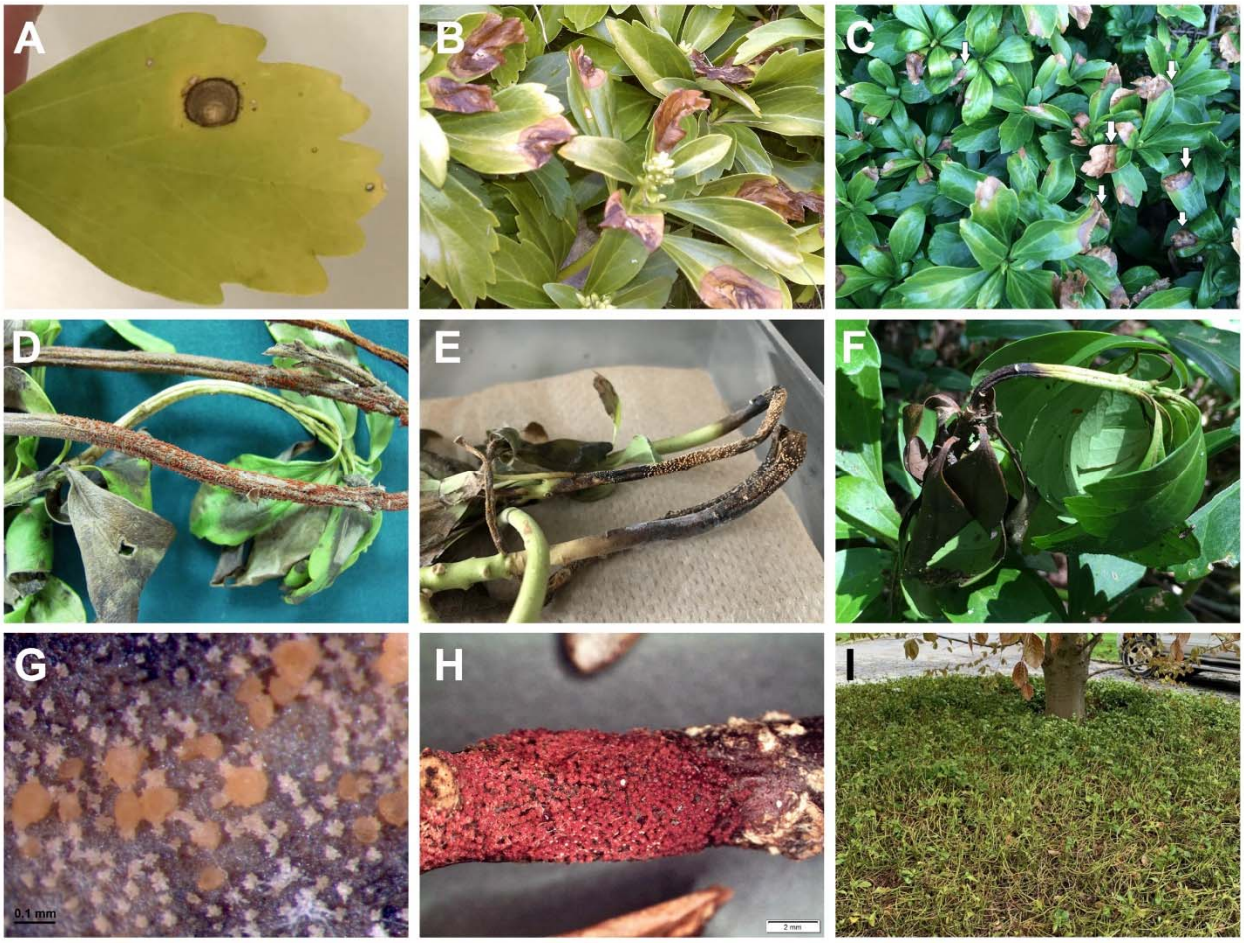

Figure 3. Symptoms and signs of Volutella blight caused by *Coccinonectria pachysandricola* on pachysandra. **(A)** A circular leaf lesion at the early stage of infection. **(B, C)** Circular and irregular lesions on leaves. Note the characteristic zonate pattern of the lesions (arrows). **(D)** Greenish brown cankers, salmon-colored sporodochia, and orange perithecia on infected stems. **(E)** Circular salmon-colored sporodochia on blighted leaves and shoots and girdling stems and stolons with dark brown to black cankers. **(F)** Girdling on an infected pachysandra stem. **(G)** Salmon-colored sporodochia and orange perithecia on the underside of an infected leaf. **(H)** Orange to carmine-red perithecia on a diseased stem. **(I)** Volutella blight observed during winter at a landscape site, showing characteristic thinning of the stand of

pachysandra. Panels B, D, and H courtesy of Chad A. Vrany, Lorraine Graney, and Meg McConnell (Bartlett Tree Experts Company), respectively. Panel I courtesy of Vincent A. Simeone (Planting Fields Arboretum).

