

COST ACTION FP1102

Determining Invasiveness and Risk of Dothistroma

Training School May 2012, Brno, Czech Republic

Detection and Diagnostics of Dothistroma

Dothistroma

Isolation and molecular identification methods

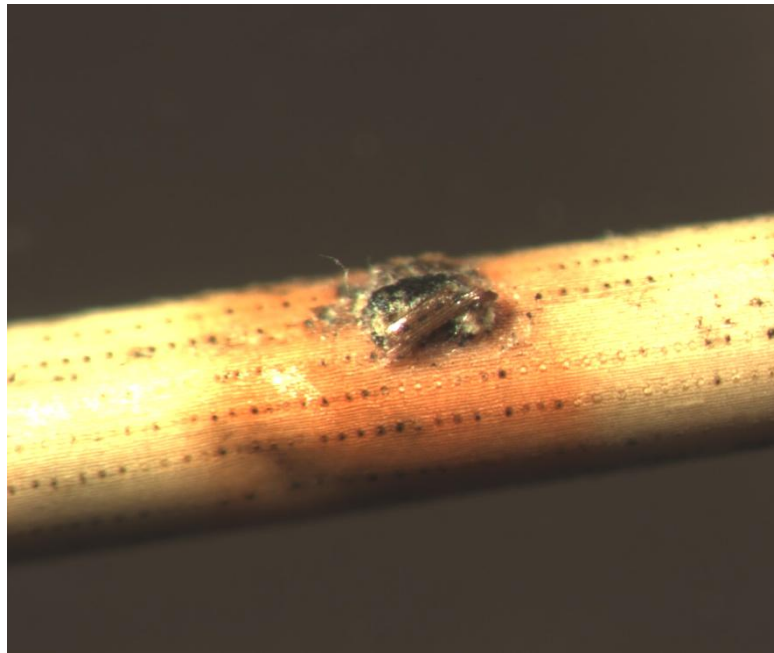


Photo I.B.

Compiled by Martin Mullett and Irene Barnes, June 2012

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1 Isolation of Dothistroma

1.1 Preparation of growth media

Various media can be used to isolate and grow Dothistroma, the most common are MA (Malt Agar), MEA (Malt Extract Agar), DM (Dothistroma Medium), DSM (Dothistroma Sporulating Medium), WA (Water Agar), PNA (Pine Needle Agar), PMMG (Pine Needle Minimal Medium with Glucose), PDA (Potato Dextrose Agar). These can be made up with or without antibiotics. The most commonly used antibiotic is streptomycin.

The most commonly used media for initial isolation include MA, MEA, DM, and PNA supplemented with streptomycin. During initial isolation, it is recommended that an antibiotic is added to the media to reduce bacterial and some yeast contamination. Once clean and pure cultures are obtained, antibiotics are not necessary.

Other media that promote sporulation of the fungus include DSM, WA, PMMG.

1.1.1 Dothistroma Medium (DM)

| | |
|-----------------|--------------|
| Malt extract | 50g |
| Nutrient agar | 23g |
| Distilled water | up to 1000ml |

Autoclave for 15 min at 121°C/15 lb psi.

1.1.2 Dothistroma Medium + streptomycin (DM+S)

| | |
|-----------------|--------------|
| Malt extract | 50g |
| Nutrient agar | 23g |
| Distilled water | up to 1000ml |

Autoclave for 15 min at 121°C/15 lb psi.

When media is cool enough to touch (~55°C), add 20-25ml of 1% streptomycin solution to the medium and shake well before pouring. Store the agar in a dark fridge to prevent breakdown of streptomycin.

A standard 1% streptomycin solution can be made up by mixing 1g streptomycin with 100ml of sterile distilled water. The standard solution should be kept in a dark fridge for storage.

1.1.3 Dothistroma Sporulating Medium (DSM)

| | |
|----------------------|--------------|
| Malt extract | 20g |
| Yeast extract | 5g |
| Agar Technical no. 3 | 15g |
| Distilled water | up to 1000ml |

Autoclave for 15 min at 121°C/15 lb psi.

1.1.4 2% Malt Agar (MA)

| | |
|----------------------|--------------|
| Malt extract | 20g |
| Agar Technical no. 3 | 15g |
| Distilled water | up to 1000ml |

Autoclave for 15 min at 121°C/15 lb psi.

1.1.5 Malt Extract Agar (MEA)

| | |
|----------------------|--------------|
| Malt extract agar | 33g |
| Agar Technical no. 3 | 10g |
| Distilled water | up to 1000ml |

Autoclave for 15 min at 121°C/15 lb psi.

1.1.6 Water Agar (WA)

| | |
|----------------------|--------------|
| Agar Technical no. 3 | 15g |
| Distilled water | up to 1000ml |

Autoclave for 15 min at 121°C/15 lb psi.

1.1.7 Pine Needle Minimal Medium + Glucose (PMMG)

Soak fresh pine needles (10% [w/v]) in distilled water for 24-48 hours at room temperature (25°C). Discard the pine needles and use the water to prepare the minimal medium with glucose as described by Carsolio et al. (1994). The PMMG recipe was published by McDougal et al. (2011).

PMMG Medium (g/l):

| | |
|--------------------------------------|--------|
| MgSO ₄ ·7H ₂ O | 0.2g |
| K ₂ HPO ₄ | 0.9g |
| KCl | 0.2g |
| NH ₄ NO ₃ | 1.0g |
| FeSO ₄ ·7H ₂ O | 0.002g |
| ZnSO ₄ ·7H ₂ O | 0.002g |
| MnCl ₂ ·7H ₂ O | 0.002g |
| Asparagine | 2.0g |
| Glucose | 3.0g |
| Agar | 20.0g |

Adjust the volume to 1 litre with the pine-soaked water.

Adjust the pH to 4.0 to mimic the pH of the pine needles (Ivory 1967).

As some of these components are used in very small amounts, it is best to make up 1L or more at a time, or prepare a sterile stock solution of those components.

1.2 Sterile techniques

- Tools are sterilized in alcohol (IMS –Industrial Methylated Spirits, 70% ethanol). If working in a clean environment (e.g. a laminar flow cabinet) the ethanol can be left to evaporate from the tools; in this case it is best to work with two or three scalpels at a time, using one scalpel while the alcohol from the others evaporates.
- If it is undesirable to leave the tools exposed then the alcohol is flamed off quickly. Scalpel blades should not be heated excessively as this blunts the blade.
- If using a flame to sterilize the tools then make sure the blades/needles are completely cooled before use otherwise it will kill the conidia.
- Instruments should be sterilized between samples.
- Working space should be cleaned (ethanol spray and wipe) between samples.

1.3 Surface sterilization of needles

Excessive harsh sterilization of needles allows the sterilant to penetrate the acervulus and destroy the spores. It is therefore recommended to gently surface sterilize by wiping the needle with an alcohol soaked tissue. In cases where the needle is relatively clean no surface sterilization is necessary. Alternatively, one can physically “scrape” the needle clean using a blade. This should be done under a microscope so as not to destroy the fruiting body.

1.4 Isolation techniques

1.4.1 Acervuli rolling

- On the back of the Petri dish mark two to three parallel lines with a permanent marker pen. This is to keep track of where the spores will be spread on the plate. Write the information of the isolate on these lines at the bottom of the plate with the date.
- Using a binocular (stereo) microscope excise a SINGLE acervulus from the needle using a scalpel (and forceps if desired). Try not to include any plant material if possible or as little as possible as the epidermis and cuticle will inevitably cover the acervulus.
- Place the excised acervulus on the medium at the end of one of the lines (Fig.1 a). Place as many acervuli on the Petri dish as there are lines (i.e. 2-3) remembering to use sterile techniques between acervuli.
- Acervuli placed on the agar will absorb some of the moisture from the agar, facilitating spore release. Using the tip of the scalpel or a needle, roll the acervulus down the medium along the pre-drawn line. This should be done under a binocular (stereo) microscope (x30 – x60 magnification). As the acervulus is rolled, the released spores will be visible on the surface of the agar (Fig. 2).
- The idea behind the roll is that most of the contaminating plant material and other debris will be at the starting end of the line (thus no need for surface sterilization of the needles) and the clean spores will be more towards the end of the line.
- With the scalpel or a needle cut small (2-3mm²) sections of the agar with one or a small number of spores on it (from the end of the line) and place it upside down on a clean, small Petri dish (45mm diameter).
- The small agar cube can be dragged across the Petri dish surface to spread out the spores to obtain single spore isolates.

