

# *Pseudonectria buxi* causing leaf and stem blight on *Buxus* in Canada

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Accepted: 21 November 2013 / Published online: 4 December 2013  
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**Abstract** An outbreak of a boxwood disease was observed in nurseries in southern Ontario in 2008, but the disease appears to have been present in Ontario for at least 15 years. From 2008 to 2010, over 300 fungal isolates were obtained from diseased samples, which included cuttings and whole plants. Almost half of the isolates (144) were found to belong to a single cultural morphotype which was confirmed as *Pseudonectria buxi* by DNA sequencing of several isolates. The remaining isolates were grouped into another seven major morphotypes, the largest of which accounted for 11 % of the isolates. Single representatives of all eight morphotypes were identified to species using morphological and molecular techniques, and were subjected to pathogenicity testing. Among them, only *Pseudonectria buxi* successfully satisfied Koch's postulates on detached leaves, small twigs and rooted cuttings, which confirmed it as the causal agent of Volutella blight. Inoculation tests showed that wounds may be the major infection courts for *P. buxi*, since non-wounded inoculated tissues did not become diseased. Among several commonly grown boxwood cultivars in Ontario, 'Green Gem' was the most susceptible compared to 'Green Velvet', 'Green Mound', 'Green Mountain' or 'Pincushion', but all were found to be susceptible. Rather than behaving just as a saprophyte or secondary invader, *P. buxi* is capable of primary invasion after wounding, and causing extensive disease on live boxwood tissues.

**Keywords** *Volutella buxi* · Volutella blight · Boxwood · Fungi · Inoculation · Wounding

## Introduction

Species of *Buxus* L., known as boxwood or box, are important landscape plants used very commonly for hedges and topiaries all over the world (Henricot and Culham 2002; Pherson 2005). Since the early 1900s, boxwood diseases have been commonly reported, with symptoms such as leaf spot, leaf and stem blight, and root rots (Jacobi et al. 2003). One of the major boxwood diseases is Volutella leaf and stem blight, hereon referred to as Volutella blight, which is caused by *Pseudonectria buxi* (DC.) Seifert, Gräfenhan & Schroers also known as *Volutella buxi* (DC.) Berk. or *Pseudonectria rousseliana* (Mont.) Seaver. This disease has been traditionally considered the primary cause of boxwood decline (Bezerra 1963), and has been found in the U.S. (White 1931), the U.K. (Berkeley and Broome 1850; Moreau 1919), Canada (Joshi and Jeffries 2006, 2010, 2011), Italy, Spain, Germany, England (Saccardo 1883) and Switzerland (Bezerra 1963). Other important fungal diseases of boxwood include the following: Macrophoma leaf spot caused by *Dothiorella candollei* (Berk. & Broome) Petr. (Dodge 1944); *Cylindrocladium* leaf spot caused by *Cylindrocladium buxicola* Henricot (Henricot and Culham 2002), boxwood rust caused by *Puccinia buxi* DC., Flore française (Preece 2000), and Phytophthora root rot caused by *Phytophthora cinnamomi* Rands and *P. parasitica* Dastur (Jacobi et al. 2003).

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Since the late 1990s, a blight of boxwood has been observed at nurseries in southern Ontario, Canada, and it has caused significant economic losses during the last few years. In 2008, a more severe outbreak was seen. Although entire plants did not die back, when leaves and branches were affected by this disease, they were not commercially acceptable, particularly since pruning out the symptomatic parts could ruin the symmetry of the plant. Over 50 % loss of potted cuttings was found for each of the following commonly grown cultivars in Ontario: ‘Green Gem’, ‘Green Velvet’, ‘Green Mound’ and ‘Green Mountain’. These cultivars are open-pollinated with the female parent *B. sempervirens* ‘Suffruticosa’ L. and the male parent *B. sinica* var. *insularis* (Nakai) M. Cheng (Batdorf 2004). In British Columbia (B.C.), this boxwood disease was also observed in several nurseries in 2009 on several cultivars, especially on *Buxus* sp. ‘Glencoe’ (commercial name ‘Chicagoland Green’) which is a hybrid of *B. microphylla* Siebold & Zucc. var. *koreana* Nakai ex. Rehder and *B. sempervirens* L.

In 2008, a single nursery in southern Ontario lost over \$60,000 due to this disease, with a similar amount in 2009. An unpublished internal report from this nursery regarding the 2008 outbreak mentioned that *Volutella* blight caused by *P. buxi* was the main boxwood disease encountered. However, this diagnosis was based on observations of the presence of fungal spore-producing structures by a local diagnostic laboratory, and the causal agent was not more rigorously investigated.

*Pseudonectria buxi* has been reported to be associated with *Volutella* blight (Bezerra 1963; Hartman 2001), but the causal agent of the boxwood disease in southern Ontario and other parts of Canada still needs further investigation. Some chemical and cultural methods of managing this disease have been reported (White 1931), but there is limited recent information. The etiology of this disease still requires further study, since the disease cycle of *Volutella* blight has not been published, nor have cultivar susceptibility tests been reported in North America. Therefore, the objectives of this study were as follows: i) to identify the causal agent associated with this disease of boxwood using morphological and molecular methods; ii) to assess optimum growth temperature of geographically disparate isolates of the causal agent; and iii) to examine the pathogenicity and susceptibility of different boxwood tissues in interactions with this pathogen.