Figure 4. Symptoms and signs of Volutella blight on sarcococca. **(A)** Sarcococca (right) infected by *Coccinonectria pachysandricola* growing near *C. pachysandricola*-infected pachysandra (left) at the U.S. National Arboretum in Washington D.C. **(B)** Brown and irregular lesions (arrows), elongated in outline on sarcococca leaves. Note the characteristic zonate pattern of the lesions often started at the leaf tip. **(C)** Blighted tips and stem cankers (arrows). **(D)** Leaves turning yellow from the brown spots at the leaf tip.

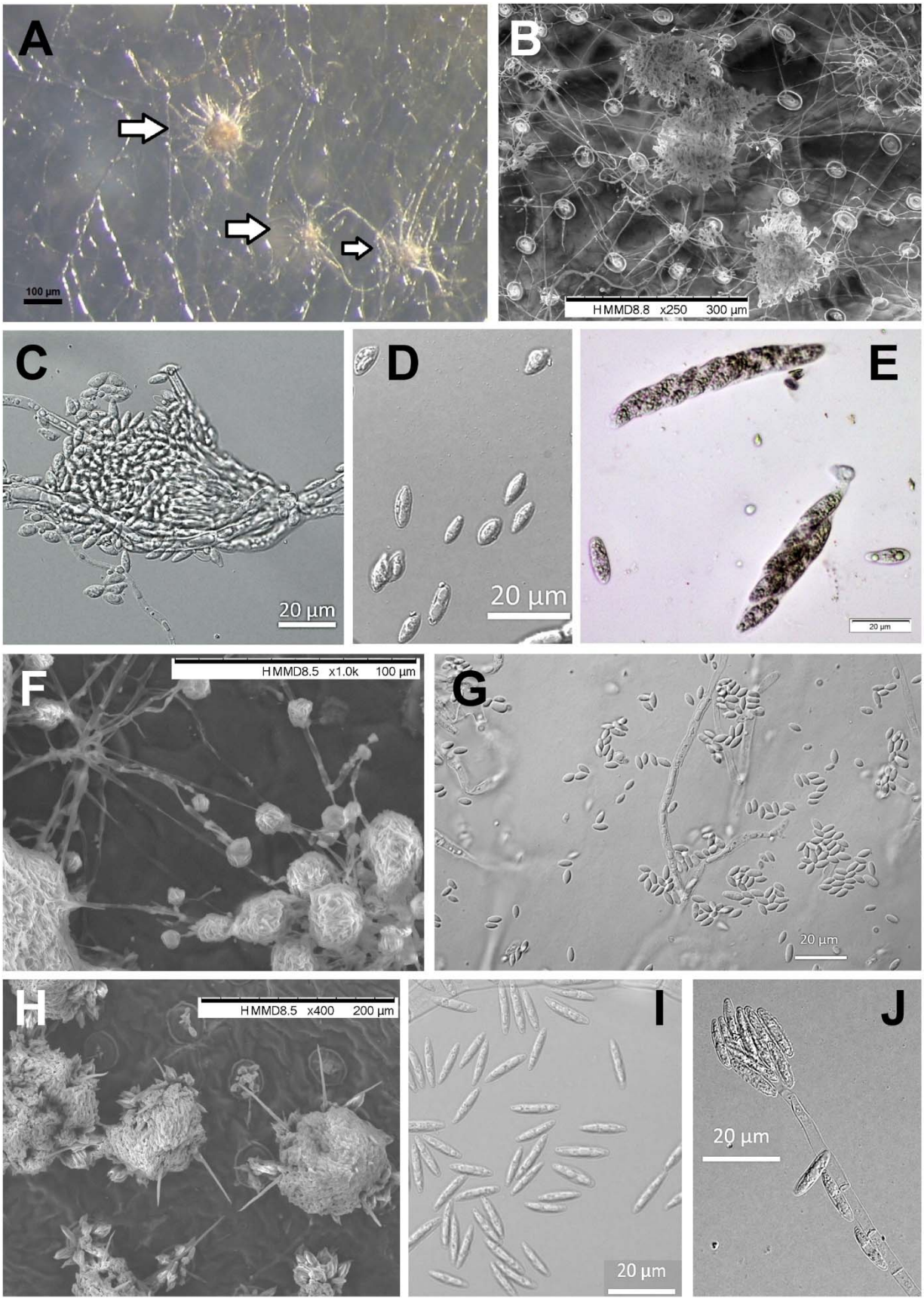
A
100 µm
B
HMMD8.8 x250 300 µm
C
20 µm
D
20 µm
E
20 µm
F
HMMD8.5 x1.0k 100 µm
G
20 µm
H
HMMD8.5 x400 200 µm
I
20 µm
J
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Figure 5. Morphological features of fungi causing Volutella blight on *Buxaceae*. **(A to E)** *Pseudonectria buxi*. **(A)** White, immature sporodochia bearing hyaline setae (arrows) produced seven days after being plated on potato dextrose agar. **(B)** Hemispherical sporodochia on infected leaf tissue observed under a scanning electron microscope (SEM). **(C)** Solitary conidiophore bearing phialides on fasciculate, aerial hyphae. **(D)** Aseptate, hyaline, ellipsoid to fusiform, and smooth-walled conidia. **(E)** Unitunicate, clavate asci, each containing eight hyaline fusoid, thin-walled, one-celled ascospores. **(F to G)** *P. foliicola*. **(F)** Conidia forming both on single conidiophores growing out of a stoma as well as ones forming sporodochia on an infected leaf under SEM. **(G)** Hyaline, aseptate, fusiform to ellipsoidal conidia. **(H to J)** *Coccinonectria pachysandricola*. **(H)** Sporodochia with hyaline setae on an infected leaf under SEM. **(I)** Ellipsoid, hyaline, and aseptate conidia. **(J)** A simple verticillate conidiophore with conidia. Panel E courtesy of Lorraine Graney (Bartlett Tree Experts Company).

