Leaf Spot and Dieback of *Buxus* Caused by *Cylindrocladium buxicola*

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Abstract

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Leaf blight symptoms were observed on potted box plants (*Buxus sempervirens* cv. Suffruticosa and *B. microphylla*) in a nursery in South Moravia in August 2010. These symptoms were suggestive of box blight on *Buxus* spp. Characteristics of the visual symptoms, microscopic features, and identification of the isolates using DNA sequencing are described. The causal agent was isolated and identified as *Cylindrocladium buxicola* Henricot by means of morphological, cultural, and molecular characters. The effect of five commercial fungicides on *C. buxicola in vitro* was studied. The most effective fungicides (kresoxim-methyl, azoxystrobin, and mancozeb) inhibited conidia germination and mycelial growth (kresoxim-methyl, myclobutanil, and penconazole) of *C. buxicola* more than 96%.

Keywords: box blight; box; fungicides

In August 2010, we received ten potted *Buxus sempervirens* cv. Suffruticosa and *B. microphylla* plants from a nursery in South Moravia. These plants were affected by disease and exhibited dark spots on the leaves. As a result of the infection, the plants defoliated and dieback ensued. The sporulation covered the abaxial surface of infected leaves and we found two independent pathogens, pink sporodochia of *Volutella buxi* (DC.) Berk. and fuzzy white spore masses of the second pathogen, which was identified as *Cylindrocladium buxicola*. *V. buxi* is usually regarded as a wound pathogen causing dieback on box or considerable damage in nurseries.

Box blight, caused by *C. buxicola,* was reported from Europe and New Zealand in the 1990s (Henricot *et al*. 2000; Henricot & Culham 2002). In 2000, it was detected in Belgium and France (Crepel & Inghelbrecht 2003), in Ireland, Italy, and the Netherlands in 2003 (Henricot 2003), in Germany in 2004 (Brand 2005), in Spain in 2008 (Varela *et al.* 2009), and in Croatia in 2009 (Cech *et al*. 2010). *C. buxicola* was detected in *B. sempervirens*, *B. microphylla, B. sinica* var. *insularis, B. macowanii, B. bodinieri, B. glomerata, B. harlandii, B. japonica,* and *B. microphylla* (Henricot 2006). In the Czech Republic the incidence of *C. buxicola* was detected on *Buxus sempervirens* cv. Suffruticosa a *B. microphylla* (Šafránková *et al*. 2012).

MATERIAL AND METHODS

The pathogen was taken from the lower side of leaf blades which had brown spots and was examined microscopically (400× magnification) in lactic acid (3%). It was isolated by plating 1 µl of conidial suspension (10^3 conidia/ml water) on the surface of potato-dextrose agar (PDA) and Malt-agar (MA) in a Petri dish.

Identification of isolates using DNA sequencing. The colony morphology and growth of our strain were analysed both on PDA and MA. The

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single conidia colonies were used for molecular identifications. DNA was extracted from freshly grown mycelia of *C. buxicola* (100 mg) using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the standard protocol. The DNA region coding for β-tubulin was sequenced from T1 (O'Donnell & Cigeknik 1997)/Bt2B (Glass & Donaldson 1995) primers. The corresponding gene area (649 bp) was amplified from the T1/Bt2B primers using the Taq PCR Core Kit (Qiagen, Hilden, Germany). The reaction mixture was prepared according to the standard protocol with the addition of 0.8mM of each primer, and 30–50 ng of genomic DNA; the concentration of Mg^{2+} in the reaction buffer was increased to 4.0mM. The PCR cycling regime consisted of an initial denaturation of 95°C/3 min, followed by 35 cycles of 94°C/40 s, 55°C/50 s, and 72°C/55 s, and terminated by a final extension of 72°C/10 minutes. The resulting amplicon was separated on 1% agarose gel and then stained by ethidium bromide. The amplified fragment was purified from the agarose gel using QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and sequenced.

Pathogenicity test. The pathogenicity of the isolates was demonstrated by inoculating the leaves of five two-year-old plants of *B. sempervirens* cv. Suffruticosa and *B. microphylla*. The leaves of box were inoculated by spraying with a suspension of conidia (10^6 conidia/ml) of *C. buxicola* and sterile distilled water in control plants. (For preparation of conidial suspension see the section Testing of fungicides.) Inoculated plants were incubated in humid chamber (95% relative humidity, 23°C).