NB. When rolling, *Dothistroma* spores appear hyaline (colourless), cylindrical and thin (Fig.3). If you see darker, greenish spores that are more thick and granular, these are probably *Lecanosticta acicola* (Fig.4).

1.4.2 Spore streaking

- Gently wipe the surface of the needle with an IMS (Industrial Methylated Spirits) or ethanol soaked tissue and place on a clean glass slide under the microscope (Fig.1 b).
- Using a binocular (stereo) microscope excise the acervulus from the needle using a scalpel and forceps.
- Place one drop of SDW (Sterile Distilled Water) on top of the acervulus and cover with a clean cover slip.
- Gently crush the acervulus under the cover slip (using forcep tips, etc...).
- View under a compound microscope to confirm the presence of *Dothistroma* conidia.
- Lift off the cover slip using fine forceps (and wipe cover slip on media if so desired). An extra drop or two of SDW can be added at this point.
- Using a sterile bacterial loop pick up a loopfull of the spore solution from the slide (Fig. 1 c) and streak onto a large (90mm) Petri dish with a medium containing streptomycin (Fig. 1 d). Four to six parallel streaks can be made per plate. A number of plates (c.3) can be made from each spore solution sample.
- Repeat until no liquid is left on the slide. Label plates with sample details and date. Single spore colonies will grow on these plates (Fig. 10).

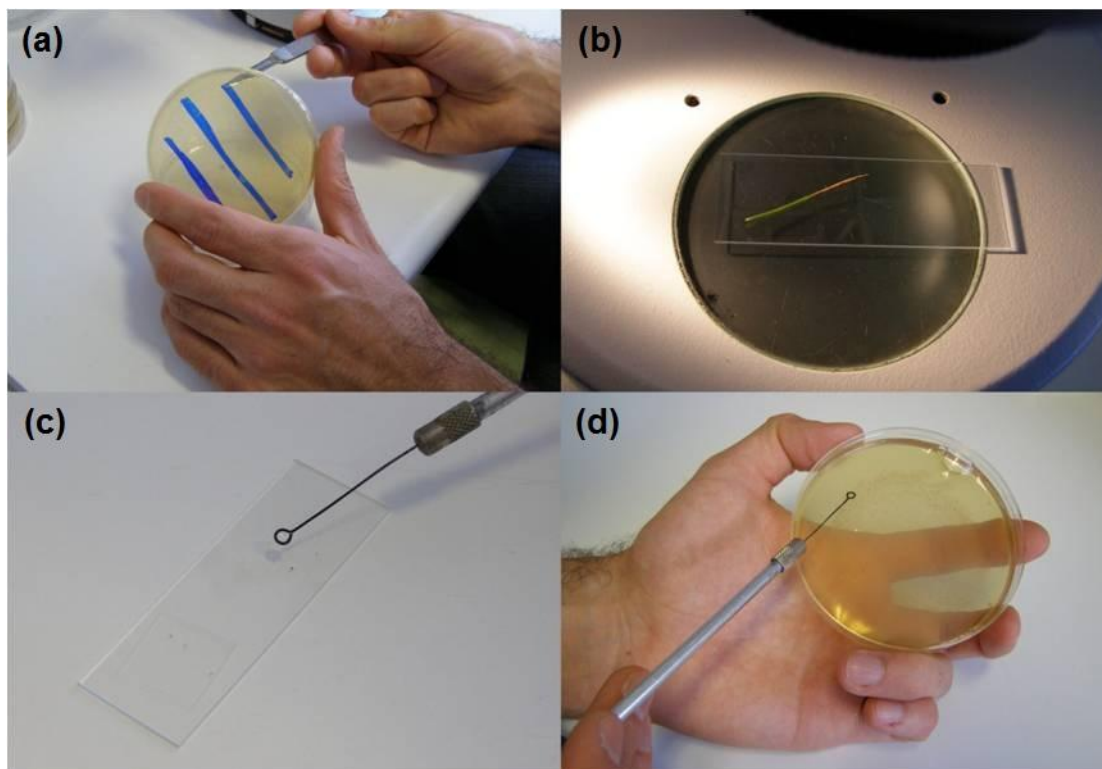


Figure 1: Isolation techniques. (a) Acervuli rolling. (b) Needle on a clean glass slide under the microscope. (c) Picking up of the spore solution. (d) Streaking spores onto a Petri dish. (Photo M.M.)

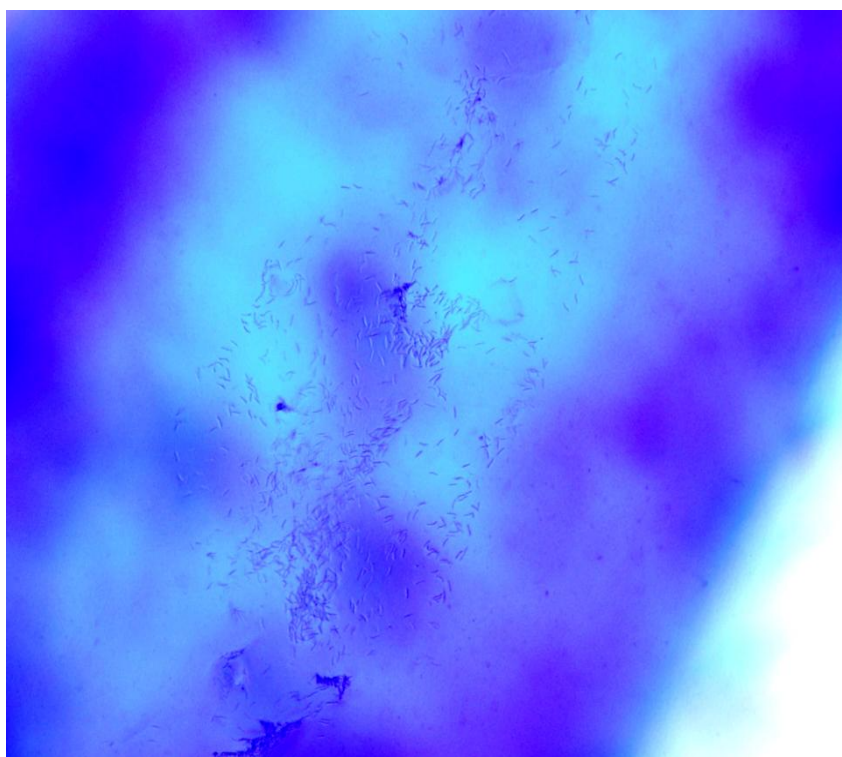


Figure 2: Conidia rolled on DM+S x40magnification. (Photo M.M.)

