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Research Papers

A fast and reliable method for *Diplodia seriata* inoculation of trunks and assessment of fungicide efficacy on potted apple plants under greenhouse conditions

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Abstract. *Diplodia seriata* is a polyphagous and widespread pathogen that infects trunks, shoots, fruit and leaves of apple plants. Fungicides applied in integrated pest management programmes can act against *D. seriata*. However, introduction of scab-resistant apple cultivars and the consequent reduction in fungicide applications under low input disease management may increase the incidence of disease caused by *D. seriata*. Despite potential outbreaks of trunk canker, no fast and reproducible protocols for artificial inoculation of *D. seriata* and fungicide efficacy tests are available for apple plants. In this study, protocols for mycelium plug- and conidium suspension-inoculation of apple stems and shoots were optimised; canker disease assessments were carried out on potted apple plants under greenhouse conditions and coupled with *D. seriata* DNA quantification with quantitative PCR (qPCR). Efficacy tests of commercial fungicides showed that captan, dithianon and fluazinam inhibited *D. seriata* mycelium growth and conidium viability *in vitro*, while penconazole and ziram did not. However, dithianon spray applications did not reduce trunk canker severity and amount of *D.* seriata DNA in artificially inoculated plants under greenhouse conditions. This optimised protocol for fast and precise assessment of fungicide efficacy is suitable for further investigating the effects of other fungicides against *D. seriata*.

Keywords. *Botryosphaeria obtusa*, *Malus domestica*, apple stem canker, *Diplodia* infection protocol, qPCR quantification.

INTRODUCTION

Diplodia seriata (synonym *Botryosphaeria obtusa*) is a plant pathogen in the Botryosphaeriaceae (Ascomycota) that has been isolated from several

different hosts worldwide (Phillips *et al.*, 2007; Úrbez-Torres and Gubler, 2009; Mondello *et al.*, 2017; Pouzoulet *et al.*, 2017; Spagnolo *et al.*, 2017). In particular, *D. seriata* causes trunk canker and shoot dieback, fruit black rot and leaf frog-eye spot on apple plants (Phillips *et al.*, 2007). Trunk canker is characterised by reddish brown lesions that each turn smoky and develop series of alternate rings, rapidly becoming elliptical in shape (Naqvi, 2007). The bark surface becomes rough and cracked with possible callus depositions around the wounds and reddish brown stains on the underlying wood tissues (Naqvi, 2007). *Diplodia seriata* fruit infection (black rot) initially appears as small raised purplish lesions on young fruits and develops into large, brown, firm lesions on ripening apples that may later rot the entire fruit (Venkatasubbaiah *et al.*, 1991). Leaf infections (frog-eye leaf spot) initially appear as reddish-brown flecks that develop into circular brown lesions, often each surrounded by a purple halo, followed by leaf chlorosis and abscission (Venkatasubbaiah *et al.*, 1991). In addition, *D. seriata* is a polyphagous pathogen and causes pear (Choudhury *et al.*, 2014), grapevine (Úrbez-Torres *et al.*, 2008), olive (Kaliterna *et al.*, 2012) and mulberry trunk canker (Arzanlou and Dokhanchi, 2013), olive (Moral *et al.*, 2008) and loquat rot (Palou *et al.*, 2013), and canker of ornamental plants (e.g. *Cotoneaster salicifolius*) (Bobev *et al.*, 2008) and forest plants (e.g. *Castanea sativa*) (Dar and Rai, 2017). Trunk cankers and infected fruit left in orchards are the main sources of pathogen inoculum (Beer *et al.*, 2015), as well as infected tissues of other plant species in close proximity (Cloete *et al.*, 2011). Thus, the removal of diseased shoots and fruit (Brown-Rytlewski and McManus, 2000; Beer *et al.*, 2015) and the use of resistant cultivars (Biggs and Miller, 2004) are recommended to limit the spread of *D. seriata*.

Conidia of *D. seriata* spread from inoculum sources to healthy trunks, shoots, fruit or leaves during rain and penetrate these through natural openings, such as wounds, lenticels and stomata (Naqvi, 2007). Trunk and shoot infections can occur mainly through pruning wounds in winter or summer, when trees are more vulnerable to canker development due to possible drought stress (Brown-Rytlewski and McManus, 2000). However, *Diplodia* spp. have consistently been found at low levels in asymptomatic apple and pear bark, acting as potential sources of new infection (Arrigoni *et al.*, 2018). Likewise, fungi in the Botryosphaeriaceae have been found as endophytes and latent pathogens in woody plants (Slippers and Wingfield, 2007), and endophytic Botryosphaeriaceae can rapidly cause disease when their hosts are under stress (Slippers and Wingfield, 2007).

Diplodia seriata black rot causes significant losses in organic orchards (up to 10% losses estimated in northern Germany), but damage is less frequent when integrated pest management (IPM) is applied (Beer *et al.*, 2015). Thus, it has been hypothesised that fungicides applied to control apple scab, flyspeck or sooty blotch can also act against *D. seriata* (Brown-Rytlewski and McManus, 2000; Beer *et al.*, 2015). Fungicides (benomyl, kresoxim-methyl and trifloxystrobin) directly applied as topical wound treatments reduced the incidence of *D. seriata* trunk canker in apple trees (Brown-Rytlewski and McManus, 2000), and fungicide sprays against apple scab can control sooty blotch and flyspeck (Weber *et al.*, 2016). This is most probably related to side effects of fungicides applied against the main pathogen (i.e. *Venturia inaequalis*), which can reduce the inoculum of some secondary pathogens. However, the introduction of scab-resistant apple cultivars can reduce fungicide applications compared with susceptible cultivars (Simon *et al.*, 2011; Didelot *et al.*, 2016), and the consequent reduction in fungicide applications under low-input disease managements of scab-resistant apple cultivars may cause outbreaks of several secondary emerging pathogens, *D. seriata* included (Ellis *et al.*, 1998). An increasing risk of canker outbreaks was also hypothesised due to climate change, which exposes plants to the risk of abiotic stresses (Slippers and Wingfield, 2007), indicating that specific fungicide applications may be required to control canker agents in the future (Ellis *et al.*, 1998).