## Materials and methods

### Sample collection and fungal isolation

Diseased boxwood cuttings and small symptomatic potted plants were obtained from several nurseries in southern Ontario and southern B.C. To obtain isolates, tissues were cut into 5-mm-long segments including diseased areas bordering on live areas of stems or leaves. The 5-mm-long pieces were dipped into 75 % ethanol for 2 s to wet the tissues, and then cut into 1 mm<sup>2</sup> pieces with surface-sterilized tools. These pieces were placed into 1 % sodium hypochlorite for 30 s and rinsed in autoclaved deionized water. Four pieces were then placed in each 9-cm-diameter Petri dish containing potato dextrose agar (PDA; Difco, Sparks, MD, USA) amended with 100 µg/ml each of tetracycline hydrochloride (Fisher AC23310, Ottawa, Canada) and streptomycin sulphate (Fisher BP910). The cultures were incubated at room temperature (~25 °C). If an isolate was capable of sporulation, a single-spore culture was obtained following Choi et al. (1999). In addition to foliar isolates, attempts were also made to obtain isolates from roots of symptomatic plants.

Healthy potted 1-year-old boxwood plants of cultivars ‘Green Velvet’, ‘Green Gem’, ‘Green Mountain’ ‘Green Mount’, up to 30 cm tall in 1-l pots, were provided courtesy of Sheridan Nurseries, Georgetown, Ontario, Canada. The cultivar ‘Pincushion’ was provided by a nursery in St. Catharines, Ontario, Canada. Cuttings were propagated from ‘Green Velvet’ in September and October, 2009 and 2010. The leaves at the bottom half of twigs up to 15 cm long were stripped off and a fresh slanted cut was made at the base of the twig. The bottom 2 cm of each twig was dipped into growth hormone powder (0.1 % indole-3-butylic acid, Stim-Root, Brantford, Ontario, Canada), and were replanted in 3-inch pots with Sunshine mix #1 (SunGro Horticulture Canada Ltd., Seba Beach, Alberta, Canada). The cuttings and whole plants were placed at 25 °C under low level continuous fluorescent lighting (50 µmol/m<sup>2</sup>/s). The plants were watered weekly until soil saturation, with a fertilizer solution prepared with granules of 20-8-20 (N-P-K) at 1.25 g/l water and pH adjusted to 6.0.

### Morphological identification

After careful observation and repeated subculturing for up to 6 months, fungal colonies were grouped into major

morphotypes on the basis of growth rate, colour and macroscopic cultural characteristics on PDA. Multiple representatives from each morphotype were also examined with light microscopy to observe the structure of mycelia, conidiophores and spores. These features were compared to those in diagnostic keys, such as that of Barnett and Hunter (1972) for tentative identification, and single representatives from each major morphotype were chosen for molecular confirmation of identification.

### Molecular identification

Isolates were grown at room temperature on a sterile cellophane film (Flexel, Atlanta, GA) overlying PDA until the colony occupied most of the cellophane. The mycelia were scraped off the cellophane, and the DNA was extracted following Edwards et al. (1991). The internal transcribed spacer (ITS) region of ribosomal DNA has been commonly used to distinguish fungal species (Doolittle 1999; Hillis and Dixon 1991), and it was used here to identify major morphotypes from diseased boxwood. The PCR reactions were performed in a total volume of 15 µl containing 1× PCR buffer (50 mM Tris-HCl, pH 8.5); 2.5 mM MgSO<sub>4</sub>; 0.2 mM of each dNTP; 0.5 µM of each primer separately (ITS1 and ITS4, White et al. 1990); 0.6 U Tsg DNA polymerase (Biobasic, Scarborough, Ontario, Canada); and 1 µl of DNA (1–10 ng/µl) extract, all gently mixed by finger vortexing. Amplifications were done in a MyCycler thermal cycler (Bio-Rad, Mississauga, Ontario, Canada), with an initial denaturation step of 94 °C for 2 min, followed by 35 cycles of 94 °C for 30 s, annealing at 55 °C for 1 min, extension at 72 °C for 1 min, and a final extension at 72 °C for 10 min. Electrophoretic separation of PCR products was done in 1 % agarose gels and visualized on an ultraviolet (UV) transilluminator (Syngene, Cambridge, U.K.). Each PCR product was sequenced with primers ITS1 and ITS4 at the Laboratory Services Division, University of Guelph. The sequencing results were subjected to similarity analysis against the GenBank NR database using BLASTN (Altschul et al. 1990). The highest scoring matches with annotated genus and species were selected as potential matches for each sequence.

