Végéphyl – SCIENTIFIC SYMPOSIUM ON BOXWOOD PESTS
TOURS – 16 AND 17 OCTOBER 2018

BIOLOGY AND MANAGEMENT OF VOLUTELLA BLIGHT CAUSED BY *PSEUDONECTRIA BUXI*

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ABSTRACT

A severe boxwood disease has been observed in Southern Ontario nurseries since the late 1990s. Symptoms ranged from yellow leaves on green stems to entirely dead shoots. Among 80 symptomatic plants, 90% had pink spore-bearing bodies on the leaves or stems, and approximately 20% of the stems had epidermal black streaks on the petioles or the stems attached to the petioles. Half of over 300 isolates obtained from symptomatic tissues belonged to a pink-orange cultural morphotype and were identified as *Pseudonectria buxi* using morphological and molecular techniques. In inoculation studies, wounds were the major penetration points for *P. buxi* since non-wounded inoculated tissues did not become diseased. The *Buxus* cultivar 'Green Gem' was the most susceptible compared to 'Green Velvet', 'Green Mound', or 'Green Mountain'. Six fungicidal treatments showed strong preventive activity and some curative activity against Volutella blight.

Keywords: disease, boxwood, fungicide, inoculation, wounding;

RÉSUMÉ

BIOLOGIE ET GESTION DE VOLUTELLA BLIGHT CAUSÉ PAR *PSEUDONECTRIA BUXI*

Une grave maladie du buis a été observée dans les pépinières du sud de l'Ontario depuis la fin des années 1990. Les symptômes correspondaient à des feuilles jaunissantes et des rameux entièrement morts. Parmi 80 plantes symptomatiques, 90% portaient des fructifications (spore) rosâtres sur les feuilles et les tiges et environ 20% des plantes présentaient des stries noires épidermiques sur les pétioles ou les tiges.. Les analyses morphologiques et moléculaires ont permis de metre en evidence que plus de la moitié des 300 isolats obtenus (à partir de tissus symptomatiques) appartenaient à un morphotype rose-orange identifié comme *Pseudonectria buxi.* Les études d'inoculation, ont montré que les plaies étaient les principaux points de pénétration de *P. buxi.* En effet les inoculations sur les tissus non blessés ont échoué. . Le cultivar *Buxus* 'Green Gem' était le sensible par rapport à 'Green Velvet', 'Green Mound' ou 'Green Mountain'. Six traitements fongicides ont montré une forte efficacité préventive et une certaine éfficacité curative contre le Volutella.

Mots clés: maladie, buis, fongicide, inoculation, blessure

INTRODUCTION

The fungus *Pseudonectria buxi* (DC.) Seifert, Gräfenhan & Schroers also known as *Volutella buxi* (DC.) Berk. is the cause of Volutella blight of *Buxus* species (Bezerra, 1963). This fungus has been reported from Europe (Saccardo, 1883), North America (White, 1931) and Asia (Shi and Hsiang, 2014a), and is probably found wherever *Buxus* is grown and environmental conditions permit disease development. Volutella blight has commonly been considered a secondary disease of boxwood, since the pathogen is often found on dead or previously killed tissues. With Volutella blight, diseased leaves and stems turn yellow and die back, and although diseased plants can stay alive, the aesthetic appearance is adversely affected. In nurseries, management of the disease has required extensive culling, especially of recently rooted cuttings propagated under warm, humid, enclosed conditions, which favour infection and disease spread. In addition to the necrotic leaves and twigs, pink spore masses of *P. buxi* may be found on stems and lower surfaces of leaves, and black streaks are also sometimes found on petioles and stems. Since the late 1990's, significant economic losses have been observed due to this disease in local nurseries, with over 50% loss of potted cuttings. The purpose of this work was to examine the biology and management of Volutella blight.

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 British Columbia in Canada. To obtain isolates, tissues were cut into 5-mm-long segments including diseased areas bordering on live areas of stems or leaves, and surface sterilized. 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 sulfate (Fisher BP910). The cultures were incubated at room temperature (~25 °C). If an isolate was capable of sporulation, a single-spore culture was obtained. Cultures were tentatively identified as *P. buxi* based on comparisons with confirmed isolates, and a few were subjected to ITS sequencing (White et al., 1990) to confirm identity.