Although the risk of emerging secondary pathogens is real, no fast and reproducible protocols are available to assess the efficacy of conventional spray applications against *D. seriata* canker under greenhouse conditions. Most of the studies on *D. seriata* infection and disease management have focused on apple black rot (Biggs and Miller, 2004; Beer *et al.*, 2015) and grapevine trunk disease (Úrbez-Torres and Gubler, 2009; Pitt *et al.*, 2012; Mondello *et al.*, 2017). Trunk canker diseases are rarely studied due to the long incubation times and difficulties in reproducing infection under controlled conditions. The aim of the present study was to optimise a fast and reliable protocol to inoculate and assess *D. seriata* on potted apple plants and to evaluate fungicide efficacy against trunk canker, using conventional spray applications under greenhouse conditions.

MATERIALS AND METHODS

Fungal isolates

Two strains of *D. seriata* (S and VT) were isolated as single conidium cultures from trunks of *Malus domesti-*

ca plants with canker symptoms in the 'Scurelle' (latitude, N46.0647636; longitude, E11.510519599999952; altitude, 482 m) and 'Vigo di Ton' orchards (latitude, N46.2655852; longitude, E11.085680799999977; altitude, 375 m) in the Trento province (northern Italy). The isolates were stored in glycerol at -80°C in the fungal collection of the Fondazione Edmund Mach, and are freely available upon request. The strains were identified by morphology and molecular methods. For molecular characterization, *D. seriata* was grown on potato dextrose agar (PDA, Oxoid) for 14 d at 27°C, and DNA was extracted form 0.2 g of mycelium using the FastDNA spin kit for soil (MP Bimedicals) according to the manufacturer's instructions. DNA was quantified using the Quant-IT PicoGreen dsDNA assay kit (Thermo Fisher Scientific) with a Synergy2 microplate reader (BioTek), and the internal transcribed spacer (ITS) region was amplified using the forward primer ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') and reverse primer ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White *et al*., 1990; Halwachs *et al*., 2017). PCR amplification was carried out using the FastStart HighFidelity PCR system (Roche) with 1 μL of extracted DNA (10 ng), 0.25 mM deoxynucleoside triphosphates, 1% (w:v) bovine serum albumin, 4% (v:v) dimethyl sulphoxide, 0.3 μM of each primer and 2.5 U of FastStart High-Fidelity DNA polymerase (Roche), in 50 μL of reaction, and the following programme: denaturation at 95°C for 5 min, 32 cycles of amplification at 95°C for 30 s, annealing at 60°C for 1 min, extension at 72°C and final extension at 72°C for 10 min. The PCR product was purified with the Illustra ExoProStar reaction (GE Healthcare Life Sciences), and single strand sequences were obtained with an ABI PRISM 3730xl DNA analyser (Applied Biosystems, Thermo Fisher Scientific), using the forward primer ITS5 according to the manufacturer's instructions (Sequencing Service of the Fondazione Edmund Mach). Sequences were aligned against the National Center for Biotechnology Information database (NCBI; http://www.ncbi.nlm.nih.gov) to confirm the identity of *D. seriata*, and they were deposited at the Genbank database of NCBI (https://www.ncbi.nlm.nih.gov/genbank/) under the accession numbers MH174673 (*D. seriata* S) and MH174674 (*D. seriata* VT).

Growth conditions for Diplodia seriata strains S and VT

Mycelium growth of *D. seriata* S and *D. seriata* VT was assessed on four growth media: i) 15 g L^{-1} technical agar (Sigma-Aldrich) in distilled water (water agar, WA), ii) 39 g L^{-1} PDA (Oxoid), iii) 2.4 g L^{-1} potato dextrose broth (Fluka, Sigma-Aldrich) supplemented with 15 g L^{-1} technical agar (PDB+A, Oxoid), and iv) 2.4 g L^{-1} potato dextrose broth (Fluka, Sigma-Aldrich) supplemented with 15 g L^{-1} technical agar (Oxoid) and 10 g L^{-1} of carboxymethyl-cellulose (PDB+A+C, Sigma-Aldrich). Plates were incubated at 27°C for 28 d under two photoperiod conditions: complete darkness or 16:8 h light:dark photoperiod. Three double-sterilised pine needles (5 cm long) were added to each WA plate, in order to stimulate the production of pycnidia, as previously reported for Botryosphaeriaceae species (Slippers and Wingfield, 2007; Amponsah *et al*., 2008). A mycelium plug (5 mm in diam.) of a 7-d-old culture of *D. seriata* S or *D. seriata* VT grown on PDA was placed in each Petri dish (90 mm diam.). The resulting colony diameters were measured after every 3 d of incubation at 27°C, and the daily colony growth rates were then calculated by dividing the maximum colony diameter (that was measured before complete plate coverage) by the number of days of incubation. Pycnidium production on PDA, PDB+A and PDB+A+C was assessed visually as percentage of dish surface covered by *D. seriata* pycnidia (pycnidia density) after 28 d incubation at 27°C. Pycnidium production on WA supplemented with pine needles was expressed as the number of pycnidia produced per cm of pine needle length by counting under a stereoscope (Nikon model SMZ800) after 28 d incubation at 27°C. Four replicates (plates) were assessed for each fungus strain, growth medium, and photoperiod condition, and the experiment was carried out twice.