Because *P. buxi* was the primary fungal species isolated from diseased boxwood plants, three isolates were further assessed with ITS-PCR: 08126 from Georgetown, Ontario, 09012 from St. Catharines,

Ontario, and 10113 from Abbotsford, B.C. Amplified ITS products from isolates 08126, 09012 and 10113 were sequenced with both forward and reverse ITS primers at the Laboratory Services Division, and consensus sequences were generated and compared with each other and against the GenBank NR database.

Because of inconsistencies in the ITS database records on GenBank for *Isblutella* species, and to further confirm the identity of the putative *P. buxi* isolate 10113, the beta-tubulin gene was partially sequenced. To amplify this gene, a set of primers was designed based on the alignment of the partial beta-tubulin sequences of the following Hypocreales: *Gibberella fujikuroi* (GFU27303); *Gibberella zeae* (Schwein.) Petch (HQ141668); *Fusarium lunatum* (Ellis & Everh.) Arx (EU926357); *Fusarium langsethiae* Torp & Nirenberg (HQ141666); *Fusarium merismoides* var. *violaceum* Gerlach (EU860012); *Fusarium domesticum* (Fr.) H.P. Bachm. (EU926355), *Fusarium* sp. (EU926356), and *Pseudonectria rousseliana* (DQ522522). The multiple sequences were aligned with MUSCLE version 3.6 (Edgar 2004) using default parameters, and the alignment viewed using CLUSTAL X version 1.81 (Thompson et al. 1997). Conserved regions were chosen for primer design with Gene Runner (v 3.04; Hastings Software, USA) while avoiding potential dimer formation and other priming issues. Primers were synthesized by the Laboratory Services Division at the University of Guelph.

### Fungal growth temperature test

Mycelial growth of nine isolates of *P. buxi* of diverse origin was evaluated at six different temperatures. Agar plugs, 5 mm in diameter, were taken from actively growing colonies on PDA and transferred into screw-cap test tubes (21×150 mm) containing 6 ml of PDA. The agar had been allowed to set with test tubes placed horizontally so that a flat thin even layer of agar solidified inside the length of each tube. Inoculated tubes were placed in incubators at 10, 15, 20, 25, 30 and 35 °C in the dark. Three replicate tubes were used for each combination of isolate and temperature. The extent of growth was measured every 2 days for up to 24 days.

### Inoculation tests

Detached leaves from asymptomatic 1-year-old ‘Green Gem’ plants were used for initial assessment of the

pathogenicity of single representatives from the eight major fungal morphotypes. Each isolate was transferred onto fresh PDA and grown for 7 days to produce inoculum, in the form of spore suspensions if spores were present, or as mycelial plugs. Leaves were washed with autoclaved deionized water, and placed abaxial or adaxial side up in 9-cm-diameter Petri dishes lined with filter paper (Qualitative P5, Fisher). The size of leaves ranged up to 1 cm by 2.5 cm. Autoclaved deionized water (2 ml) was added to each dish to maintain high humidity for infection. For fungal isolates which sporulated, aliquots of 0.14 ml of spore suspensions of  $10^6$  spores/ml were spread over both top and bottom surfaces of leaves. For isolates which did not sporulate, a 5-mm-diameter mycelial plug from a 7-day-old PDA culture was placed on the top of a boxwood leaf. Four detached leaves were placed in each Petri dish with three dishes for each isolate. All cultures were sealed with Parafilm (Fisher) and placed at 25 °C with 24 h light ( $50 \mu\text{mol}/\text{m}^2/\text{s}$ ). These experiments were repeated at least three times, and negative controls (water spray or PDA plugs without fungal inoculum) were also used as treatments. The leaves were observed daily up to 7 days, and if any disease symptoms or signs were present, attempts were made to re-isolate the causal agent.

### Susceptibility tests

Three major factors were examined with respect to susceptibility of *P. buxi* on detached leaves: cultivar, tissue type and age, and wounding. For the various susceptibility tests on detached leaves, spore suspensions were used for inoculation following the methods mentioned above. As a measure of disease severity, the density of sporodochia was rated from 0 (no sporodochia) to 9 (fully covered by sporodochia), and leaf discolouration was noted where present. For each test, there were between three and five replications.

To assess disease resistance of different boxwood cultivars, asymptomatic leaves of ‘Green Gem’, ‘Green Mountain’, ‘Green Velvet’, ‘Green Mount’, and ‘Pincushion’ were used. Detached leaves were placed either abaxial or adaxial side up on dishes and inoculated with spore suspensions. To assess differences in susceptibility by leaf age, 1-month-old and 1-year-old ‘Green Gem’ leaves were used. One-month-old leaves were harvested from new growth of 1-year-old boxwood plants, while 1-year-old leaves were selected as the bottommost leaves of the same plants. Four leaves of each age were

placed in one Petri dish, totalling eight detached leaves per dish, and three replicates were used. To assess whether wounded tissues were more susceptible, both intact detached leaves and cut leaves of ‘Green Velvet’, ‘Green Gem’ and ‘Green Mountain’ were placed in Petri dishes, with three replicates per cultivar, and incubated at 25 °C under light.