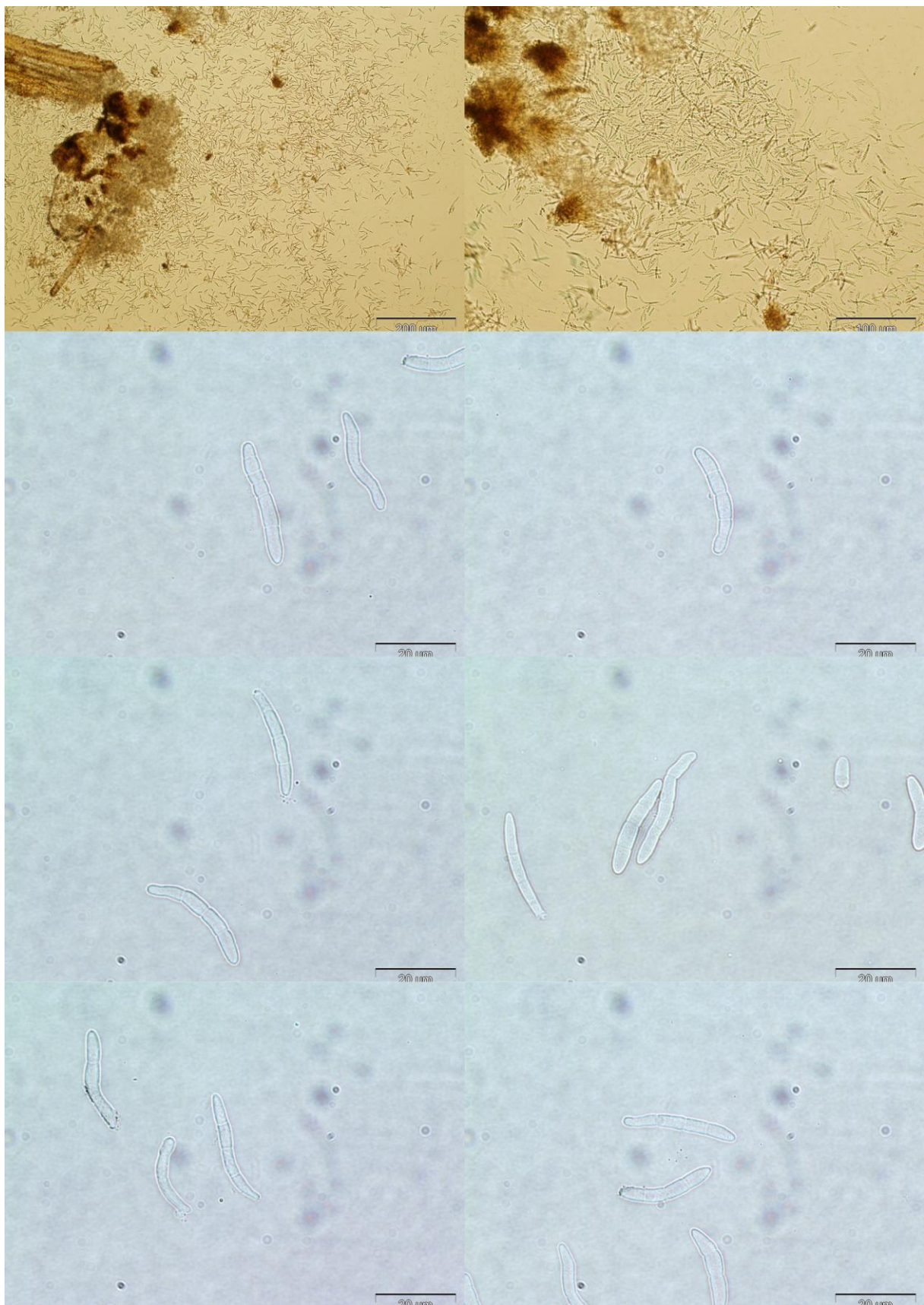


Figure 3: Conidia of *Dothistroma septosporum* at various magnifications. (Photo M.M.)

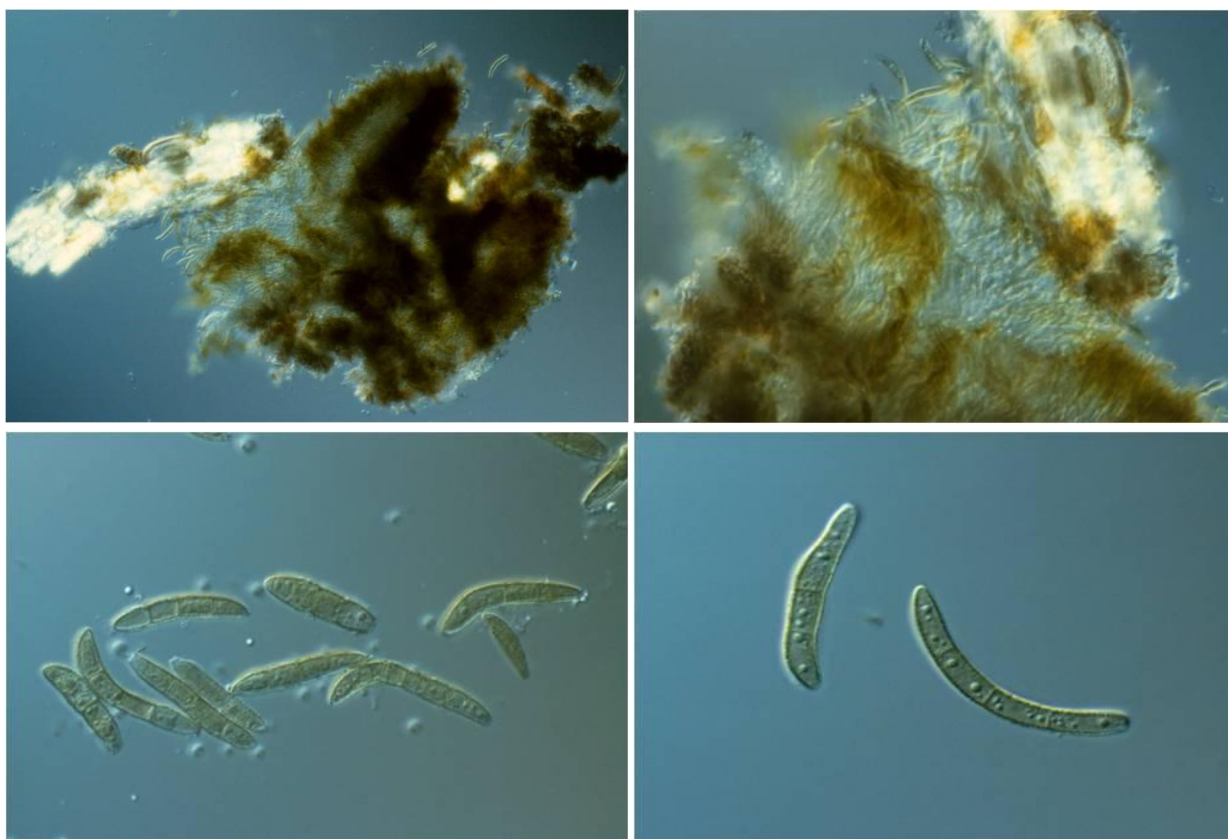


Figure 4: Acervulus and conidia of *Lecanosticta acicola* at various magnifications. (Photo T.K.)

1.4.3 Lesion isolation (not recommended)

The standard plant pathological approach is to cut a segment of plant tissue from the dead-live junction and plate it out onto media. For foliar material surface sterilization is necessary prior to this (see section 3). With *Dothistroma* infected needles this equates to chopping out infected needle sections and placing onto agar. This method is not very successful as many faster growing saprophytes and endophytes outgrow the *Dothistroma*. This method is not recommended due to its low success rates and because it produces mixed cultures.

1.4.4 Incubation and growth

Incubate the Petri dishes at 20°C (17-22°C) and sub-culture from a single germinating colony as soon as possible. Good germination is usually achieved after two days. Cultures are visible with the naked eye after ~7days. Depending on the isolates, density of cultures and type of media used, dothistromin production will be visible after >7days (see Fig. 10 and 11). *Dothistroma* cultures are characteristically small (usually the smallest, slowest growing cultures on the plate) and have a distinctive zig-zagging hyphae when young (see arrows in the photos below, Fig. 5 and 6). *Lecanosticta acicola* cultures are also slow growing but usually with a whitish appearance and aerial mycelium (Fig. 11) whereas *Dothistroma* cultures are adpressed and pinkish (Fig. 12). Short-term storage of cultures is at 20°C (17-22°C). For long term storage see below.

