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- **Summary**

Dothistroma septosporum, a causal agent of Dothistroma needle blight (DNB), is a damaging fungal pathogen of pines that has recently started to affect native Scots pine woodlands in the UK. In addition to silvicultural methods, fungicide spraying of forest nursery stock can help prevent the spread of DNB. However, the effectiveness of modern single site fungicides against *D. septosporum* and the risk of fungicide resistance evolution remain largely unknown. In this project we aimed to establish sensitivity profiles of *D. septosporum* to some widely used single site fungicide classes *in vitro*, and to determine whether fungicide resistance is already present, as this could increase the spread of *D. septosporum* genotypes on planting stock in native woodlands. For this purpose we compared isolates of *D. septosporum*, originating from pine stands unexposed to fungicides, with isolates from nursery outbreaks, for sensitivity to a range of commonly applied fungicides. Most of the fungicides we tested were effective *in vitro* and we observed no significant shifts in sensitivity in forest nurseries. Although further tests *in planta* are required to confirm effectiveness of single site fungicides against *D. septosporum*, our

results suggest that they can be successfully used in DNB control, although appropriate measures to prevent the evolution of fungicide resistance are strongly recommended.

- **Introduction**

Dothistroma needle blight (DNB), caused by *Dothistroma septosporum,* is a damaging disease of pines and other conifers worldwide. DNB was originally a problem in exotic pine plantations in the Southern Hemisphere and was only sporadically reported in European countries. The situation changed in the 1990s, when the disease was found to seriously affect both native and exotic pines throughout countries in the Northern Hemisphere, including the UK (Bradshaw 2004, Fraser et al. 2015). Currently, DNB is not only found in exotic pine plantations, but also in both plantations and natural stands of native Scots pine (*Pinus sylvestris*) in the UK, raising concern about the conservation of these iconic woodlands (Brown et al. 2012).

Different management options are available for the control of DNB, including various silvicultural methods, the use of resistant pine stocks, planting of alternative host species and fungicide application (Bulman et al. 2013). Copper-based fungicides are effective and widely used in forest nurseries for control of DNB; aerial spraying of forest plantations is far less common and used only in a few countries, for instance in New Zealand and in some European countries such as Serbia and Hungary (Bulman et al. 2013). An advantage of copper-based fungicides, which belong to the class of multisite inhibitors, is that they present a low risk in terms of fungicide resistance development (Hollomon 2009, Fungicide Resistance Action Committee, FRAC, www.frac.info). However as multisite inhibitors they are likely to target pathways shared by a wide range of non-target organisms and, as heavy metals they suffer from poor eco-toxicological profile (Hollomon 2009, Wightwick et al. 2010) and therefore are likely to cause serious environmental damage. In addition, their poor efficacy against a range of target organisms other than *D. septosporum* means that their use is limited in practice.

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In the UK, chemical control of forest diseases is only permitted on forest nursery stock and ornamental plants (Chemicals Regulation Directorate, CRD, www.hse.gov.uk/crd). Currently, in addition to copper-based fungicides, a variety of newer products, belonging to the class of single site inhibitors, are used in disease control and prevention in forest nurseries. The advantage of these compounds is that they specifically target pest organisms; however the use of these fungicides also increases the risk of resistance evolution in the pathogen (Hollomon 2009). Even though *D. septosporum* might not be the direct target of these single site inhibitors, it will be exposed to the full range of any such fungicides applied in forest nurseries. It is therefore of great practical importance to know the degree to which *D. septosporum* is sensitive to, and can be controlled by, this new generation of fungicides. Furthermore it is of interest to determine whether use of single site inhibitors in forest nurseries has already selected for *D. septosporum* strains insensitive to these fungicides. This is especially pertinent, given that DNB outbreaks have recently been reported in UK forest nurseries despite the application of fungicide treatments.