To further exam the susceptibility on cut tissues, small twigs and rooted cuttings of ‘Green Velvet’ were also tested. To assess whether infection would spread along a twig, the basal cut site of each twig (5–8 cm long) retaining 5–10 leaves, was inoculated with a hyphal plug. The detached twigs were incubated as described for the detached leaves. For rooted cuttings of ‘Green Velvet’, non-wounded and wounded leaves were also tested. Wounds were made on attached leaves by cutting three leaves in half. Both wounded and non-wounded cuttings were sprayed with a suspension of  $10^6$  spores/ml until runoff or with a water control. All rooted cuttings were covered with plastic bags for 3 days to maintain high humidity for infection, and the bags were removed after 3 days. The plants were incubated under light at 25 °C. Observations were made daily up to 2 weeks.

### Statistical analyses

The mycelial growth and disease ratings of *Pseudonectria buxi* were subjected to analysis of variance with SAS PROC GLM (SAS Institute, Cary, NC, USA). When significant treatment effects were found, means were separated by the test of least significant difference (LSD;  $p=0.05$ ).

## Results

### Symptomatic samples and fungal identification

Symptoms on boxwood from nurseries ranged from yellow leaves on green stems to entirely dead shoots (Fig. 1). Although leaves and branches on some plants were dry and dead, usually the plants were still alive. Of the 80 symptomatic plants, 90 % had pink spore-bearing bodies on the leaves or stems (Fig. 2), and approximately 20 % of the stems had epidermal black streaks on the petioles or the stems attached to the petioles (Fig. 3). Pink spore-bearing bodies were sometimes found on the surface of black streaks. Approximately 10 % of diseased plants had both pink spore-bearing bodies and black streaks.



**Fig. 1** Volutella blight caused by *Pseudonectria buxi* on boxwood. Diseased leaves are bronzed and yellowed and become shrivelled

From the 80 symptomatic boxwood samples, over 400 isolates were found. Around 100 were considered common ubiquitous fungi and contaminants such as species of *Penicillium* L., *Aspergillus* P. Micheli ex Haller and *Cladosporium* Link, and these were not further tested. Root isolations from symptomatic plants also gave a variety of other fungi but without major consistent morphotypes and these isolates were not retained for further analysis.

The 312 isolates were grouped into eight major morphotypes after repeated subculturing and observations over several weeks, and based on colony characteristics



**Fig. 2** Pink sporodochia of *Pseudonectria buxi* are observed on infected leaves and stems of boxwood



**Fig. 3** Black streaking can be observed on some boxwood stems associated with Volutella blight

and spore-producing structures. Isolation frequencies for cultural morphotypes were as follows: 46 % pink-orange; 11 % pure white; 10 % light purple; 8 % red-grey; 8 % yellow-orange; 7 % red; 7 % white-yellow; and 3 % dark background with protruding black dots. The pink-orange fungus (Fig. 4) was isolated from chlorotic and necrotic areas on leaves and stems as well as from black streaks on stems, and occasionally (<15 % of the time) from asymptomatic tissues. This pink-orange fungus had verticillate conidiophores and elliptical spores,  $6-9 \times 2-2.5 \mu\text{m}$  (Fig. 5). Acervuli with setae and conidia were observed on the infected tissues, which is one of the features of *Volutella* species, but these structures are usually not found in cultures.



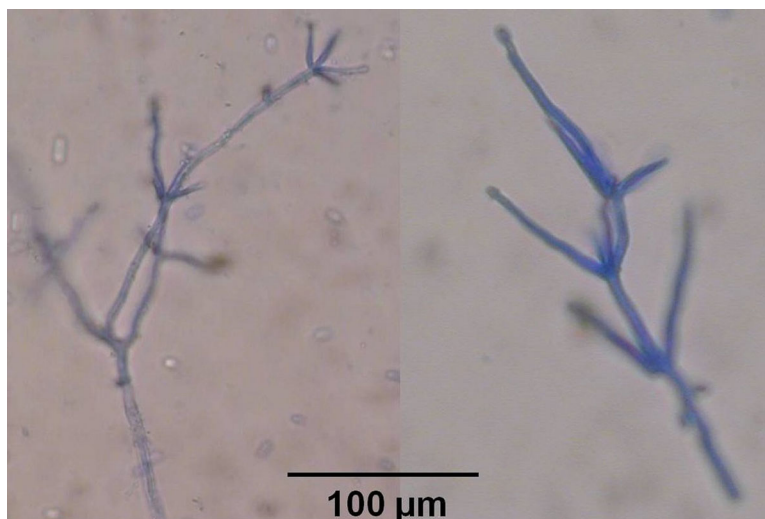
**Fig. 4** Colony of *Pseudonectria buxi* grown on PDA at 25 °C for 7 days

Single representatives of each of the eight morphotypes were then subjected to morphological identification and DNA sequencing of the ITS regions to confirm identity (Table 1). The sequencing results were subjected to similarity analysis against the GenBank NR database using BLASTN, with the following results: pink-orange (isolate 08126), 483/491 bp top match with *Pseudonectria buxi* (HQ897800); pure white, 421/421 bp top match with *Sarocladium strictum* (W. Gams) Summerbell (KC254093); light purple, 420/424 bp top match with *Fusarium oxysporum* Schltdl. (GU445374); red-grey, 481/488 bp top match with *Glomerella fiorinae* (Marcelino & Gouli) R.G. Shivas & Y.P. Tan (JQ754369); yellow-orange, 505/505 bp top match with *Epicoecum nigrum* L. (KC311470); red, 479/480 bp top match with *Fusarium tricinctum* Nees (EF611095); white-yellow, 529/529 bp top match with *Bionectria ochroleuca* (Schwein.) Schroers & Samuels (JQ782651); and protruding black dots, 504/508 bp top match with *Phoma* sp. Sacc. (HE608801). The sequencing results have been deposited in GenBank (Table 1).