PLANT CULTURE

Healthy potted one-year-old boxwood plants of *Buxus* cultivars 'Green Velvet', 'Green Gem', 'Green Mountain' 'Green Mount' and 'Pincushion', up to 30 cm tall in 1 L pots, were provided by local nurseries. These are open-pollinated seedlings with the female parent *B. sempervirens* 'Suffruticosa' and the male parent *B. sinica* var. *insularis,* except for 'Pincushion' which is *B. sinica* var. *insularis.* For plants used in rooted cutting experiments, 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-butytic acid), and were replanted in 3-inch pots with potting mix. The potted cuttings and whole plants were

placed at 25 °C under low level continuous fluorescent lighting (50 µmol/m^2/s). The plants were watered to soil saturation weekly, 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.

INOCULATION TESTS

Three major factors were examined with respect to virulence 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. To measure of disease severity, the density of sporodochia was rated from 0 (none) to 9 (fully covered by sporodochia), and leaf discoloration was noted where present. For each test, there were up to five replications.

To assess disease resistance of different boxwood cultivars, asymptomatic leaves were used from each of five *Buxus* cultivars ('Green Velvet', 'Green Gem', 'Green Mountain' 'Green Mount' and 'Pincushion'). Detached leaves were placed either abaxial or adaxial side up in dishes lined with wetted filter paper. To assess differences in susceptibility by leaf age, one-month-old and one-year-old detached leaves of highly susceptible 'Green Gem' were used. One-month-old leaves were harvested from new growth of one-year-old boxwood plants, while one-year-old leaves were selected as the bottommost leaves of the same plants. Four leaves of each age were placed in one Petri dish, totaling eight detached leaves per dish, and three replicate plates 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. The leaves were sprayed with a suspension of 10^6 spores/ml until droplets beaded on the surface, and Petri dishes were sealed with parafilm to retain moisture.

For rooted cuttings of 'Green Velvet', non-wounded and wounded leaves attached to the plants were also tested. Wounds were made on attached leaves by cutting leaves in half. Both wounded and non-wounded plant tissues 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.

FUNGICIDE TESTS

To ensure consistent rates of infection through wounding and inoculation, the tips of eight leaves per potted plant were cut off just before inoculation. Six fungicide treatments, one inoculation-only treatment and one water-only treatment (Table 1) were applied to 30-cm-tall boxwood plants until runoff (approximately 1.5 mL/pot). Spore suspensions (10^6 spores/mL, prepared as described above) of a single isolate (08147) were used as inoculum. For both pre- and post-infection fungicide assessments, plants were placed into trays (28 cm × 53 cm) where treatments were randomized, and each flat of 18 plants was covered with a transparent plastic bag for 3 days after

inoculation to maintain high humidity needed for infection.

A range of fungicides commonly used for diseases of ornamental plants was tested in growth room trials (Table 1). To assess the pre-infection efficacy of fungicides, plants were inoculated 7 days after the six fungicidal treatments were applied, with three replicate plants per treatment. To assess the post-infection efficacy of fungicides, six fungicides were applied to plants in two ways: in group one, fungicides were applied at 7 days post-inoculation (dpi); and in group two, fungicides were applied at 7 dpi and again at 14 dpi. The combination of the six different fungicide treatments by application frequencies (single or double) resulted in a total of 14 different treatments (including untreated and inoculated controls) with three replicate plants per treatment during the same time.

Table 1. Treatments used against Volutella blight caused by *Pseudonectria buxi* on boxwood plants in a growth room (25 °C) [Traitements contre leVolutella causés par *Pseudonectria buxi* sur des plants entiers de buis dans une chambre de culture (25 ° C)]

Treatment	Manufacturer	Active ingredient	Product/L water[b]
Non-inoculated control	--	Water	--
Inoculated control	--	Spore suspension (10^6 spores/mL)	--
Phyton-27 + Nova 40W	Phyton Corporation + Dow AgroSciences Canada Inc	5.5% copper[a] + 40% myclobutanil	0.3 g + 0.3 g
Dithane DG 75WP	Dow AgroSciences Canada Inc	75% mancozeb	3 g
Daconil 2787F	Syngenta Canada Inc.	40% chlorothalonil	2.4 mL
Rovral Green	Bayer CropScience Inc.	240 g/L iprodione	24 g
Senator 70WP	Nippon Soda Company Ltd.	70% thiophanate-methyl	0.14 g
Banner MAXX	Syngenta Canada Inc	14.3% propiconazole	0.35 mL

[a] The copper was in the form of picro-cupric-ammonium formate in Phyton-27

[b] These dilution rates follow label recommendations, and each plant was treated until runoff with approximately 1.5 mL of fungicide solution or water.