In this paper we report the results of *in vitro* lab assays to evaluate the sensitivity of *D. septosporum* to a number of fungicides that nurseries have reported using on coniferous trees. We evaluate whether any shifts in sensitivity have already occurred in the *D. septosporum* population, as a consequence of fungicide use, by comparing isolates from untreated forest stands with those from nursery outbreaks. We have focussed on the single site inhibitor class of fungicides to which the risk of resistance evolution is medium or high. Copper-based products belonging to multisite inhibitors were excluded from the assay because resistance outbreaks in this group are not common (Hollomon 2009, FRAC) and their effectiveness against *D. septosporum* has already been well documented in research studies (Bulman et al. 2013)

- **Materials and Methods**

- **Fungal culture preparation**

Isolates of *D. septosporum* collected from naturally regenerated and planted forest stands of native Scots pine (*Pinus sylvestris*) and plantations of lodgepole pine (*Pinus contorta*) and Corsican pine (*Pinus nigra ssp. laricio*) (N=79, years 2014, 2015), with an unknown history of exposure to fungicides, were used to measure the baseline range of sensitivity to fungicides. The sensitivity of *D. septosporum* isolates from nursery outbreaks (received from Alice Holt, Forest Research, N=50, collected over four years; 2011, 2012, 2014, 2015) were compared to this baseline range in order to identify possible shifts in sensitivity in the nursery situation.

Single spore cultures of *D. septosporum* were isolated following a slightly modified protocol described previously by Mullett et al. (2015). This modification involved the spore suspension from a single fruiting body being spread evenly across potato dextrose agar plates (Oxoid, Basingstoke, UK) amended with streptomycin 200µg ml^{-1} (Sigma-Aldrich, Saint Louis, USA). Thereafter germinating spores were transferred onto Dothistroma sporulating medium (DSM) plates (Bradshaw et al. 2000) and cultured at 12 hours light/12 hours dark at 20°C (Gallenkamp, INF 780C, Weiss Technik Konigswinter, Germany).

To induce sporulation for the fungicide inhibition assay, spread plates of *D. septosporum* cultures were grown on DSM plates (without antibiotics) for 7 days in a growth cabinet at 20°C. Spores were collected, filtered through 100µm nylon filters (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) and centrifuged at 4500 rpm for 5 minutes (20°C). The supernatant was removed and spores were re-suspended in alkyl ester (AE) broth, (Skinner et al. 1998).

- **Assay design**

Private nurseries and commercial nurseries within the Forestry Commission were surveyed to obtain information about the range of fungicides and the spraying regimes commonly used to

control fungal diseases on coniferous trees. On this basis, six single site inhibitor fungicide classes were chosen for testing against *D. septosporum*; Quinone outside Inhibitors (QoIs, azoxystrobin), Demethylation Inhibitors (DMIs, prothioconazole-desthio, propiconazole), Phenylamides (PA, metalaxyl-M), Anilinopyrimidines (cyprodinil), Dicarboximides (iprodione) and Succinate Dehydrogenase Inhibitors (SDHI, boscalid) (Sigma-Aldrich). Although SDHIs were not reported to be used by nurseries, they were included in the analysis based on their effectiveness against other pathogenic Mycosphaerellacea, such as *Zymoseptoria tritici*, a major pathogen of wheat in Europe. Phenylamides (PA, metalaxyl-M), Anilinopyrimidines (cyprodinil), Dicarboximides (iprodione) were tested only on the initial number of isolates (N=14), originating from naturally regenerated and planted forest stands with an unknown history of exposure to fungicides, and removed from further analysis due to their lack of effectiveness against *D. septosporum* in *in vitro* assays.