#### Sequence analysis of *P. buxi*

To confirm the morphological identification of the pink-orange fungus (Fig. 4) as *P. buxi*, three isolates from different locations (08126, 09012 and 10113) were subjected to ITS-PCR. The individual consensus sequences from forward (ITS1) and reverse (ITS4) primers were 491, 427 and 517 bp, respectively, and they showed 100 % identity to each other in their overlapping regions.

**Fig. 5** Verticillate phialides of *Pseudonectria buxi* grown on PDA at 25 °C for 7 days



When these sequences were compared against the GenBank NR database at the start of the study (February 2010), a sequence annotated as *Volutella buxi* (FJ555527) showed 97 % identity (496/513 bp), while a sequenced annotated as *Volutella ciliata* (AJ301967) showed 99 % identity (512/522 bp). To resolve this, sequencing of another region was done to confirm identity.

Primers were designed to amplify a part of the beta-tubulin gene based on multiple alignments with eight fungal species closely related to *P. buxi*. The designed beta-tubulin primers were btub\_F750: 5'-AACAACTGGCCAAGGGTC and btub\_R1400: 5'-GAAGAGTTCTTGTTCTGGA. The same amplification methods were used as in ITS-PCR but with an annealing temperature of 45 °C. A single band was observed from the PCR reaction, and the product was sequenced using both forward and reverse primers, giving a consensus sequence of 592 bp. The sequence results were compared against the GenBank nr database using BLASTN, and the top match was 97 % (575/592 bp) with *P. rousselliana* (DQ522522). The next 40 best matches were with species of *Fusarium* at 91 % sequence identity or less.

#### Growth temperature test

Nine isolates of *P. buxi* from Ontario nurseries were grown at various temperatures (10, 15, 20, 25, 30 and 35 °C) up to 24 days to determine their growth temperature optima. Mean growth rates of nine isolates at each temperature did not show significant differences ( $p=0.15$ ); however, differences in mycelial growth rate were

**Table 1** Number and frequency of morphotypes recorded from 312 fungal isolates associated with Volutella leaf and stem blight of boxwood

Morphotype	Frequency	Species (Accession)
Pink-orange	46 %	<i>Pseudonectria buxi</i> (KC819610)
Pure white	11 %	<i>Sarocladium strictum</i> (KC819613)
Light purple	10 %	<i>Fusarium oxysporum</i> (KC819615)
Red-grey	8 %	<i>Glomerella fioriniae</i> (KC819618)
Yellow-orange	8 %	<i>Epicoccum nigrum</i> (KC819619)
Red	7 %	<i>Fusarium tricinctum</i> (KC819616)
White-yellow	7 %	<i>Bionectria ochroleuca</i> (KC819614)
Dark background with protruding black dots	3 %	<i>Phoma</i> sp. (KC819617)

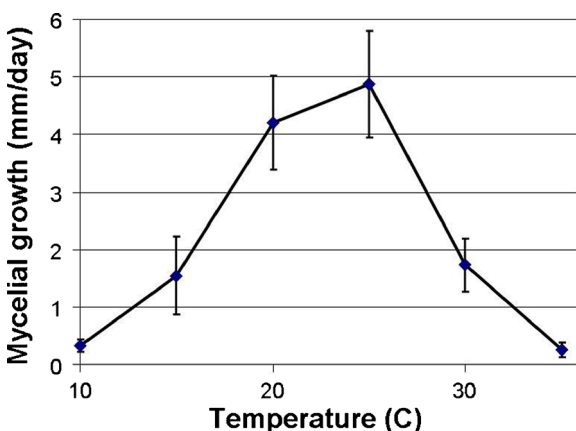
A representative isolate from each of the eight major colour morphotypes was further investigated with morphological and molecular biology techniques, and the ITS sequences have been deposited in GenBank with accession number shown

observed among various temperatures ( $p < 0.0001$ ). At the lowest (10 °C) or highest (35 °C) temperatures, growth rate was the lowest, at 0.33 or 0.25 mm/day, respectively (Fig. 6). At 15 and 30 °C, growth rate was suboptimal at 1.54 and 1.73 mm/day, respectively (Fig. 6). Isolates grew the fastest at 20 or 25 °C, with 4.21 or 4.88 mm/day, respectively, which were not significantly different (Fig. 6). A growth temperature of 25 °C was used in the subsequent tests.