Diplodia seriata inoculation of apple plants

Two protocols for inoculation (mycelium plugs and conidium suspensions) and two plant tissues (trunks and shoots) were compared. To prepare the mycelium plugs, *D. seriata* S was grown on PDA for 28 d at 27°C in complete darkness, and plugs (5 mm diam.) were obtained with a flame-sterilised cork borer under sterile conditions. Conidium suspensions were prepared by growing *D. seriata* S on WA plates, each supplemented with three double-sterilised pine needles, for 28 d at 27°C with a 16:8 h light:dark photoperiod. Thirty sporulated pine needles were then transferred to 30 mL of distilled sterile water, ten sterile stainless steel spheres were added to each vial which was then shaken by vortexing at the maximum speed for 2 min, in order to break the pycnidia and allow the release of conidia. The conidium suspension was vacuum-filtered using a membrane filter (160 μm pore size, NY6H, Millipore) to remove intact pycnidia and pine needle fragments. The conidium concentration was adjusted to 1×10^5 conidia mL^{-1} using a Thoma cell counting chamber under a light micro-

scope (Nikon model Eclipse 80i), and the suspensions were applied to apple plants with a compressed air hand sprayer.

Three-year-old apple plants of the apple scab-resistant cv. Fujion were planted in 6 L capacity pots and grown under greenhouse conditions for 3 months, at 25±1°C with a 16:8 h light:dark photoperiod and relative humidity (RH) of 60 ± 10%, until each plant reached the petal fall phenological stage (Chapman and Catlin, 1976). Longitudinal wounds (20 mm long, 8 mm wide and 2 mm deep) were then made on 3-y-old trunks and 1-y-old shoots of the plants, using a flame-sterilised scalpel without totally removing the outer bark. Each wound on the *D. seriata*-inoculated plants was inoculated with a mycelium plug (5 mm diam.) or sprayed with 300 μL of conidium suspension (1×10^5 conidia mL^{-1}). On each plant, eight wounds were made alternately on opposite sides on the trunk, with more than 30 cm between wounds longitudinally along each trunk and to avoid canker lesions overlapping. For each plant, four wounds were inoculated with mycelium plugs and four were sprayed with the conidium suspension. Likewise, eight wounds were made on four separate shoots, four of them inoculated with the mycelium plug and four sprayed with the conidium suspension. As controls, a second group of plants was prepared, and each wound was mock inoculated with a sterile PDA plug (5 mm diam.) or with 300 μL of sterile distilled water. Five replicates (plants) were prepared as *D. seriata* S-inoculated and as control plants.

Each wound was covered with the outer bark and wrapped with laboratory plastic film (Parafilm, Bemis) for 7 d, to maintain high humidity and reduce the risk of contamination, as reported for *D. seriata* inoculations on the grapevine (Spagnolo *et al.*, 2017). Plants were incubated at 95±5% RH and 25±1°C for 48 h, and then maintained under greenhouse conditions in a randomised complete block design.

Sixty days after inoculation, trunks and shoots were cut into sections (15 cm long; sufficient to include whole cankers) containing the inoculation site, and surface-sterilised by placing in 1% v:v sodium hypochlorite solution for 45 sec, then washed twice in sterile distilled water for 60 sec. Trunk sections were peeled to remove 2-3 mm of superficial tissue layers and a calliper was used to measure the length of each canker. Disease severity and incidence were assessed for each wound of mycelium plug- or conidium suspension-inoculated trunks and shoots. For each replicate (plant), disease severity was assessed as the mean of canker length (mm) of four inoculated wounds. Disease incidence was expressed as the percentage (%) of infected wounds. In order to remove the effects of the wound scar, the canker lengths of mock-inoculated plants were subtracted from the disease severity scores of mycelium plug and conidium suspension *D. seriata*-inoculated plants.

In vitro fungicide efficacy tests against Diplodia seriata

Five commercial fungicides commonly used in apple protection programmes (Table 1) (Longo *et al.*, 2017) were tested against *D. seriata* mycelium growth and conidium viability *in vitro*. To assess effectiveness against mycelium growth, a small mycelium portion (2 × 1 mm) was collected with a flame-sterilised bacteriological needle from a 28-d-old *D. seriata* S culture on PDA maintained in darkness, and was transferred to a 2 mL sterile plastic tube containing 1 mL of sterile distilled water (control) or fungicide solution at the concentration recommended on the product label (Table 1). To assess activity against conidium viability, a conidium suspension was prepared as for apple plant inoculations (see above), and 500 μL of suspension (1×10^5 conidia mL^{-1}) was added to 500 μL of sterile distilled water (control) or a 2-fold concentrated fungicide solution in 2 mL collection tubes, to give the recommended concentration for each fungicide.

Samples were incubated at room temperature for 5 h under orbital shaking at 80 rpm and centrifuged at 8000*g* for 3 min. The supernatants were discarded and the pellets were washed three times by suspend-

Table 1. Fungicides tested against *Diplodia seriata*.

Active ingredient	Main apple pathogen target	Commercial name	Manufacturer	Dosage (g L^{-1})
Captan	*Venturia inaequalis*	Merpan 80 WDG	Adama	1.5
Dithianon	*V. inaequalis*	Delan 70 WG	Basf Crop Protection	0.4
Fluazinam	*V. inaequalis*	Banjo	Adama	0.7
Ziram	*V. inaequalis*	Diziram 76 WG	FMC Foret S.A.	2.0
Penconazole	*Podosphaera leucotricha*, *V. inaequalis*	Topas 10 EC	Syngenta	0.3

ing in 1 mL of sterile distilled water and centrifuging at 8000*g* for 3 min, to remove fungicide residues. Each mycelium portion was plated on PDA and diameters of resulting colonies were measured after 3 d incubation at 27°C. Pelleted conidia were resuspended in 200 µL of sterile distilled water, 100 µL of each suspension was plated on PDA and conidium viability was assessed as colony forming units per unit of suspension volume (CFU mL^{-1}) after 2 d incubation at 27°C. Six replicates (tubes) were prepared for each fungicide and inoculation procedure, and the experiment was carried out twice.