Testing of fungicides. Five commercial fungicides (Dithane DG Neotec – a. i. mancozeb, 0.2%; Discus – a. i. kresoxim-methyl, 0.025%; Horizon 25 EW – a. i. penconazole, 0.1%; Ortiva – a. i. azoxystrobin, 0.1%; Talent – a. i. myclobutanil, 0.06%) were tested *in vitro* for their effect on conidial germination and mycelial growth of *C. buxicola.* All fungicides are commonly used in the Czech Republic for the control of leaf spots in ornamental plants.

The PDA + fungicide medium was prepared by adding 1 ml of fungicide suspension at the relevant concentration to an Erlenmeyer flask (500 ml) containing 299 ml of PDA liquid cooled to 45°C. The mixture was agitated and poured into 90 mm Petri dishes. Concentrations were prepared on the basis of the recommended field application rates of the fungicides. Petri dishes without fungicides were prepared for the control.

Conidial suspension was prepared by growing the fungus in Petri dishes on PDA. After 14 days of incubation at 25 ± 1°C, 3 ml of 0.01% Tween 80 in distilled water was added to the Petri dish, and the conidial suspension (1 ml) was pipetted into a bottle (20 ml) containing distilled water (9 ml). This was adjusted to a final suspension (10^6 conidia ml). The final suspension (0.1 ml) was transferred to Petri dishes containing the PDA + fungicides at different concentrations (10^0, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}) and spread using a glass rod. The Petri dishes were incubated at 23 ± 1°C in dark. The number of germinated conidia was counted 24 h after incubation under a light microscope at 100× magnification. All conidia with an apparent germ tube were considered as germinated. There were four replicates per each fungicide treatment, and one hundred conidia were evaluated in each Petri dish.

To test the mycelial growth, mycelial plugs (3 mm^2) of PDA with *C. buxicola*, cut from a 10-day-old culture, were placed ("up down") in the centre of the Petri dish containing PDA + fungicides (five replicates for each treatment). The Petri dishes were incubated at temperature of 25 ± 1°C with 12 h photoperiod, and mycelial growth was assessed by measuring the colony diameter 14 days post inoculation.

RESULTS AND DISCUSSION

The pathogen was isolated and identified using traditional and molecular methods. The sequence (590 bp) was deposited in GenBank (Accession No. JN616287) and was 100% identical to published sequences of *C. buxicola* in GenBank (Nos. AY078123.1 and AY078118.1).

Typical symptoms of *C. buxicola* infection on the leaves were observed 13 days after inoculation, but no symptoms were visible on the control plants. *C. buxicola* was successfully re-isolated from the inoculated plants.

The observed symptoms on leaves matched the symptoms produced by *C. buxicola,* described on box in other countries (Henricot & Culham 2002).

Description of our strain
Cylindrocladium buxicola

Macroconidiophores (89–147 µm) were penicillate with broadly ellipsoidal hyaline vesicles on the top

(6.5–10.5 µm) with papillate apex. Primary branches were aseptate or one-septate (10–32.5 × 3–5.5 µm), secondary braches were aseptate (12–20.5 × 3–5.5 µm), and tertiary branches were rare. Each terminal branch produced 2–5 phialides (7.5–18 × 2.2–4.5 µm). Conidia (49–63 × 3.5–5.5 µm) were hyaline, straight, cylindrical, rounded at both ends, and one-septate. Chlamydospores were not found.

After 7 days of growth (on PDA and MA at 20°C to 22°C) colonies were approximately 15–18 mm in diameter. The colour of each colony was characterised by a brown central area surrounded by a white to cream mycelial growth.

Due to the popularity and expansion of boxwood and the spread of *C. buxicola* in neighbouring countries, infection is likely to spread in the Czech Republic. At risk are mainly thick bushes and plants irrigated by spraying, because at higher levels of air humidity and leaf wetness conidia grow after a few hours. This pathogen has a rapid disease cycle that can be completed in one week. The fungus can survive as mycelium on fallen leaves or chlamydospores on decomposing fallen leaves for at least 5 years (Henricot *et al*. 2008). The minimum temperature required for growth is 5°C, the optimum 25°C; growth is inhibited at 30°C, and at temperatures above 33°C the fungus dies (Henricot & Culham 2002). Germination of the conidia occurs as early as 3 h after infection, and penetration follows 5–7 h post-infection. The fungus directly penetrates the cuticule of the box plant, or the mycelium resurges through the leaf stomata 48 h after infection (Henricot 2003). It is necessary to follow strict hygiene measures. Tools and equipment, as well as hands and shoes, should be thoroughly cleaned after work and, where appropriate, disinfected. Badly affected plants should be disposed of; in fast-growing and less susceptible plants, infected parts may be removed. Because chlamydospores can survive for several years, the surface layer of soil must be removed.