#### Inoculation tests

Detached boxwood leaves were inoculated with representative isolates of each of the eight morphotypes for which the ITS regions had been sequenced. Except for representatives of the morphotype identified as *P. buxi*, none of the other seven fungi were found to cause disease symptoms on detached boxwood leaves using

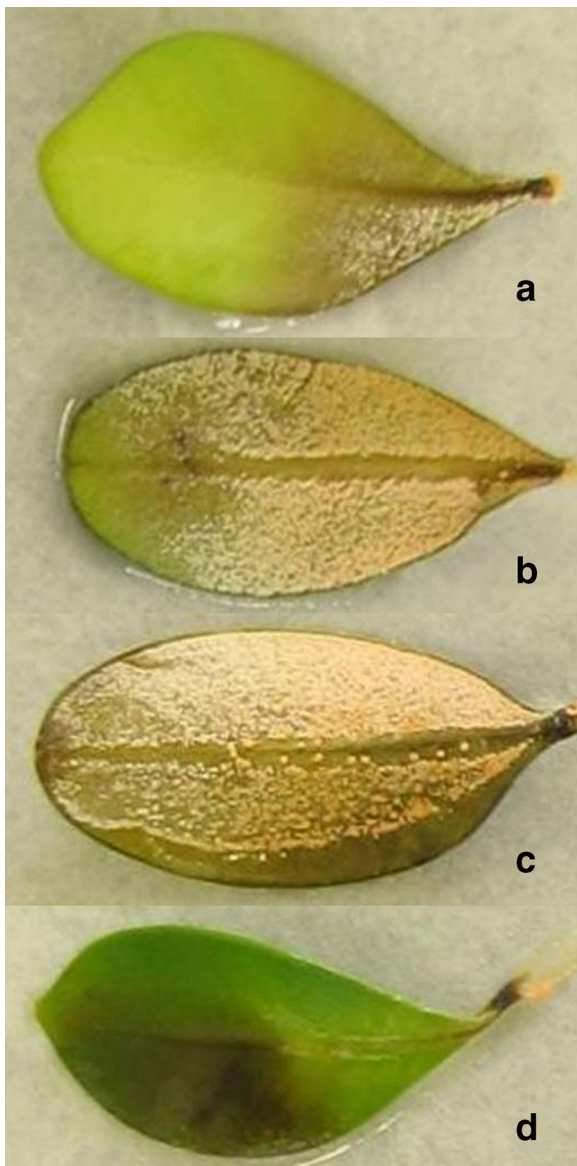
spore inoculations or mycelial plug tests even weeks after inoculation and continuous incubation at 100 % relative humidity. On detached leaves that were inoculated with spore suspensions of *P. buxi*, verticillate structures and pink sporodochia were first observed on the abaxial leaf surfaces 3 days later, first appearing on the cut ends of the petioles and then spreading toward the distal part of the leaves (Fig. 7a–c). After 7 days, yellowing and other discoloration was observed on inoculated leaves, but not on mock-inoculated leaves. By day 10, the abaxial surfaces of detached leaves, whether intact or cut in half were fully covered with sporodochia. Yellowing and other discoloration was also observed by day 10, but the leaves were not desiccated and shrivelled as seen in the field, probably because of the high relative humidity conditions in Petri dish. On adaxial surfaces, yellowing could be observed, but no sporodochia were seen (Fig. 7d). Isolation from inoculated detached leaves gave cultures of *P. buxi*, while isolations from mock-inoculated leaves which remained asymptomatic gave occasional common airborne fungi, but never *P. buxi*. The yellowed leaf symptoms were fully reproduced and the pathogen re-isolated to successfully complete Koch's postulates.



**Fig. 6** Mean mycelial growth rates of *Pseudonectria buxi* at 10, 15, 20, 25, 30 and 35 °C on PDA. Each isolate by temperature combination was replicated three times. Error bars indicate standard error

#### Boxwood susceptibility

In susceptibility tests on different tissue types, the same process of disease development of Volutella blight was observed as in the inoculation test described above. Different cultivars also had differential susceptibility to infection. Among five boxwood cultivars ('Green Gem', 'Green Velvet', 'Green Mound', 'Green Mountain' and 'Pincushion'), 'Green Gem' was found to be the most susceptible cultivar (disease rating 3.2 at day 8 after inoculation, on a scale of 0=no disease to 9=100 %



**Fig. 7** Detached leaves of boxwood ‘Green Velvet’ inoculated with spore suspensions of *Pseudonectria buxi*. Day 3 (**a**), day 5 (**b**) and day 10 (**c**) show the abaxial side of the leaves where disease developed from the wounded petiole, and sporodochia spread toward the leaf tip. Day 10 (**d**) shows the adaxial surface with yellowing, but sporodochia did not develop even when the other side was fully covered with sporodochia

disease), while ‘Pincushion’ was the least susceptible (disease rating 0.8). The leaf age had a significant impact on susceptibility, where 1-month-old boxwood leaves were more susceptible to infection than 1-year-old leaves, where the disease ratings at 7 days after inoculation were 5.9 and 1.6, respectively (LSD=1.3 at  $p=0.05$ ).