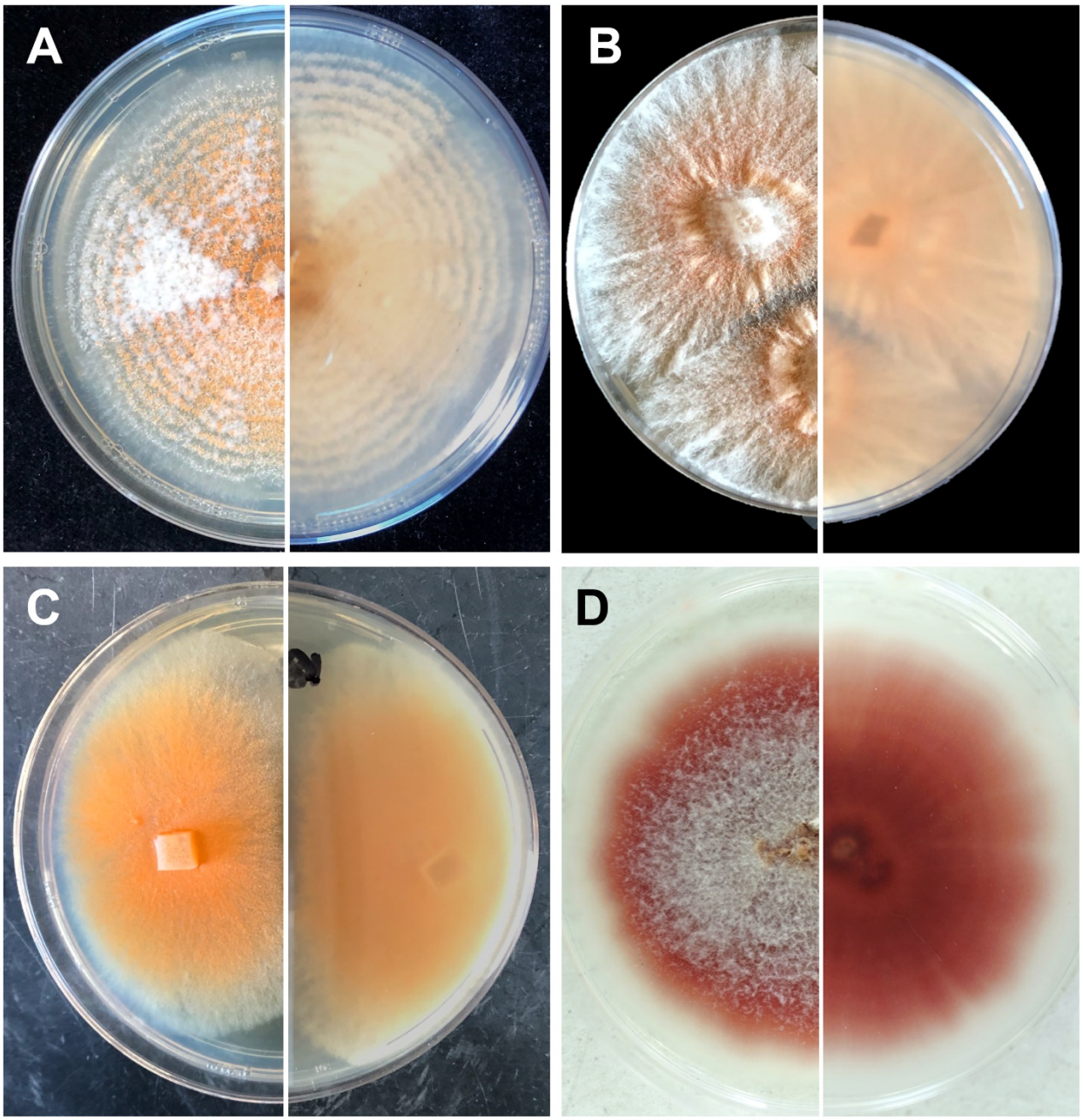

Figure 6. Colony morphology of **(A)** *Pseudonectria buxi*, **(B)** *P. foliicola*, **(C)** *Coccinonectria pachysandricola*, and **(D)** a *Fusarium* species isolated from boxwood. All cultures were grown in potato dextrose agar at 25ºC under fluorescent light. The *P. buxi* culture was maintained under a 12-hour photoperiod for 16 days, while others were under a 24-hour photoperiod for 10 days.