The fungicide sensitivity assay was performed in 96 well plates (BD Falcon, Franklin Lakes, USA), each well containing 5×10^4 spores ml^{-1}. Three replicate wells were used per isolate. Final concentrations of fungicides tested were for prothioconazole-desthio and propiconazole 1, 0.5, 0.1, 0.05, 0.01, 0.005, 0.001, 0 mg litre^{-1}, for azoxystrobin 5, 1, 0.5, 0.1, 0.05, 0.01, 0.005, 0 mg litre^{-1}, for boscalid 50, 10, 5, 1, 0.5, 0.1, 0.05, 0 mg litre^{-1} and for cyprodinil, iprodione and metalaxyl-M 100, 50, 10, 5, 1, 0.5, 0.1, 0 mg litre^{-1}. Fungicide stocks of 5000 mg litre^{-1} were prepared in DMSO (Dimethyl sulfoxide, Sigma-Aldrich) and all of the subsequent fungicide dilutions were prepared in AE broth. Plates were incubated in the dark at 20°C, with shaking at 170 rpm. Optical density was measured at day zero and seven on a FLUOstar Omega (BMG Labtech, Offenburg, Germany) spectrophotometer (wavelength= 600 nm, 20 flashes per well). Concentrations causing a 50% reduction of colony growth (EC$_{50}$

values) were calculated from the 4-parameter fit of the standard curve in the Omega software. Spore suspensions with no added fungicides were used as controls in the experiment. The significance of differences in mean EC_{50} values between forest and nursery populations of isolates were tested using a two-sample t-test of log transformed data (to normalise residuals) in MINITAB 17 (Minitab Inc., State College, USA).

- **Results**

Fungicides belonging to QoIs (azoxystrobin) and DMIs (prothioconazole-desthio, propiconazole) were the most effective of the fungicides tested in inhibiting the growth of *D. septosporum in vitro*, with mean EC_{50} values of 0.009 mg litre^{-1} for azoxystrobin, 0.002 mg litre^{-1} for prothioconazole-desthio and 0.012 mg litre^{-1} for propiconazole. Fungicides belonging to SDHIs (boscalid) also reduced growth of *D. septosporum*, with a mean EC_{50} value of 0.236 mg litre^{-1}. The mean EC_{50} values for the remaining three single site inhibitors classes were 28.4 mg litre^{-1} for Anilinopyrimidines (cyprodinil) and 100 mg litre^{-1} for both PAs (metalaxyl-M) and Dicarboximides (iprodione). The overall conclusion is that three out of six tested fungicide classes were effective in reducing *D. septosporum* growth in lab assays (QoIs, DMIs and SDHIs) whilst the remaining three classes, Anilinopyrimidines, PAs and Dicarboximides were ineffective in *in vitro* tests.

In most cases the distribution of EC_{50} values in forest and nursery populations overlapped (Figure 1). In the case of azoxystrobin and boscalid there was a small shift in EC_{50} values in nursery samples caused by a single isolate with increased EC_{50} value (Figure 1). For both compounds the small increases in mean EC_{50} values of the nursery population were statistically significant (QoIs *P* =0.049, T-Test, T-Value = -2.00, DF =92 and SDHIs *P* =0.024, T-Test, T-Value = -2.30, DF =83). No significant differences in mean EC_{50} values were indicated for either

of the DMIs tested, prothioconazole-desthio (P =0.463, T-Test, T-Value = -0.74, DF =102) or propiconazole (P =0.146, T-Test, T-Value = -1.47, DF =78) (Figure 1).

- **Discussion**

This study presents the first assessment of *D. septosporum* sensitivity *in vitro* to several classes of modern fungicides. Although statistically significant differences in sensitivity between forest and nursery isolates were found for QoI and SDHI actives, the EC_{50} values recorded in all nursery samples were very low and the differences in mean values between nursery and control (forest) samples were very small (QoIs EC_{50} highest: forest 0.024 mg litre^{-1}, nursery 0.042 mg litre^{-1}; SDHIs EC_{50} highest: forest 0.514 mg litre^{-1}, nursery 0.598 mg litre^{-1}). In confirmed reports of resistance development in other plant pathogens the EC_{50} values for resistant phenotypes are much higher. For instance in QoI and SDHI resistant isolates of *Botrytis cinerea* EC_{50} values of > 30 mg litre^{-1} for QoIs and > 8 mg litre^{-1} for SDHIs were recorded (Kim and Xiao 2011). Thus differences in sensitivity found between nursery and forest samples in this study are unlikely to result in any differences in *D. septosporum* control *in vitro*. The observed differences are more likely to result from the normal range of sensitivity expected in any natural population, together with sampling effects. All isolates tested from nursery outbreaks were within the range of baseline sensitivity determined in isolates from untreated forests and there were no biologically significant shifts in sensitivity compared to these untreated forest isolates for the fungicide classes tested. This suggests that at present there is no fungicide resistance development in nurseries to the fungicide classes tested. Although one possible explanation for disease outbreaks in the nurseries could have been *D. septosporum* shifts in sensitivity to fungicides applied, our results suggest that the DNB outbreaks recorded in the UK are not due to evolution of fungicide resistance.