Fungicide treatments of apple plants inoculated with Diplodia seriata

Three-year-old apple plants (cv. Fujion) grown under greenhouse conditions were inoculated on the trunks with the *D. seriata* S mycelium plugs (5 mm diam.), and conidium suspensions (300 µL of 1×10^5 conidia mL^{-1}), as described above. Eight wounds were made on each plant on diametrically opposite sides of the trunk: four wounds were inoculated with mycelium plugs and four wounds were sprayed with the conidium suspension. Plants were treated with dithianon 7 and 1 d before inoculation, and 1 d and 7 d after inoculation, at the manufacturer's recommended dose rate (equivalent to 0.1 kg ha^{-1} for young plants; Table 1), using a compressed air hand sprayer. As experimental controls, plants were sprayed with water 1 d before *D. seriata* S inoculation. Plastic film was removed from each wound before fungicide or water treatments, and a new plastic film cover was then applied once the plant tissues had completely dried. A group of mock-inoculated plants with sterile PDA plugs (5 mm diam.) or with 300 µL of sterile distilled water was prepared to assess the effects of wounding, as described above. Plastic films were removed 7 d after inoculation, and the plants were kept for 60 d under greenhouse conditions. Trunks were then cut into sections (each 15 cm long), surface-sterilised and peeled to assess disease severity and incidence lesions. Four wood pieces (each 20 mm long, 5 mm wide and 1 mm thick) were collected with a flame-sterilised scalpel from each trunk section. Two wood samples were obtained from each plant, one from each mycelium plug-inoculated trunk (16 wood pieces) and one from each conidium suspension-inoculated trunk (16 wood pieces). These wood pieces were stored at -20°C for DNA extraction and *D. seriata* S quantification. Five replicates (plants) were analysed for each treatment in a randomised complete block design and the experiment was carried out twice.

DNA extraction and Diplodia seriata quantification with real-time quantitative PCR

Each wood sample was ground in a sterile stainless steel jar with 2.5 mL of a cold (4 °C) sterile isotonic solution (0.85% NaCl), using a mixer-mill disruptor (MM 400, Retsch) at 25 Hz for 45 s, as described by Arrigoni *et al.* (2018). DNA was extracted from 500 µL of each ground wood sample using the FastDNA spin kit for soil (MP Bimedicals) according to the manufacturer's instructions, and was quantified using the Quant-IT PicoGreen dsDNA assay kit (Thermo Fisher Scientific) with a Synergy2 microplate reader (BioTek). *Diplodia seriata* was quantified with real-time quantitative PCR (qPCR) using the primer pair DseCQF (5'-CTCTGCAATCGCTGACCCTTG-3') and DseCQR (5'-ACGTGTTTGTCTAACTAGTAGAGAGTACC-3'), that previously showed high sensitivity and positive correlation between the qPCR-quantified *D. seriata* DNA amount and *D. seriata* incidence (Pouzoulet *et al.*, 2017). qPCR assays were carried out using the KAPA SYBR FAST qPCR Master Mix at the concentration recommended in the manufacturer's instructions (1×; Kapa Biosystems), with 0.5 µM of each primer and DNA template ranging in concentration from 0.5 to 4 ng μL^{-1} in 25 µL of reaction volume. Amplification was performed using a Roche Light Cycler 480 (Roche Diagnostics GmbH) with the following programme: 95°C for 3 min; 40 cycles of 95°C for 3 sec, 62°C for 40 sec and an additional melting analysis from 60°C to 95°C, according to Pouzoulet *et al.* (2017). Negative and standard controls containing, respectively, nuclease-free water and 0.01 ng of pure *D. seriata* DNA, were included in every run. A standard curve for absolute quantification of *D. seriata* in apple wood samples was obtained in triplicate by mixing serial dilutions of *D. seriata* S DNA (1 ng, 0.1 ng, 0.01 ng, 1 pg, 0.1 pg) with 10 ng of apple DNA extracted from axenic apple plants grown *in vitro*. Wood samples of five replicates (plants) were analysed for each treatment and inoculation procedure, and the experiment was carried out twice.

Statistical analyses

Data were analysed with Statistica 13.1 software (Dell). Normal distribution (Kolmogorov-Smirnov test, $P > 0.05$) and variance homogeneity of the data (Levene's or Chochran's tests, $P > 0.05$) were checked, and non-parametric tests were used when these parametric assumptions were not respected. Each experimental repetition was analysed individually, and the Kruskal–Wallis test was used to demonstrate equivalent results in the two repetitions of each experiment ($P > 0.05$, non-significant

differences between the two experimental repetitions). For each experiment, data from the two experimental repetitions were pooled and the Kruskal–Wallis test ($P \leq 0.05$) was used to detect significant differences among treatments. Interactions among factors affecting the *D. seriata* growth, pycnidium production, severity and incidence of disease were assessed according to the Kruskal–Wallis test ($P \leq 0.05$), merging data for growth media, fungal strains, inoculated plant organ (trunks or shoots) or inoculation procedures (mycelium plug or conidium suspension).