The rate of disease development was much faster on cut leaves than detached leaves which only had a small wound at the petiole detachment. By day 5 after inoculation, sporodochia covered 80–100 % of the abaxial surfaces of cut leaves, but less than 40 % area was covered by sporodochia on intact detached leaves starting from the wound at the petiole.

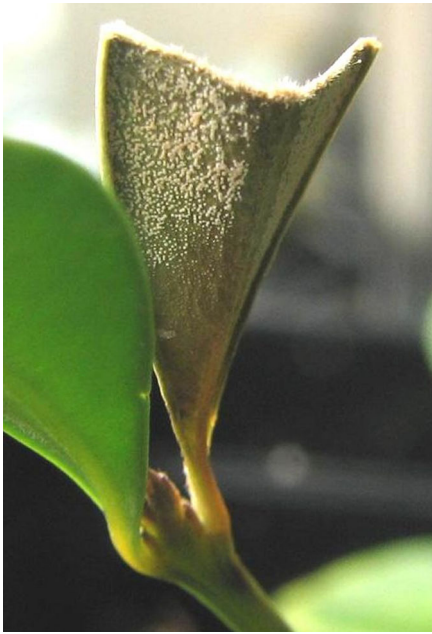
To further examine the susceptibility of wounded tissues, other tests were done with detached twigs, and rooted cuttings. On detached small twigs which were inoculated with spore suspensions over the entire twigs, pink sporodochia were first observed near the bottom cut ends of the twigs by 3 days after inoculation. Sporodochia continued to form up the twig from the cut site, and onto leaves; and by 2 weeks after inoculation, sporodochia could be seen on leaves and stems of the lower half of the 5–8 cm-long twigs.

For rooted cuttings which were not artificially injured, but were inoculated with spore suspensions, disease did not develop even a month after inoculation. On rooted plants where several leaves were cut, infection started from the cut edges, with sporodochia appearing usually within 3 days, accompanied by yellowing, with the remaining parts of the cut leaves developing abundant sporodochia by day 7 (Fig. 8). Pink sporodochia were observed on wounded leaves 3 days after inoculation, but not on the neighbouring non-wounded leaves even a month after inoculation. Between 1 and 2 weeks after inoculation, cut leaves began to yellow, and by 2 weeks after inoculation, leaves became further discoloured and began to wither and yellow when not incubated under high humidity conditions.

## Discussion

For over 15 years now, a blight of boxwood has been observed in nurseries in southern Ontario. This is not the box blight disease caused by *Cylindrocladium buxicola* which was first detected in North America in 2011, and is currently causing great concern in the nursery industry in the U.S. (Ivors and LeBude 2011) and Canada (Elmhirst et al. 2013). Our intensive sampling from several nurseries between 2008 and 2009 and subsequent sampling in 2010 and 2011 did not reveal the presence of *C. buxicola*. Instead, we encountered mainly a fungus associated with dieback of leaves and twigs, and bearing pink sporodochia concentrated on the underside of leaves.





**Fig. 8** This leaf on a rooted cutting of ‘Green Velvet’ was cut and inoculated with a spore suspension of *Pseudonectria buxi*. Pink sporodochia first formed on the cut edge by day 3, and yellowing and discoloration were observed on the wounded leaf by 10 days after inoculation

Most of the isolates obtained in this study were from foliage, but there were attempts at root isolations to assess the possibility of predisposition by a root pathogen with subsequent invasion by *P. buxi*. The roots of symptomatic plants were usually healthy without visible lesions or dieback. From root isolations, there were no consistent major fungal morphotypes isolated after surface sterilization procedures. Whether inoculation of plants with a root pathogen would lead to greater infection by *P. buxi* was not tested, but inoculation of artificially wounded leaves of healthy plants with *P. buxi* did lead to heavy disease levels, so the occurrence of Volutella blight did not seem to require pre-existing infection by other pathogens, although wounds were required for infection. Almost half of the 312 isolates from 80 symptomatic samples obtained between 2008 and 2009 from southern Ontario nurseries were found to belong to the pink-orange morphotype which was identified as *P. buxi* based on morphological and molecular techniques.

Comparisons of the ITS sequence of three isolates of *P. buxi* showed matches with sequences in GenBank annotated as *P. buxi*, *P. rousseliana* or *Volutella buxi* which are all considered synonyms of the same fungus.