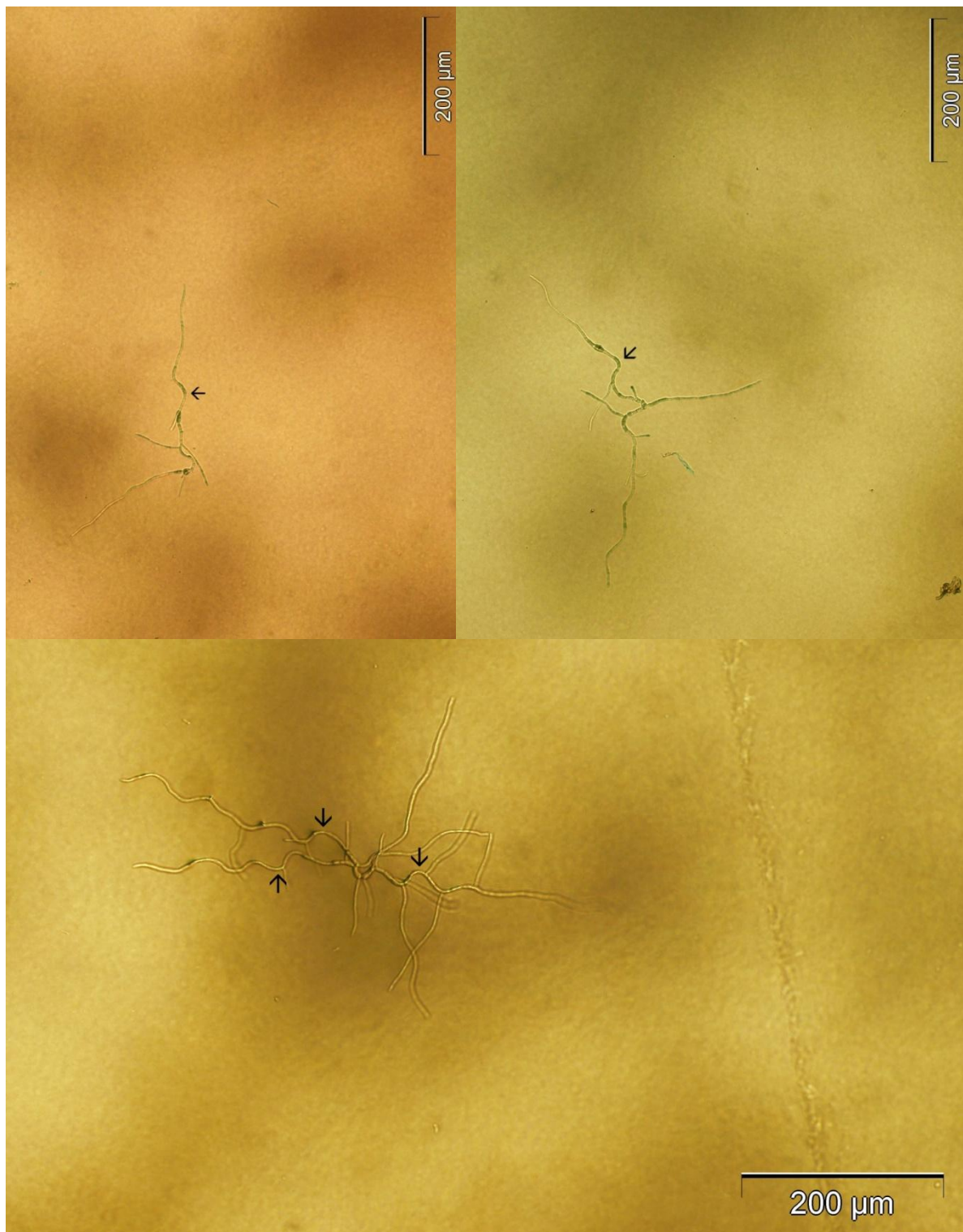


Figure 5: Three day old *Dothistroma* cultures on DM+S at 20°C. (Photo M.M.)

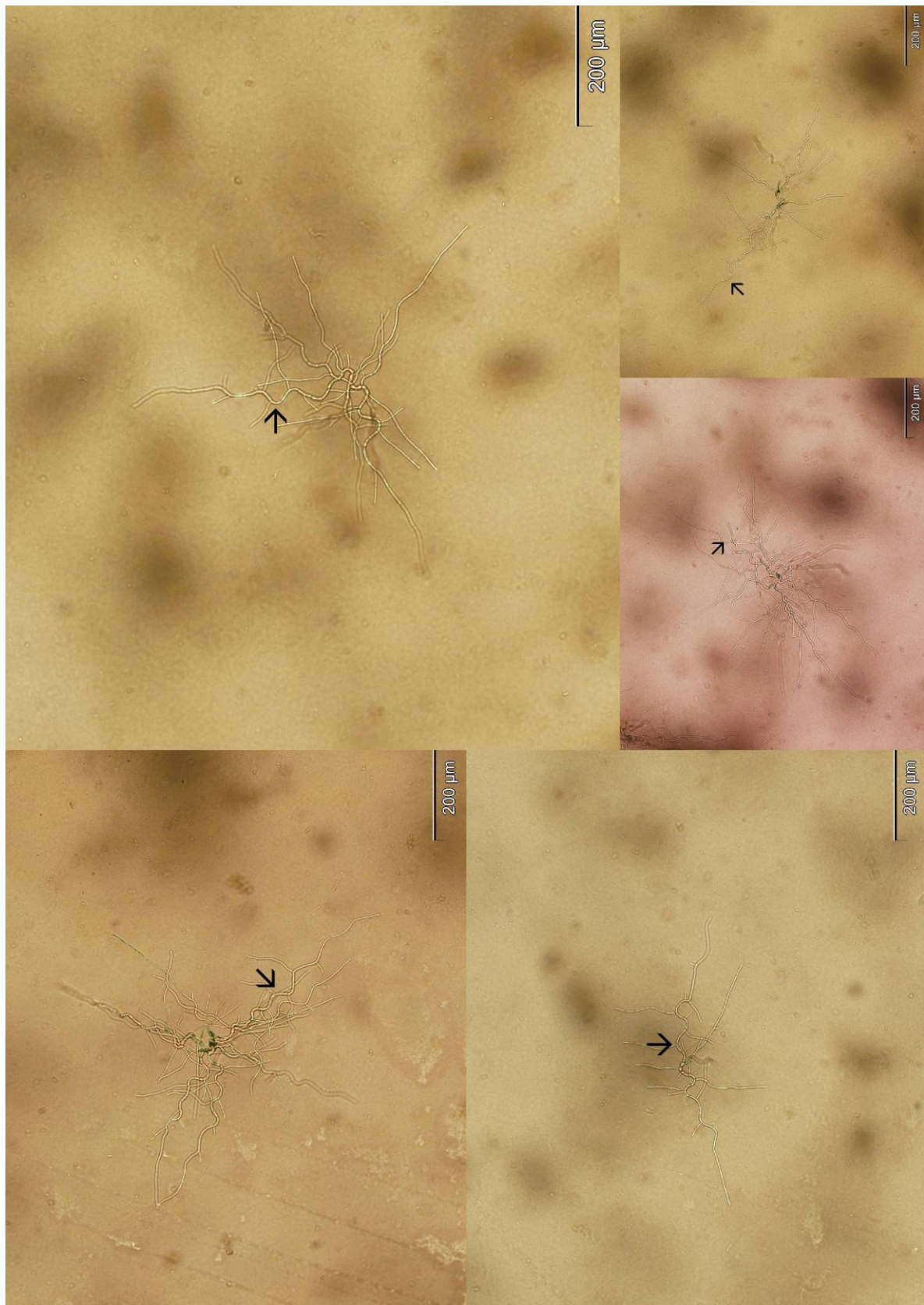


Figure 6: Four day old *Dothistroma* cultures on DM+S at 20°C. (Photo M.M.)