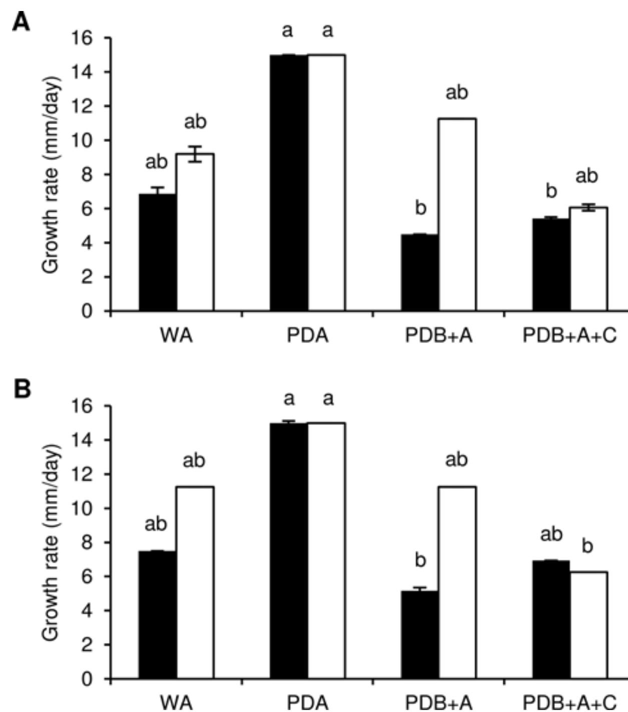

Figure 1. Mean mycelium growth rates of the *Diplodia seriata* strain S (black) and *D. seriata* strain VT (white), grown a) in complete darkness and b) with a 16:8 hour light:dark photoperiod on water agar (WA), potato dextrose agar (PDA), potato dextrose broth supplemented with agar (PDB+A) and PDB+A supplemented with cellulose (PDB+A+C) for 28 d at 27°C. The Kruskal-Wallis test showed no significant differences between the two experimental repetitions ($P > 0.05$, n = 4 replicates per experiment) and data from the two experiments were pooled. Means and standard errors of eight replicates (dishes) from the two experiments are shown for each fungal strain and growth medium. Different letters indicate significant differences (Kruskal-Wallis test; $P \leq 0.05$).

RESULTS

Diplodia seriata propagation and inoculation under greenhouse conditions

The greatest growth rate was found for *D. seriata* S and *D. seriata* VT on PDA (Figure 1), and no differences were detected between the two photoperiod conditions after merging data for growth media and fungal strains (Kruskal-Wallist test, $P = 0.264$). Pycnidium production, expressed as pycnidium density and numbers of pycnidia, was comparable among growth media and fungal strains (Figure 2), while production was greater with a 16:8 h light:dark photoperiod compared with complete darkness, after merging data for growth media and fungal strains (Kruskal-Wallis test, respectively, $P = 3.9 \times 10^{-8}$ and 9.4×10^{-4}).

Severity of disease was greater from mycelium plug inoculations than from conidium suspension inoculations (Figure 3), while no differences were found between the two inoculation procedures on shoots (Figure 4a). Statistically significant effects of the inoculation procedure (Kruskal–Wallis test, $P = 0.002$), but not plant organ (Kruskal–Wallis test, $P = 0.449$), were detected for disease severity after merging data for trunks and shoots and for conidium suspension and mycelium plug inoculations. Likewise, the inoculation procedure (Kruskal–Wallis test, $P = 0.002$), but not plant organ (Kruskal–Wallis test, $P = 0.307$), affected disease incidence. The mycelium plug inoculations on shoots resulted in greater disease incidence compared to conidium suspension inoculation on trunks (Figure 4b).

Effects of commercial fungicides against Diplodia seriata

Incubation of *D. seriata* S with dithianon, captan or fluazinam reduced mycelium growth (Figure 5a) and conidium viability *in vitro* (Figure 5b). However, penconazole or ziram did not affect *D. seriata* S mycelium growth or conidium viability.

Dithianon was applied before or after *D. seriata* S inoculation of greenhouse-grown plants, and this fungicide did not reduce disease severity (Figure 6a), disease incidence (Figure S1) or amounts of *D. seriata* DNA (Figure 6b) after mycelium plug or conidium suspension inoculation of trunks. Disease severity, disease incidence and amounts of *D. seriata* DNA were affected by the inoculation procedure (Kruskal–Wallis tests, respectively, $P = 4.0 \times 10^{-7}$, $P = 7.1 \times 10^{-7}$ and $P = 3.5 \times 10^{-5}$) and they were greater in the mycelium plug-inoculated plants than in in those inoculated with conidium suspensions, after merging of data for water- and dithianon-treated plants.

DISCUSSION

Diplodia spp. have been found in asymptomatic apple bark (Arrigoni *et al.*, 2018) and Botryosphaeriace-