In general, *Cylindrocladium* diseases are very difficult to be controlled with fungicides. Box blight is a foliar and stem disease and thorough coverage of the entire plant foliage is necessary to protect the plant.

The most effective fungicides in our tests *in vitro* were kresoxim-methyl, azoxystrobin, and mancozeb. These fungicides inhibited conidia germination of *C. buxicola* more than 96% (Figure 1). Jayasinghe and Wijesundera (1995) recorded 100% inhibition of conidial germination *Cylindrocladium quinqueseptatum* after mancozeb treatment. Bolland *et al*. (1985) noted mancozeb as the most effective fungicide against *Cylindrocladium quinqueseptatum.*

All the fungicides tested inhibited mycelial growth of *C. buxicola* but only the effectiveness of kresoxim-methyl, penconazole, and myclobutanil was higher than 96% (Figure 2). Our results are consistent with other published data (Brand 2006; Henricot *et al*. 2008). Henricot *et al*. (2008) looked at the effect of 13 fungicides on *C. buxicola* and found that some of the active substances inhibit spore germination (mancozeb, carbendazime, azoxystrobin, kresoxim-methyl) or mycelium growth (penconazole, prochloraz, carbendazim, kresoxim-methyl, epiconazole + pyraclostrobin or boscalid + pyraclostrobin). Brand (2006) tested nine fungicidal compounds for their activity against *C. buxicola*. Conidial germination was inhibited by low concentrations of tolylfluanid, mancozeb, chlorothalonil, and fludioxonil + cypro-

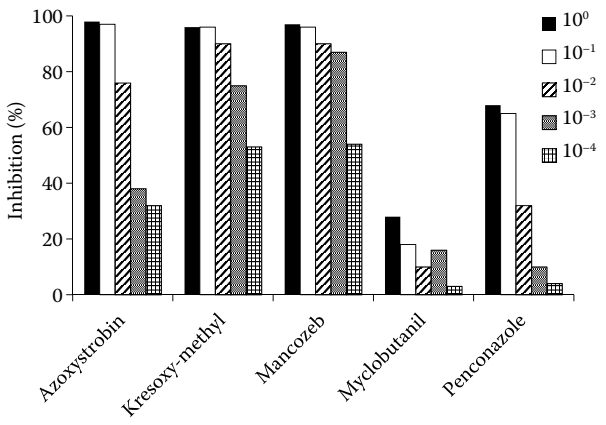

Figure 1. Inhibition of conidia germination (%)

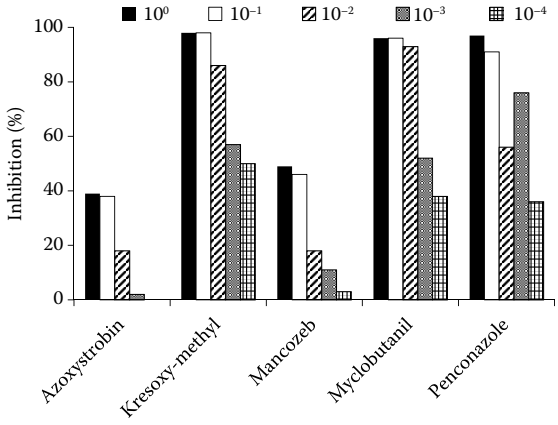

Figure 2. Inhibition of mycelial growth (%)

dinil. Mycelial growth was completly prevented by prochloraz, propiconazole, thiophanate-methyl, and carbendazim + flusilazole. Fludioxonil is one of the most effective chemicals against other *Cylindrocladium* species (Haralson *et al*. 2009). Chlorothalonil, which was the most inhibiting to spore germination *in vitro*, has also been reported as effective in the field (Henricot *et al.* 2008). Fungicides should be applied on both sides of the leaves to prevent both germination and penetration of the fungus. When conditions favour disease development, spray intervals should not exceed 7–10 days. Chemical control fungicide management has not been studied in the Czech Republic and there are currently no registered fungicides to protect box against *Cylindrocarpon buxicola*.

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