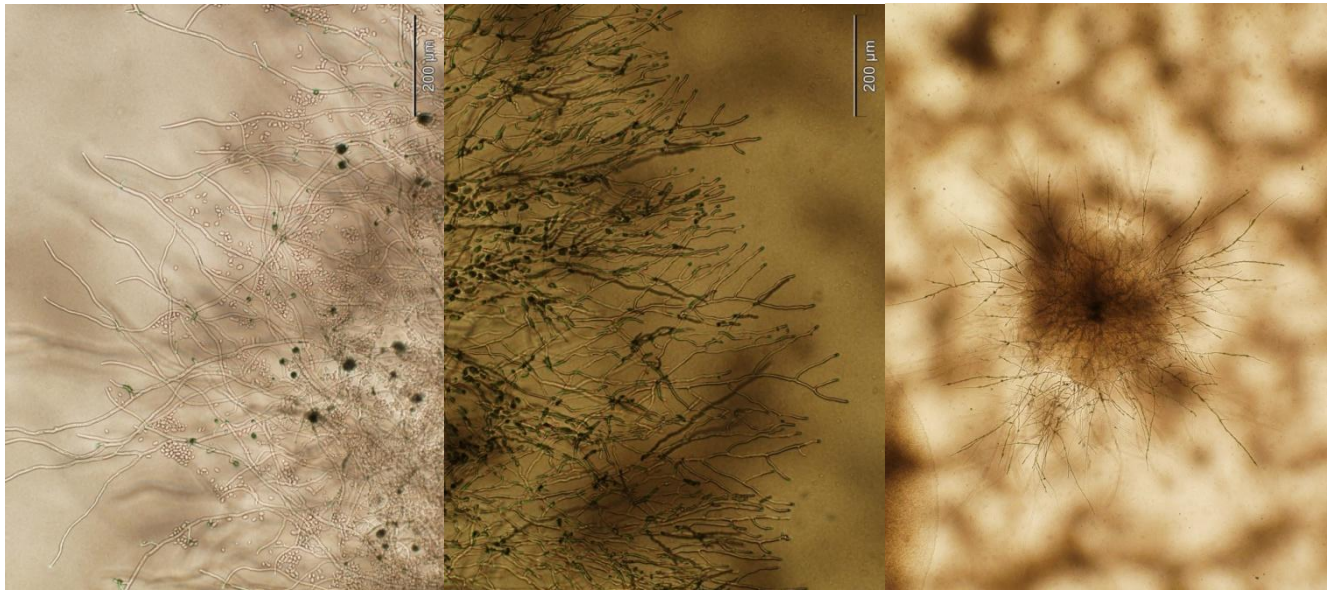


Figure 8: Non *Dothistroma* cultures at 3 days at 20°C on DM+S. (Photo M.M.)

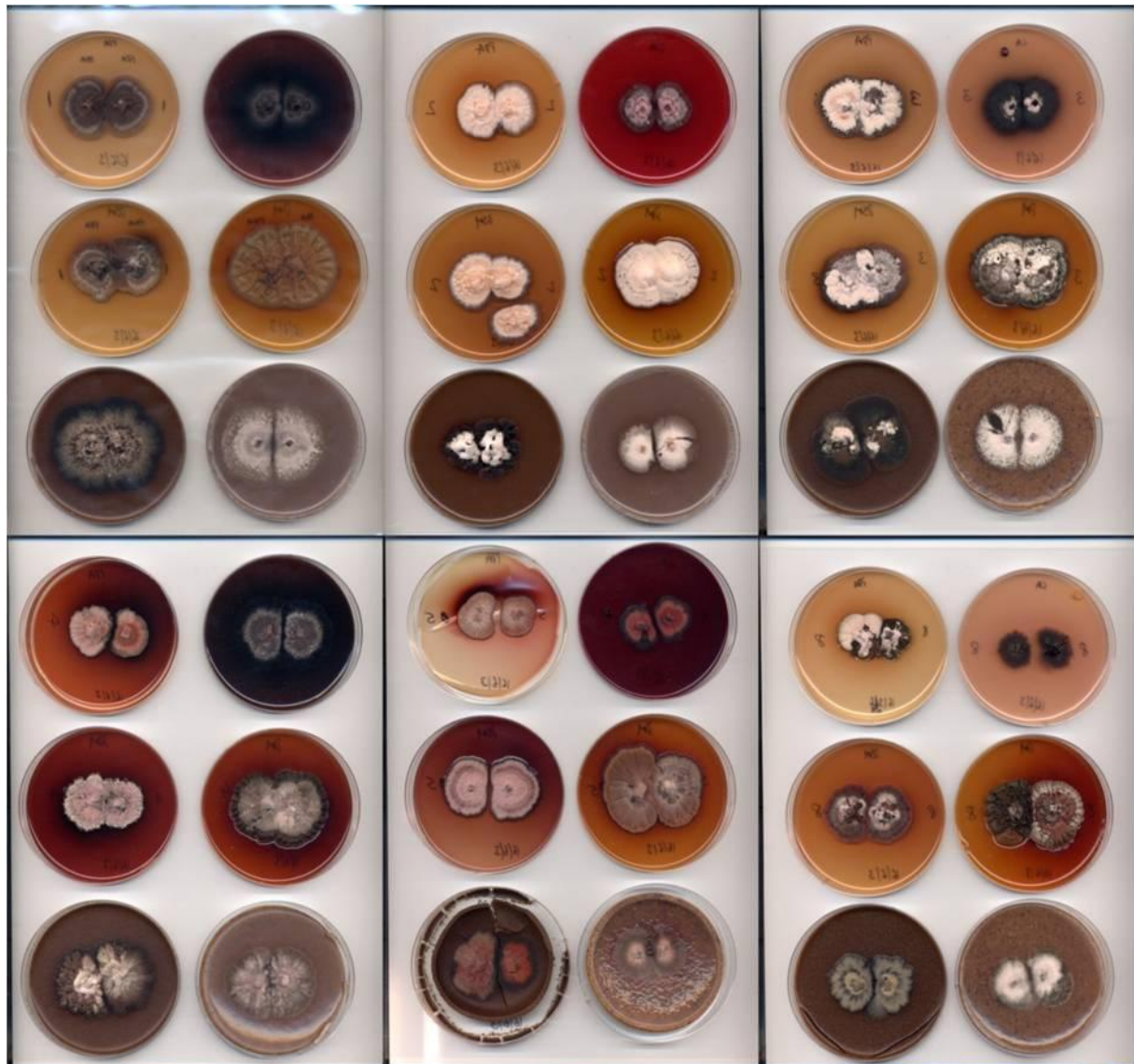


Figure 7: *Dothistroma* culture variation from around the world on various media. (Photo F.R.)



Figure 9: *Dothistroma* isolate variability (UK isolate D233). (Photo R.B.)



Figure 10: Streak isolation method onto DM+S. Cultures after 20 days at 20°C. Note dothistromin production. (Photo M.M.)

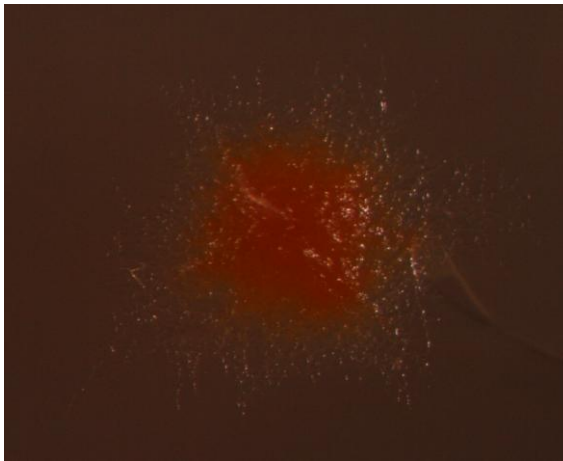


Figure 11: Nine day old culture of *Dothistroma septosporum* on MEA. (Photo M.M.)