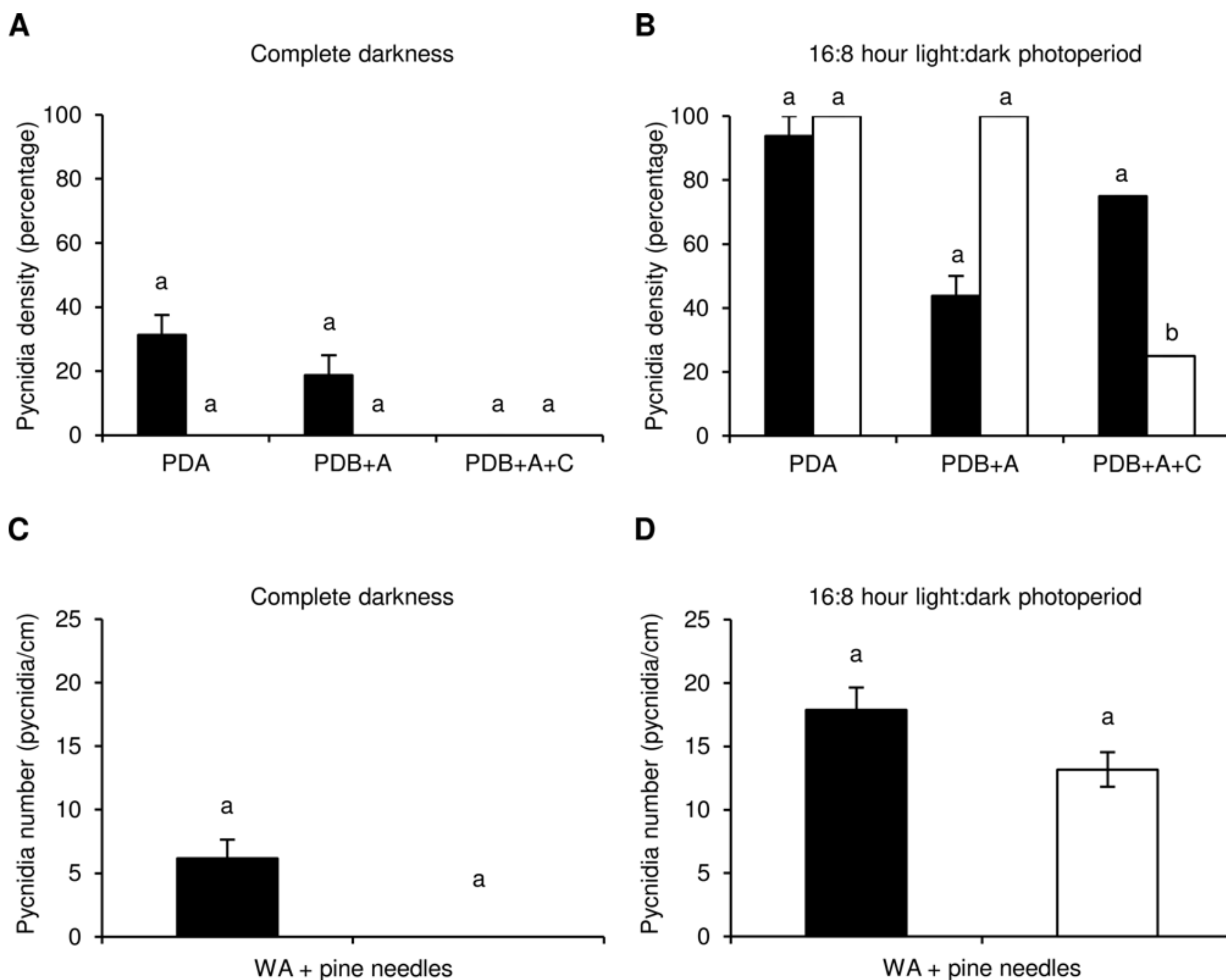

Figure 2. Pycnidium production from *Diplodia seriata* S (black) and *D. seriata* VT (white) grown on potato dextrose agar (PDA), potato dextrose broth supplemented with agar (PDB+A), PDB+A supplemented with cellulose (PDB+A+C) and water agar supplemented with pine needles (WA + pine needles). Pycnidium production was assessed, a, b) as the percentage of Petri plate surface covered by *D. seriata* pycnidia, or c, d) as the numbers of pycnidia per centimetre of pine needles 28 d after growth in complete darkness or with a 16:8 hour light:dark photoperiod at 27°C. The Kruskal-Wallis test showed no significant differences between two experimental repetitions ($P > 0.05$, n = four replicates per experiment) and data from the two experiments were pooled. Means and standard errors of eight replicates (dishes) from the two experiments are shown for each fungal strain and growth medium. Different letters indicate significant differences (Kruskal-Wallis test; $P \leq 0.05$).

ae genera can persist as latent pathogens of trees (Slippers and Wingfield, 2007). This suggests these fungi could be linked to possible plant infections and disease. In apple orchards, *Diplodia* spp. inoculum is probably controlled by fungicide applications in plant protection programmes (Ellis *et al*., 1998). Therefore, the reduction in chemical fungicides under low-input disease management of apple scab-resistant cultivars (Simon *et al*., 2011; Didelot *et al*., 2016) may lead to canker outbreaks (Ellis *et al*., 1998). In order to investigate fungicide efficacy against apple trunk canker, fast and reproducible methods were developed for *D. seriata* inoculation, disease assessment and *in vitro* fungicide efficacy testing. *Diplodia seriata* S mycelia and pycnidia were obtained on PDA in complete darkness and WA supplemented with pine needles with a 16:8 hour light:dark photoperiod. This agrees with previous studies on Botryosphaeriaceae species (Kim *et al*., 2005; Slippers and Wingfield, 2007; Amponsah *et al*., 2008; Phillips *et al*., 2013; Dheepa *et al*., 2018). Pycnidium production of *D. seriata* was greater with a 16:8 hour light:dark photoperiod than in complete darkness, confirming the role of photoperiod in

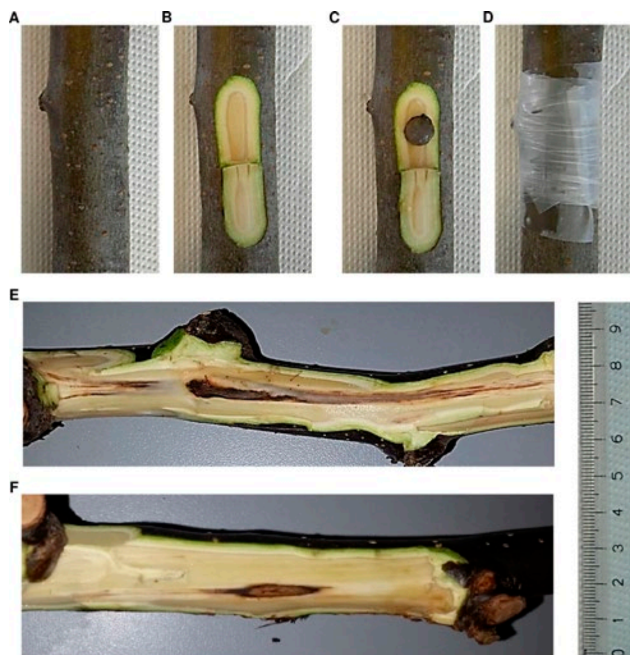

Figure 3. Inoculation procedure of a) 3-y-old apple trunk and b) cut to obtain a longitudinal wound. Each wound was c) inoculated with a mycelium plug or sprayed with conidium suspension (example as in the panel b), and d) covered with laboratory plastic film. Disease severity was assessed on e) mycelium plug-inoculated and f) conidium suspension-inoculated trunks 60 d after incubation under greenhouse conditions. Length (cm) is specified by the ruler on the right.