The QoI and DMI fungicide classes proved to be very effective in *D. septosporum* control in lab assays. SDHIs also showed good effectiveness. Boscalid in mixtures is approved on forest nursery stocks; however it is possible that newer SDHI compounds such as isopyrazam, bixafen or fluxapyroxad, which currently are not authorised in forest nurseries, would show higher effectiveness than boscalid. Although the remaining three fungicide classes, PA, Anilinopyrimidines and Dicarboximides were ineffective in inhibiting *D. septosporum* growth *in vitro*, it is possible that they still could be useful in spray programs for control of diseases other than DNB. Although this study gives an understanding of the efficacy of a number of fungicides against *D. septosporum in vitro*, it does not provide the data on effectiveness of commercial products against the disease in the field. Laboratory assays may not be an entirely accurate predictor of field efficacy in that fungicide could be more or less active when taken up or metabolised by plants compared to in lab assays. Thus this study should be rather seen as an indication and further field trial studies are needed to confirm field performance of commercially formulated products.

There are important practical lessons to be taken from this study, especially if the fungicides tested are shown to be effective *in planta* and widely adopted in DNB control. Forest nurseries are using high risk fungicides in terms of resistance development such as QoIs and DMIs (FRAC). Resistance to fungicides can evolve rapidly and suddenly. For example, resistant pathogens appeared only two years after the commercial launch of QoIs for use on cereal crops (Bartlett et al. 2002). Additionally, both mating type loci are present within some of the UK populations of *D. septosporum*, emphasising its potential for sexual reproduction (Mullett 2014) and the rapid incorporation of resistance mutations into the population through recombination (McDonald and Linde 2002). It is strongly recommended that fungicide management guidelines

should be adopted to reduce the risk of fungicide resistance developing for *D. septosporum* and other pathogens present in nurseries. These could draw on experience with effective fungicide management strategies in agriculture. More information on fungicide resistance management guidelines can be found on the FRAC website (www.frac.info). The chemical products authorised for use in forest nurseries and the statutory requirements for their use are available at the CRD website (www.hse.gov.uk/crd).

Copper-based fungicides are, and are likely to remain one of the key products in DNB control in the nurseries in the UK. The significance of single site inhibitors however is likely to increase in the near future due to their increased effectiveness and improved environmental profile (Hollomon 2009, Wightwick et al. 2010). This study has established the range of baseline sensitivity to major single site fungicide classes for *D. septosporum in vitro* which can be used for reference in any future monitoring programs. These programs, designed to detect and manage fungicide resistance early, would ideally be run on a yearly basis, rather than waiting until problems with control occur. Regular monitoring of sensitivity combined with the application of anti-resistance measures are key points for successful and long term disease management. Additionally it is widely known that fungicides have a negative impact on the environment, including aquatic organisms (Bartlett et al. 2002, Hollomon 2009, Wightwick et al. 2010). Thus there is an important rationale, both in agriculture and even more in forest conservation, for using fungicides that are effective against the disease and for minimising the use or potential use of any that provide poor control or for which resistance has developed.

- **Acknowledgements**

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- **Figures**

Figure 1 Distribution of EC_{50} values of four fungicides tested against *D. septosporum* isolates from forest and nursery populations.