Figure 12: Nine day old culture of *Lecanosticta acicola* on MEA. (Photo M.M.)

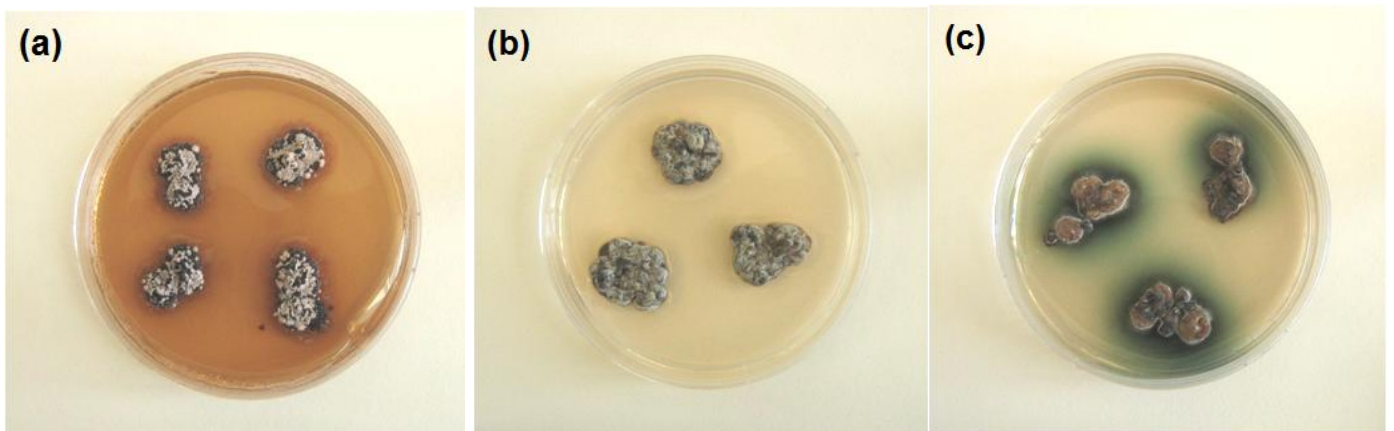


Figure 13: Pigment production on 2% MEA. (a) Characteristic orange agar discoloration from the production of dothistromin. (b) No pigment or agar discoloration (c) A blue pigment is sometimes observed in some cultures. (Photo I.B.)

1.5 Mite prevention

- Seal Petri dishes with Parafilm.
- Place plates in sealed Tupperware containers. Cardboard boxes should be avoided as these harbour insects that might carry mites.
- Place plates on 'mite traps' – a container with a thin layer of paraffin oil in the bottom with the plates on a stand above the oil.
- Use furniture spray to prevent mites travelling.

1.6 Storage of cultures

There is still a lack of knowledge on how long the cultures will survive using the various methods. However, successful recovery has been possible using the first 3 methods after a period of 8 years.

All cultures should be stored (at least) in duplicate using each method and preferably using more than one method.

1.6.1 Agar cubes

Small cubes ($\sim 5\text{mm}^3$) of the culture with agar are cut from the plate and placed into a cryovial or microeppendorf tube ($\sim 1.5\text{ml}$). These are stored at $\sim 4^\circ\text{C}$.

1.6.2 Oil storage

Cubes ($\sim 5\text{mm}^3$) of the culture are cut from the agar and placed into a cryovial or microeppendorf tube ($\sim 1.5\text{ml}$). This is covered with high viscosity sterile paraffin oil, centrifuged briefly to dislodge any air bubbles and stored at $\sim 4^\circ\text{C}$.

1.6.3 Water storage

Several cubes ($\sim 5\text{mm}^3$) of the culture are cut from the agar and placed into a cryovial or microeppendorf tube ($\sim 1.5\text{ml}$). This is covered with sterile distilled water and stored at $\sim 4^\circ\text{C}$.

1.6.4 Glycerol

Several small cubes ($\sim 5\text{mm}^3$) of the culture (preferably a sporulating culture) are cut from the agar and placed into a cryovial or microeppendorf tube ($\sim 1.5\text{ml}$). This is covered with 15% glycerol and kept at -80°C .

1.6.5 Frozen cultures

Cultures are grown on small bijou bottle agar slants until established. While growing the caps must be left as loose as possible. When fully grown the caps are tightened and the cultures stored at -20°C .

1.6.6 Freeze dried

Viable cultures have been known to be recovered from freeze dried material. However, this has not been tested for *Dothistroma*.

2 Molecular identification methods

2.1 ITS region (White et al. 1990)

2.1.1 Sequencing of the ITS region

Table 1: Primer sequences of the ITS primers

| Primer | Sequence |
|----------------|------------------------------|
| ITS1 (forward) | 5' TCCGTAGGTGAACCTGCGG 3' |
| ITS2 (reverse) | 5' GCTGCGTTCTTCATCGATGC 3' |
| ITS3 (forward) | 5' GCATCGATGAAGAACGCAGC 3' |
| ITS4 (reverse) | 5' TCCTCCGCTTATTGATATGC 3' |
| ITS5 (forward) | 5' GGAAGTAAAAGTCGTAACAAGG 3' |

Table 2: Sequences of the ITS region of *D. septosporum*, *D. pini* (Russia and Ukraine), *D. pini* (USA) (Barnes et al. 2008)

| Species | Sequence of ITS region |
|---------------------------------|--|
| <i>D.septosporum</i> | CTGAGTGAGGGCGAAAGCCCGACCTCCAACCCTTTGTGAACCAACT CTGTTGCTTCGGGGGCGACCCGCGCGTTTCGGCGACGGCGCCCC CGGAGGTCATCAAACTGTCATCTTGCGTCGGAGTCTTAAGTAA ATTTAAACAAAACCTTTCAACAACGGATCTCTTGGTTCTGGCATCGAT GAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCA GTGAATCATCGAATCTTTGAACGCACATTGCGCCCCGTGGTATTCC GCGGGGCATGCCTGTTTCGAGCGTCATTTCACTCAAGCCTGCT TGGTATTGGGCGTCGCGGTTCCGCGCGCCTTAAAGTCTCCGGCTG AGCAGTTCGTCTCTAAGCGTTGTGGCATATATTTTCGCTGAAGAGTTC GGACGGCTTTTGGCCGTAA |
| <i>D.pini</i> Russia_Ukraine | CTGAGTGAGGGCGAAAGCCCGACCTCCAACCCTTTGTGAACCAACT CTGTTGCTTCGGGGGCGACCCGCGCGTTTCGGCGACGGCGCCCC GGAGGTCATCAAACTGTCATCTATGCGTCGGAGTCTTAAGTAA TTTAAACAAAACCTTTCAACAACGGATCTCTTGGTTCTGGCATCGATG AAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCA TGAATCATCGAATCTTTGAACGCACATTGCGCCCCGTGGTATTCCG CGGGGCATGCCTGTTTCGAGCGTCATTTCACTCAAGCCTAGCTT GGTATTGGGCGTCGCGGTTCCGCGCGCCTTAAAGTCTCCGGCTGA GCAGTTCGTCTCTAAGCGTTGTGGCATATATTTTCGCTGAAGAGTTC GGACGGCTTTTGGCCGTAA |
| <i>D.pini</i> USA | CTGAGTGAGGGCGAAAGCCCGACCTCCAACCCTTTGTGAACCAACT CTGTTGCTTCGGGGGCGACCCGCGCGTTTCGGCGACGGCGCCCC GGAGGTCATCAAACTGTCATCTATGCGTCGGAGTCTTAAGTAA TTTAAACAAAACCTTTCAACAACGGATCTCTTGGTTCTGGCATCGATG AAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCA TGAATCATCGAATCTTTGAACGCACATTGCGCCCCGTGGTATTCCG CGGGGCATGCCTGTTTCGAGCGTCATTTCACTCAAGCCTAGCTT GGTATTGGGCGTCGCGGTTCCGCGCGCCTTAAAGTCTCCGGCTGA GCAGTTCGTCTCTAAGCGTTGTGGCATATATTTTCGCTGAAGAGTTC GGACGGCTTTTGGCCGTAA |

2.1.2 ITS-RFLP (Barnes et al. 2004)

Use *A*/ul restriction enzyme to digest PCR product (e.g. amplified with ITS1 and 4 primers) subsequently visualizing results on an agarose gel. *Dothistroma septosporum* sequence is not cut by the enzyme resulting in a band at ~550 bp. *Dothistroma pini* sequence is cut and results in two fragments: ~170 bp and ~350 bp.