Symptoms for pre-infection efficacy tests were rated based on the extent of foliar yellowing and shriveling, as a product of the number of symptomatic leaves (0 to 8 among the eight cut leaves) and the average proportion of symptomatic leaf area (ranging from 0 to 1 representing 0% to 100%). The range of disease ratings was from 0 (no disease) to 8 (all eight leaves fully symptomatic). Prior to statistical analyses, the ratings were transformed to percent where 0 = 0% disease to 8 = 100% disease. In the pre-infection efficacy test, plants were rated 7 days after inoculation.

For post-infection efficacy tests, the effects of fungicides on sporodochial production were rated at 7 days after last fungicide treatment using a similar scale to the one described above, but based on the number of sporodochia rather than yellowing and shrivelling. For single 7 dpi treatments in the post-infection fungicide test, disease was rated 14 days after inoculation, and for

the double application test, disease was rated at 21 days after inoculation which was 7 days after the last treatment.

STATISTICAL ANALYSES

The mycelial growth, disease ratings, and sporodochial counts 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.

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.

Among the remaining 312 isolates, almost half produced a pink-orange culture on PDA. This 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 fungus had verticillate conidiophores and elliptical spores, 6-9 × 2-2.5 µm (Fig. 5), and acervuli with setae and conidia were observed on the infected tissues, which is one of the features of *Volutella* species, but acervuli were not observed in culture. Four representatives of the pink orange fungus were then subjected to ITS sequencing with both forward (ITS1) and reverse (ITS4) primers. The sequencing results were subjected to similarity analysis against the GenBank NR database using BLASTN, and all four matched *Pseudonectria buxi* (HQ897800) with less than 9 mismatches in the 421 bp length. More details on isolation and characterization of the different morphotypes can be found in Shi and Hsiang (2014b).

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% disease), while 'Pincushion' was the least susceptible (disease rating 0.8). Leaf age had a significant impact on susceptibility, where one-month-old boxwood leaves were more susceptible to infection than one-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).

Figure 1: Volutella blight on *Buxus* (Volutella sur *Buxus*)

Figure 2: Pink spore-bearing bodies of *P. buxi* on leaves and stems of *Buxus* (Fructifications porteusess de spores roses de *P. buxi* sur *Buxus*)

Figure 3: Black streaks on petioles associated with *P. buxi* (Stries noires sur les pétioles engendrées par *P. buxi*)

Figure 4: Pink-orange colony on PDA after 7 days at 25 C (Colonie rose-orange sur milieu de culture PDA)

Figure 5: Verticillate conidiophores and elliptical spores of *P. buxi* (Conidiophores verticillaires et spores elliptiques de *P. buxi*)

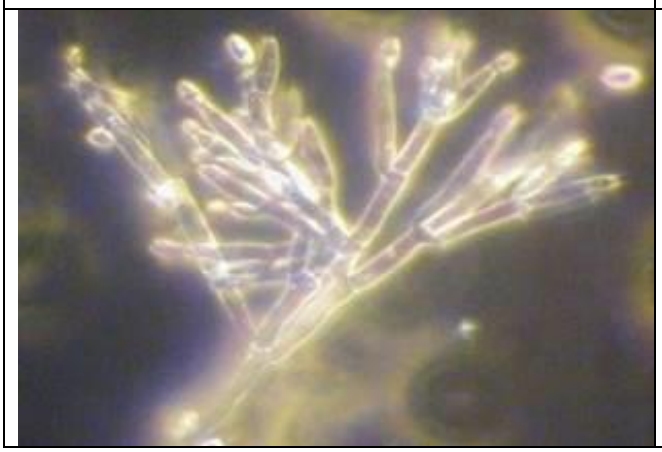

Figure 6: Cut leaves on rooted cuttings of *Buxus* prepared for inoculation (Feuilles coupées sur des boutures enracinées de *Buxus* servant à l'inoculation)

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 exam 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 to 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 leaves were cut (Figure 6), 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. Pink sporodochia were observed on wounded leaves 3 days after inoculation, but not on the neighboring non-wounded leaves even a month after inoculation. Between 1-2 weeks after inoculation, cut leaves began to yellow, and by two weeks after inoculation, leaves became further discolored and began to wither and yellow when not incubated under high humidity conditions.

FUNGICIDE TESTS

On control plants which were wounded and inoculated with no fungicide treatment, the first symptoms were observed 3 days after inoculation, but no symptoms were observed on non-wounded inoculated leaves. The wounded inoculated leaves became yellowed and were covered

with sporodochia by day 7. In the pre-infection efficacy test where each fungicide was applied 7 days before inoculation, all six fungicidal treatments significantly suppressed disease development compared to the inoculated control (Table 3). Among the pre-infection treatments, significantly more disease was observed on plants treated with iprodione (13%) or mancozeb (6.3%), compared to the other four fungicides (1% to 5% disease), which were not significantly different from the water check with no observable disease at day 7 (Table 3).