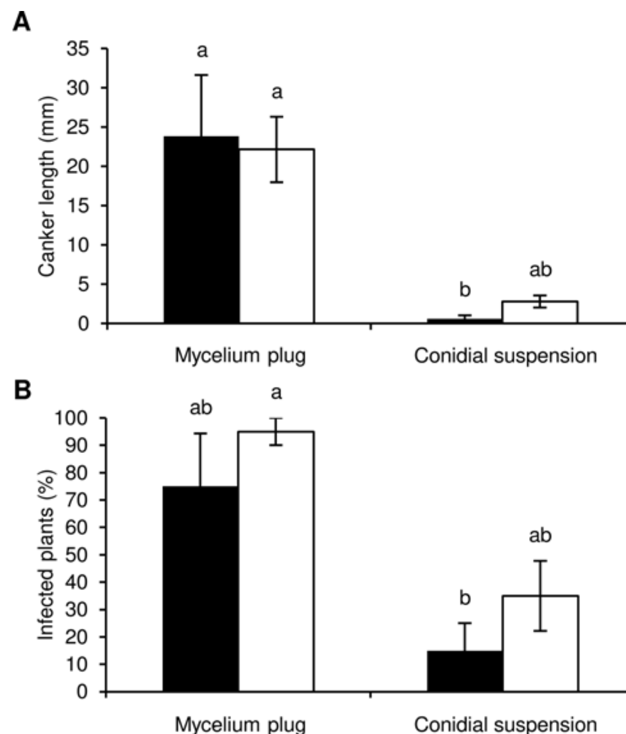

Figure 4. a) Disease severity and b) incidence caused by *Diplodia seriata* S, assessed, respectively, as canker lengths and as percentage of infected plants 60 d after incubation under greenhouse conditions. Three-y-old trunks (black) and 1-y-old shoots (white) of apple plants were inoculated with a *Diplodia seriata* S mycelium plugs or conidium suspensions. Means and standard errors are shown for five replicates (plants) for each plant organ and inoculation procedure. Different letters indicate significant differences (Kruskal-Wallis test; $P \leq 0.05$).

spore production for Botryosphaeriaceae species (Kim *et al.*, 2005; Dheepa *et al.*, 2018), particularly with pine needles (Slippers and Wingfield, 2007; Amponsah *et al.*, 2008; Phillips *et al.*, 2013).

In a previous study, a method for the inoculation of apple plants with mycelium plugs of *Botryosphaeria dothidea* and *B. obtusa* was developed under field conditions, resulting in high variability between experiments (Brown-Rytlewski and McManus, 2000). The lack of reproducibility was attributed to the fluctuating environmental conditions in the field (Brown-Rytlewski and McManus, 2000), so to overcome this variability we developed a method for artificial inoculation of *D. seriata* and fungicide efficacy testing on apple trunks under greenhouse conditions. Mycelium plug inoculations produced severe canker symptoms on the trunks and shoots of potted apple plants, and disease severity was greater in mycelium plug-inoculated plants compared with conidium suspension-inoculated plants, in agreement with Botryosphaeria dieback (e.g. *D. mutila* and *Neofusicoccum* spp.) in grapevine (Amponsah *et al.*, 2011). Severity of disease caused by *D. seriata* on apple trunks and shoots was comparable, indicating that tissue age did not affect disease development under controlled conditions, as occurred for grapevine shoots and trunks inoculated with *Neofusicoccum luteum* (Amponsah *et al.*, 2012a). In the *in vitro* fungicide efficacy tests, three commercial fungicides (fluazinam, dithianon and captan) reduced *D. seriata* viability and growth, while penconazole and ziram did not. Dithianon was reported to have little effect against *D. mutila* on grapevine plants (Amponsah *et al.*, 2012b) and demethylation-inhibiting fungicides showed different efficacy levels against *D. seriata* and *D. mutila in vitro* (Torres *et al.*, 2013), indicating their species-specific susceptibility to fungicides. Penconazole has previously been reported as an inhibitor of *in vitro D. seriata* mycelium growth (Pitt *et al.*, 2012; Mondello *et al.*, 2017), and this fungicide showed lower efficacy than fluazinam (Pitt *et al.*, 2012). Differences in penconazole efficacy could be related to the specific *D. seriata* strains used in this study, and/or to the efficacy of the test protocols used. In particular, mycelia