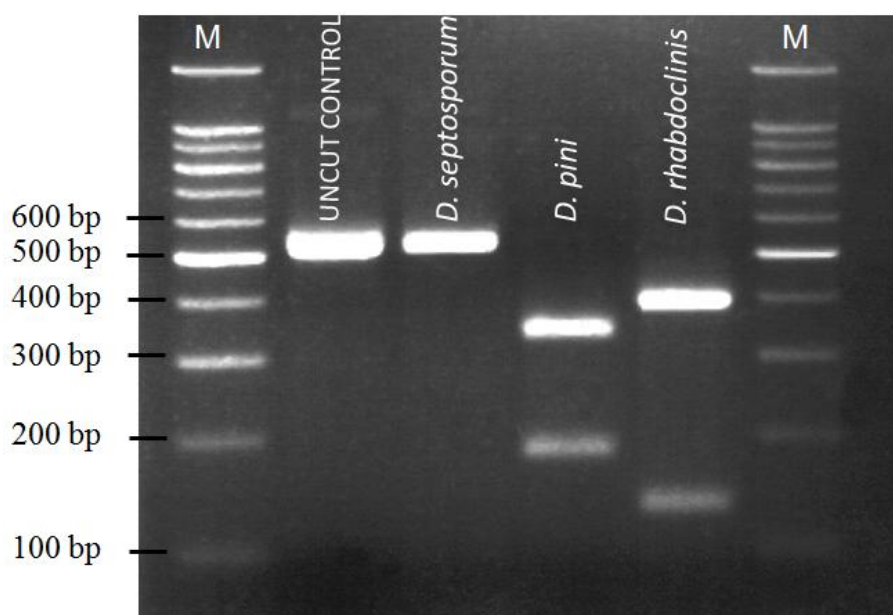


Figure 14: Agarose gel of *Dothistroma* ITS-RFLP PCR products. Reproduced and edited from Barnes et al. (2004).

2.1.3 ITS-RFLP (Pehl et al. 2004)

Use *Hinf*I, *Hae*III, *Hha*I, *Nci*I, *Hpa*II for differentiation of various needle pathogens. Probably also between *D. septosporum* and *D. pini* but this has not been tested yet.

2.2. Sequencing of β -tubulin or Elongation Factor

Table 3: Primer sequences of the β -tubulin primers (Glass and Donaldson 1995).

| Primer | Sequence |
|----------------|--------------------------------|
| Bt1a (forward) | 5' TTCCCCCGTCTCCACTTCTTCATG 3' |
| Bt1b (reverse) | 5' GACGAGATCGTTCATGTTGAACTC 3' |
| Bt2a (forward) | 5' GGTAACCAAATCGGTGCTGCTTTC 3' |
| Bt2b (reverse) | 5' ACCCTCAGTGTAGTGACCCTTGGC 3' |

Table 4: Primer sequences of the Elongation Factor 1 alpha primers (Carbone and Kohn 1999).

| Primer | Sequence |
|-------------------|----------------------------|
| EF1-728 (forward) | 5' CATCGAGAAGTTCGAGAAGG 3' |
| EF1-986 (reverse) | 5' TACTTGAAGGAACCCTTACC 3' |

2.3 Determination of Mating Type (Groenewald et al. 2007)

Table 5: Primer sequences. * = primers to use in *D. pini* reactions, ** = primers to use in *D. septosporum* reactions, *** = primers to use in both *D. septosporum* and *D. pini* reactions

| Primer | Sequence | Use |
|-------------|-----------------------------|-----|
| DpiniMat1f2 | 5' AGTAAGCGACGCGCTCCCATG 3' | * |
| DpiniMat2f | 5' GTAAGTGATCGTTGAACATGC 3' | * |
| DseptoMat1f | 5' CGCAGTAAGTGATGCCCTGAC 3' | ** |
| DseptoMat2f | 5' GTGAGTGAACGCCGCACATGG 3' | ** |
| DotMat1r | 5' TTGCCTGACCGGCTGCTGGTG 3' | *** |
| DotMat2r | 5' CTGGTCGTGAAGTCCATCGTC 3' | *** |

Table 6: Four primers in each reaction

| <i>D. septosporum</i> | <i>D. pini</i> |
|-----------------------|----------------|
| DseptoMat1f | DpiniMat1f2 |
| DseptoMat2f | DpiniMat2f |
| DotMat1r | DotMat1r |
| DotMat2r | DotMat2r |

Table 7: PCR reaction set-up for determination of Mating Type

| | |
|-----------|------------------------------------|
| 1x | PCR buffer |
| 48 µM | each of the dNTPs |
| 4 pmol | of each primer |
| 1 µM | MgCl ₂ |
| 0.7 units | Taq polymerase |
| x µl | of diluted genomic DNA (10ng-20ng) |
| 12.5µl | Total volume |

Examples:

Table 8: PCR mix *D. pini*

| Volume | Component |
|---------|---|
| x µl | DNA (10ng-20ng) |
| 1.25 µl | 10x PCR Buffer |
| 0.25 µl | MgCl ₂ (50mM) [end concentration of 1mM] |
| 0.6 µl | dNTP's (1mM) |
| 0.25 µl | Dpini Mat1f2 (10µM) |
| 0.25 µl | Dot Mat1r (10µM) |
| 0.25 µl | Dpini Mat2f (10µM) |
| 0.25 µl | Dot Mat2r (10µM) |
| 0.08 µl | Taq (Bioline 5U/µl) |
| x µl | dH ₂ O |
| 12.5 µl | Total volume |

Table 9: PCR mix *D. septosporum*

| Volume | Component |
|---------|---|
| x µl | DNA (10ng-20ng) |
| 1.25 µl | 10x PCR Buffer |
| 0.25 µl | MgCl ₂ (50mM) [end concentration of 1mM] |
| 0.6 µl | dNTP's (1mM) |
| 0.25 µl | Dsepto Mat1f (10µM) |
| 0.25 µl | Dot Mat1r (10µM) |
| 0.25 µl | Dsepto Mat2f (10µM) |
| 0.25 µl | Dot Mat2r (10µM) |
| 0.08 µl | Taq (Bioline 5U/µl) |
| x µl | dH ₂ O |
| 12.5 µl | Total volume |

Table 10: PCR cycling conditions for determination of Mating Type

| Temperature | Time | Phase |
|-------------|------|----------------------|
| 94°C | 5min | initial denaturation |
| 94°C | 20s | 40 cycles |
| 65°C | 20s | |
| 72°C | 40s | |
| 72°C | 5min | final elongation |