In post-infection assessments, because leaves were wounded and inoculated with spore suspensions before fungicide treatment 7 days later, they all became infected and started turning yellow and shrivelling by 7 days after inoculation which was the time of fungicide application. After the fungicide application, the number of sporodochia appeared fewer, but the yellow and shriveled leaves did not recover. Therefore, the extent of sporodochial production was used to indicate the disease level for post-infection treatments. For both types of post-infection treatments with either single (day 7) or double (day 7 and day 14) fungicide applications, significantly fewer sporodochia were observed on the plants treated with any of the six fungicides. The most efficacious treatments (Table 4) involved chlorothalonil (14 or 8% disease) and propiconazole (16 or 11% disease) for either the single or double applications, respectively; and the least efficacious were iprodione (38 or 29% disease) and mancozeb (43 or 24% disease), respectively. Significantly more sporodochia were seen with post-infection treatments of iprodione than other treatments. More details on fungicide tests can be found in Shi and Hsiang (2015).

DISCUSSION

The inoculation tests showed the following: (1) one-month-old boxwood leaves were more susceptible to infection than one-year-old leaves; (2) 'Green Gem' was the most susceptible cultivar, 'Green Velvet', 'Green Mound' and 'Green Mountain' were moderately susceptible, and 'Pincushion' was the least susceptible among these tested; (3) wounding was required for infection; and (4) adaxial leaf surfaces were much less susceptible to infection than abaxial (upper) surfaces.

The life cycle of *Volutella buxi* and the disease cycle of Volutella blight have not been thoroughly investigated. Based on published details of the disease cycle of *Volutella pachysandrae* (Safrankova 2007, Plant Protection Science 43:10), and observations of Volutella blight and *P. buxi* from the current work, the putative life cycle of *P. buxi* in an outdoor environment is described as follows. As temperatures warm up in the spring, spores of *P. buxi* are produced by overwintered mycelium in dead tissues such as fallen leaves and attached dead tips and shoots. These spores are produced throughout the spring and dispersed by air or splashing water (rain or irrigation). They can infect boxwood tissues, especially younger new foliage which is more susceptible than older overwintered foliage. The fungus will grow through foliar tissues and into woody tissue. In summer with drier warmer conditions, spore production is reduced, but with wet weather of over several days duration, the fungus can grow out from infected tissues, produce spores and cause more infections when plant surface wetness periods exceed 18 hr. In the fall, with wetter cooler conditions, the fungus is again active and will produce spores to cause more infections. In winter,

the fungus becomes dormant in fallen leaves and in stems of dead tissues until the following spring.

The disease cycle under controlled environment conditions may differ because enclosed environments offer protection from desiccation as well as more humid or wet and warmer conditions that are favorable for plant growth, but also for fungal growth. During this propagation cycle, there are various stages where Volutella blight might enter the system, and even become enhanced by cultural operations. These should be taken into consideration during such operations.

(1) For rooted cuttings, mother plants may have non-symptomatic infections that carry hyphae of *P. buxi* inside plant tissues, even if care is made to remove visibly diseased tissue.

(2) When cuttings are dipped in fungicides before propagation, the foliar portions are not protected. However, *P. buxi* is almost strictly restricted to entry through wounds, so if wounding of above ground parts is minimized during these controlled environment operations, infection levels might be decreased.

(3) With latent or non-symptomatic infections, *P. buxi* still can grow out from infected live tissues, produce spores, cause infections and be transported by air and splashing water. The infection process is faster in controlled environments between 20 to 25 °C and under high humidity in propagation rooms than in the external environment, since in lab studies with detached leaves, the entire disease cycle from infection to spore production can occur in three days. Furthermore, we found that from dead dried leaves, the fungus is still viable after six months under dry storage conditions at room temperature, and hence debris can be a source of infection, in addition to infected non-symptomatic live tissues.

(4) In cases where there is a high risk of disease, repeated applications of fungicides are likely needed because spores will be constantly produced and released under the moist warm growth conditions in controlled environments.

The following active ingredients have commonly been used against woody ornamental diseases: copper, myclobutanil, mancozeb, chlorothalonil, iprodione, thiophanate-methyl, and propiconazole. In preventive and curative tests on potted plants in growth rooms, all fungicides tested here were found to have the potential to be used in an integrated control program to reduce infection by *P. buxi* and losses due to Volutella blight. However, more testing of fungicides and other control agents is required under commercial operating conditions in the field and in greenhouses.

ACKNOWLEDGEMENTS

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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