However, one of the top matches was with a fungus annotated as *V. ciliata* (AJ301967). There were other ITS sequences in GenBank annotated as *V. ciliata*, but they did not match AJ301967, probably indicating some errors in identification and annotation. To investigate this further, we found a beta-tubulin sequence for *P. buxi* in GenBank (DQ522522), and designed conserved primers to amplify a ~600 bp portion of the gene. The use of these primers with isolates which we identified as *P. buxi* yielded a 592 bp fragment which had 97.1 % identity with DQ522522. This was an unexpectedly high level of variation, so to examine the variation of the beta-tubulin gene within a fungal species, another nectriaceous fungus *Neonectria ditissima* (Tul. & C. Tul.) Samuels & Rossman was selected for closer examination. Among the 24 beta-tubulin sequences of *N. ditissima* found in GenBank, a multiple alignment showed 11 polymorphic sites in a 600 bp portion of the beta-tubulin gene with some isolates showing this full range of variation (e.g. JF268726 vs. DQ789870) at 98.2 % identity. In comparison, the *P. buxi* beta-tubulin gene sequenced here of isolate 10113, was found to have 17 differences in this 600 bp region compared to the sequence on GenBank (DQ522522). At this level of divergence (97.1 % identity), the two *P. buxi* sequences were judged to be of the same species. The ITS sequences (isolate 08126, 09012 and 10113) of *P. buxi* have been deposited in GenBank as accessions KC819610, KC819611, KC819612, and the beta-tubulin sequence as accession KC819609.

To characterize the biological activity of *P. buxi*, growth tests at different temperatures with nine isolates revealed that the fungus showed the fastest growth between 20 and 25 °C, but could also grow at 10 and 35 °C, the lowest and highest temperatures tested. The growth at these suboptimal temperatures was surprisingly vigorous at up to 30 % of the optimal growth rate. This implies that this fungus has a broad ecological range, and could be causing infections when the plants may be dormant or growing slowly.

After inoculation of non-wounded leaves, either abaxial (many stomates) or adaxial (no stomates), the leaves did not become infected, implying that this fungus does not penetrate through natural openings such as stomates. Bezerra (1963) described germ tubes of *P. rousseliana* (sexual stage of *P. buxi*) penetrating boxwood leaves through stomates, but this was not observed in the current study. Sporodochia of *P. buxi* were produced only on abaxial surfaces and adjacent areas, but not on adaxial surfaces, although spores would form

on the abaxial side of adaxially inoculated leaves. Perhaps sporodochia do not form regularly on adaxial surfaces because of the thicker cuticle compared to abaxial surfaces, or perhaps the presence of stomata on the abaxial surfaces allow egress of the fungus and subsequent sporodochial formation.

One-month-old boxwood leaves were found to be more susceptible to infection than 1-year-old leaves. This result is in agreement with previous studies where tissues from young tissues were found to be more susceptible (Agrios 2005). Possibly older boxwood leaves have more lignin in their cell walls or thicker cuticles to prevent pathogen penetration into plants (Mamza et al. 2008). Among the five common Ontario-grown cultivars tested, all were found to be susceptible, with ‘Green Gem’ the most susceptible, and ‘Pincushion’ the least. The results of susceptibility on different cultivars in this study were consistent with nursery reports of losses due to *Volutella* blight where ‘Green Gem’, ‘Green Velvet’, ‘Green Mound’ ‘Green Mountain’ had losses of 58 %, 40 %, 30 % and 10 %, respectively, in 1-year-old rooted cuttings at a nursery in Ontario in 2009. *Buxus sinica* var. *insularis* ‘Pincushion’ was reported as resistant to insects (Batdorf 2004), and we found the least amount of *Volutella* blight on leaves of ‘Pincushion’. Further assessment with more isolates of *P. buxi* from more locations (other provinces or countries) may provide better confirmation of greater resistance toward *Volutella* blight by ‘Pincushion’ than other cultivars tested.

In inoculation tests on detached leaves, small twigs, and rooted cuttings, only wounded sites were found to be susceptible to infection. Differential susceptibility of wounded tissues of other plants to related fungi has been previously reported. Wounded stems of *Pachysandra terminalis* Siebold & Zucc. showed differential susceptibility to infection by *Volutella pachysandricola* B.O. Dodge, depending on the time of year that wounds were inflicted, since *P. terminalis* was more susceptible after wounding in April or May than in June, July or September (Hudler et al. 1990). More research is needed on seasonal differences in susceptibility of boxwood tissues to *Volutella* blight.

In Ontario, boxwood cuttings are often made from older mother plants in September to October for propagation, and kept in propagation rooms (25 °C and 85 % humidity) over winter. After 1 month, careful attention may reveal the presence of small pink spore-bearing bodies. At the end of May of the following year, cuttings are usually out-planted in the field or in the lathe-house,

including ones that are infected. Serious losses can occur at all stages where wounding of boxwood tissues occurs and the tissues are kept under moist warm environments. There has been surprisingly little work done on the etiology of *Volutella* blight, which is likely the most commonly observed disease on boxwood. Because of its frequency on dead tissues, it has been considered a saprophyte or secondary invader. However, rather behaving just as a saprophyte or secondary invader, *P. buxi* in this study was found to be capable of primary invasion after wounding, and causing extensive disease on susceptible boxwood tissues. Further research is needed on its interactions with other pathogens such as the recently introduced *C. buxicola*, and whether there is synergistic activity leading to greater disease severity. Further research is also needed on cultural and chemical methods to control this disease.

**Acknowledgments** This research was supported by funding from Landscape Ontario and the Ontario Ministry of Agricultural, Food and Rural Affairs. Donation of plant material from nurseries in Ontario is also gratefully acknowledged.

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