Visualize product on gel

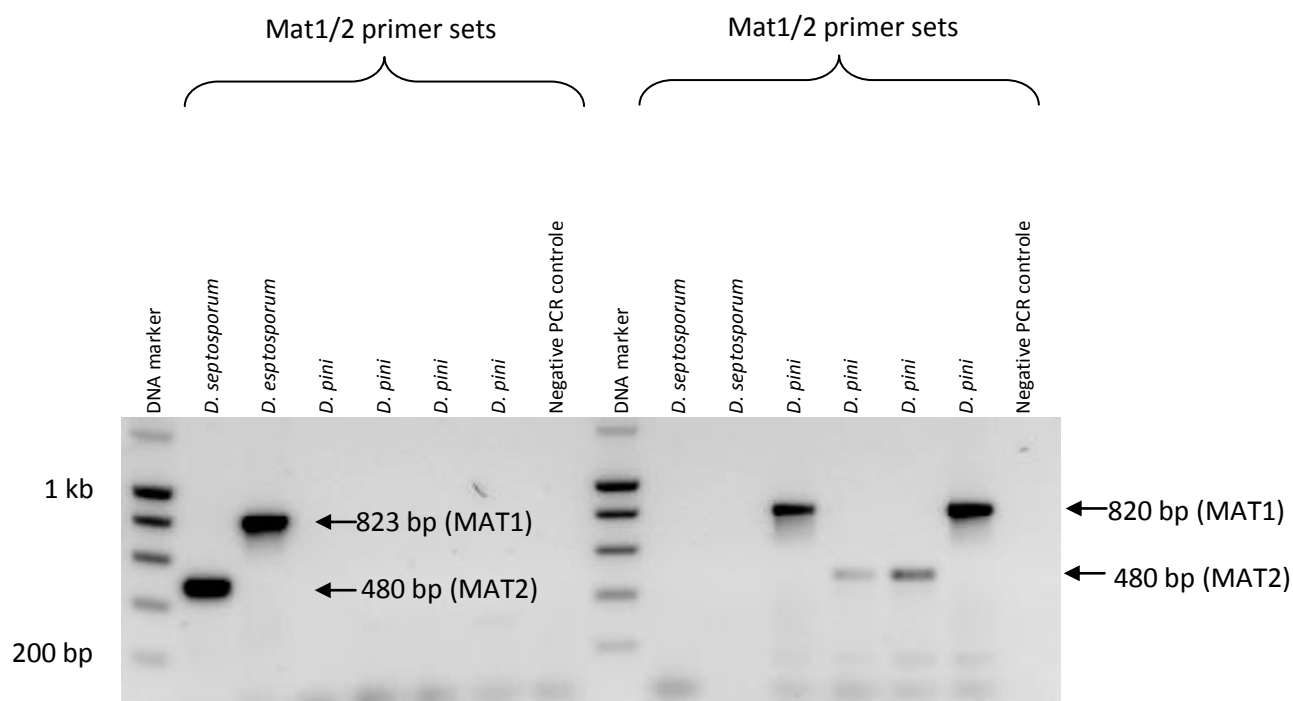


Figure 15: Agarose gel of *Dothistroma* mating type PCR products. Reproduced from Groenewald et al. (2007). (Photo M.G.)

2.4 Conventional PCR (Ioos et al. 2010)

Table 11: Primers for conventional PCR

| Target primer or probe | Sequence (5' to 3') | DNA region |
|------------------------------------|-----------------------------|------------------|
| <i>D. septosporum</i> | | |
| Dstub2-F | 5' CGAACATGGACTGAGCAAAAC 3' | β -tubulin |
| DStub2-R | 5' GCACGGCTCTTTCAAATGAC 3' | β -tubulin |
| <i>D. pini</i> | | |
| DPtef-F | 5' ATTTTTCGCTGCTCGTCACT 3' | EFI- α |
| DPtef-R | 5' CAATGTGAGATGTTCGTCGTG 3' | EFI- α |
| <i>Lecanosticta acicola</i> | | |
| LAtef-F | 5' GCAAATTTTCGCCGTTTATC 3' | EFI - α |
| LAtef-R | 5' TGTGTTCCAAGAGTGCTTGC 3' | EFI - α |

Table 12: PCR reaction set-up for conventional PCR

| | |
|-------------|--|
| 1 X | reaction buffer (Hotgoldstar PCR buffer, Eurogentec) |
| 2 mM | MgCl ₂ |
| 0.2 mM | each of the dNTP's |
| 0.4 μ M | of forward and reverse primers |
| 0.5 U | Hotgoldstar (=taq polymerase) (Eurogentec) |
| 2 μ l | template DNA (0.8-20ng) |
| x μ l | Molecular grade water |
| 20 μ l | Total volume |

Table 13: PCR cycling conditions for conventional PCR

| Temperature | Time | Phase |
|-------------|--------|----------------------|
| 95°C | 10 min | initial denaturation |
| 95°C | 30s | 35 cycles |
| 60°C | 30s | |
| 72°C | 60s | |
| 72°C | 10min | final elongation |

2.5 Real time PCR (Ioos et al. 2010)

Table 14: Primers and probes for real time PCR. F = forward primer; R = reverse primer; P = probe. BHQ1 = Black hole quencher 1, Biosearch Tech. Inc.; BHQ2 = Black hole quencher 2, Biosearch Tech. Inc.; BHQ3 = Black hole quencher 3, Biosearch Tech. Inc.

| Target primer or probe | Sequence | DNA region |
|------------------------------------|---------------------------------------|------------------|
| <i>D. septosporum</i> | | |
| DStub2-F1 | 5' CGAACATGGACTGAGCAAAA 3' | β -tubulin |
| DStub-R1 | 5' TGCCTTCGTATCTGCATTTTC 3' | β -tubulin |
| DStub2-P1 | 5' ROX-TGGAATCCACAGACGCGTCA-BHQ2 3' | β -tubulin |
| <i>D. pini</i> | | |
| DPtef-F1 | 5' ACAGCAATCACACCCTTGC 3' | EFI- α |
| DPtef-R1 | 5' TCATGTGCTCAATGTGAGATGT 3' | EFI- α |
| DPtef-P1 | 5' FAM-CCCCAGCCGATTACACGACG-BHQ1 3' | EFI- α |
| <i>Lecanosticta acicola</i> | | |
| LAtef-F1 | 5' CCTCCTTCATCTTCCCCTTC 3' | EFI- α |
| LAtef-R1 | 5' TGTGGGAGATAGCGTTGTCA 3' | EFI- α |
| LAtef-P1 | 5' Cy5-CAAGCACTCTTGAACACACCGC-BHQ3 3' | EFI- α |
| POSITIVE CONTROL | | |
| 18S uni-F | 5' GCAAGGCTGAACTTAAAGGAA 3' | 18S rDNA |
| 18S uni-R | 5' CCACCACCCATAGAATCAAGA 3' | 18S rDNA |
| 18S uni-P | 5' JOE-ACGGAAGGGCACCACCAGGAGT-BHQ1 3' | 18S rDNA |

Table 15: PCR reaction set-up for real time PCR

| | |
|-------------|--|
| 1 X | reaction buffer |
| 5 mM | MgCl ₂ |
| 0.2 mM | each of the dNTP's |
| 0.3 μ M | of each of 4 forward and reverse primers |
| 0.1 μ M | each of 4 DLP's |
| 0.5 U | hotgoldstar |
| 2 μ l | template DNA (0.8-2ng) |
| x μ l | Molecular grade water |
| 20 μ l | Total volume |

Use the qPCR core kit No ROX (Eurogentec).

Each reaction contains equal concentrations of forward primers, reverse primers, and probes for each of the 3 pathogens and positive control (18S rDNA target). A bulk mix of all the primers and probes can be premade and stored frozen. This bulk primer/probe mix has proved stable over time, easy to use, and reduces pipetting errors.

Table 16: PCR cycling conditions for real time PCR

| Temperature | Time | Phase |
|-------------|-------|----------------------|
| 95°C | 10min | initial denaturation |
| 95°C | 15s | 40 cycles |
| 60°C | 55s | |

2.6 Nested PCR (Langrell 2011)

Nested PCR (i.e. two rounds of PCR). Minimum detection limit of 10fg of DNA per μ l, but does not distinguish between *D. septosporum* and *D. pini*.

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