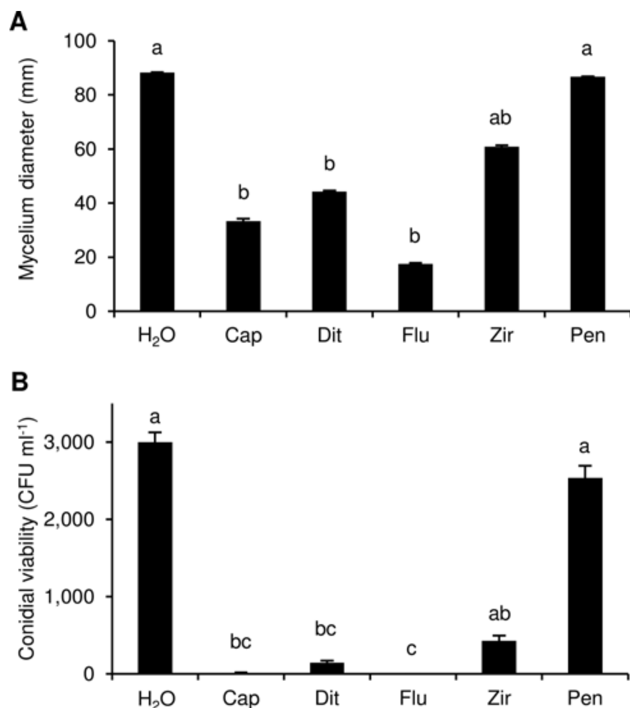

Figure 5. *Diplodia seriata* S a) mycelium growth and b) conidium viability after incubation in water (H_2O) or in presence of the fungicides: captan (Cap), dithianon (Dit), fluazinam (Flu), ziram (Zir) or penconazole (Pen). Mycelia and conidia were incubated for 5 h with the different treatments and then plated onto potato dextrose agar. Mycelium growth and conidium viability were assessed, respectively, as the colony diameter and colony forming units per unit of volume (CFU mL^{-1}) 3 and 2 d after incubation at 27°C. The Kruskal-Wallis test showed no significant differences between the two experimental repetitions ($P > 0.05$, n = 6 replicates per experiment) and data from the two experiments were pooled. Means and standard errors for 12 replicates (dishes) from the two experiments are shown for each treatment. Different letters indicate significant differences (Kruskal-Wallis test; $P \leq 0.05$).

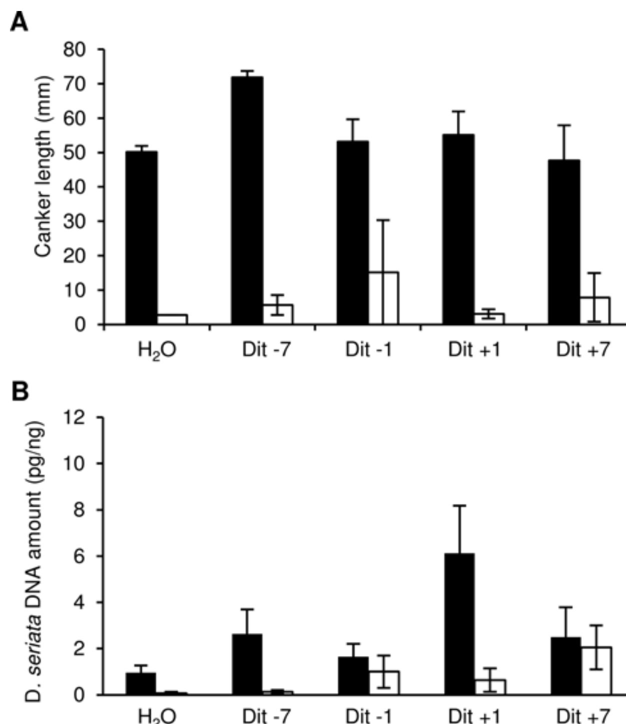

Figure 6. Disease severity a) caused by *Diplodia seriata* S and amount of pathogen DNA for 3-y-old trunks of apple plants inoculated with a mycelium plugs (black) or conidium suspensions (white) 60 d after incubation under greenhouse conditions. Plants were treated with water 1 d before inoculation (H_2O) or with dithianon 7 d before inoculation (Dit -7), 1 d before inoculation (Dit -1), 1 d after inoculation (Dit +1) or 7 d after inoculation (Dit +7). Kruskal-Wallis test showed no significant differences between the two experiments ($P > 0.05$, n = five replicates per experiment), and data from the two experiments were pooled. Means and standard errors for ten replicates (plants) from the two experiments are shown for each plant organ and inoculation procedure. No significant differences among treatments of mycelium plug- or conidium suspension-inoculated plants were found (Kruskal-Wallis test; $P > 0.05$).

and conidia were incubated in the fungicide solution for 5 h and then plated on PDA in our assays, while mycelium plugs were plated on PDA supplemented with fungicide in the protocol of Pitt *et al.* (2012), possibly leading to greater exposure to penconazole. Due to its frequent applications in IPM apple orchards (Blommers, 1994), dithianon was selected for efficacy tests in the present study. This fungicide gave no reduction in *D. seriata* severity, incidence or amounts of *D. seriata* DNA, after mycelium plug- or conidium suspension inoculations of apple plants. Discrepancies between *in vitro* and *in planta* efficacy tests have also been described for Botryosphaeria dieback agents (Mondello *et al.*, 2017), indicating that fungicide deposition, adhesion and/or penetration in host tissues can influence their efficacy (Zabkiewicz, 2007). Moreover, topical wound treatments of benomyl, kresoxim-methyl and trifloxystrobin reduced the incidence of *D. seriata* trunk canker under field conditions (Brown-Rytlewski and McManus, 2000), indicating that the mode of fungicide application and the active ingredient affected efficacy.

In the present study, methods for *D. seriata* inoculation and quantification were optimised on a scab-resistant apple cultivar (cv. Fujion), and efficacy tests of commercial fungicides were carried out *in vitro* and with conventional spray application under greenhouse conditions. Although *in vitro D. seriata* mycelium growth and conidium viability were inhibited by three fungicides (dithianon, captan and fluazinam), trunk canker symptoms were not affected by spray applications of dithianon under greenhouse condi-

tions, suggesting that application of this fungicide did not significantly contribute to indirect *D. seriata* control in IPM. The optimised methods described in this paper can be used to accurately assess fungicide efficacy against *D. seriata*, and to precisely estimate the impacts of reduced fungicide applications in low-input disease management systems for scab-resistant apple cultivars.

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