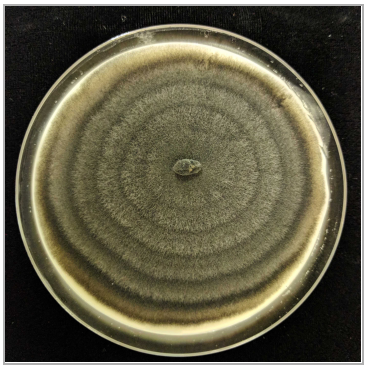

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Culturing of infective agents from infected wheat leaves

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ABSTRACT

This protocols is part of the ANU Biosecurity mini-research project #1 "Plant Pathogen Diagnostics: Visuals, subcultures, and genomics".

You will be provided four pots of 3-4 week old wheat plants that have been infected with different wheat pathogens. Each pot has been infected with one major pathogen. You will not know which pot has been infected with which pathogen. However, you will be provided a compendium of 10-15 wheat pathogens that will guide you to identify the infective agent for each treatment group. The fifth treatment group will be uninfected wheat plants which will be clearly identified. You can use treatment group #5 as negative control for your experiments.

In total, each group will obtain five pots each:

	A	B
	Treatment group 1	Unknown infective agent
	Treatment group 2	Unknown infective agent
	Treatment group 3	Unknown infective agent
	Treatment group 4	Unknown infective agent
	Treatment group 5	Uninfected control

This specific protocol is a step by step guide to culture infective agents out of infected wheat leaves. This process will take three weeks to complete, therefore the protocol is applicable for week 2-4.

The final goal is to isolate the disease causing agent, if at all possible, via culture media. Of course not all pathogens are culturable and the protocol is geared towards

filamentous pathogens which are the most severe pathogens of wheat in Australia. So the specific approach to culturing and the identity of the disease causing agent defines the possibility of the organism being cultured. Something to keep in mind when interpreting the results.

Protocols progress overview:

- Week 2 you will identify suitable infected leaf material, surface sterilise it, and culture cut leaf pieces on water agar to enable microbial growth.
- Week 3 you will identify organisms that have grown out of the leaf onto the agar and subculture them onto high nutrient agar plates.
- Week 4 you will take pictures of the subcutlured organisms and try to classify it them based on morphological features.

GUIDELINES

You must have read, understood, and follow the health and safety instructions provided in the "Overview Mini-Research Project #1 BIOL3106/6106" provided on Wattle (ANU learning portal).

You must have signed and returned one copy of the "Student Safety Declaration Form For Practical Class Work" before starting the any laboratory work.

You must have read and understood the Hazard Sheets (Risk assessment) of all chemicals listed bellow in the "Safety Warnings" section. These Hazard Sheets are provided on Wattle as part of the "Overview Mini-Research Project #1 BIOL3106/6106" document.

MATERIALS

As always you need to bring a lab notebook, a printed version of this protocol, and a pen to record your adventures in the lab.

Consumables and culture material needed:

- 15 ml Faclon tubes with 10ml 1% hypochlorid solution; 5 per group; week 2 only.
- TWA (15 g/L agar in distilled water) + 100 µg/ml ampicillin and 30 µg/ml streptomycin; 5 per group; week 2 only.
- Cling-film; week 2 only.
- Microtape; week 3 only.
- PDA plates; 5 per group; week 3 only.
- White cardboard sheets to take pictures of cultures in week 3 and week 4.

Equipment needed:

- Scissors to cut leaves in week 2.
- Scalpel to cut microbial outgrowth in week 3.
- Sterile forceps to handle cut leaves in week 2; to handle cut out microbial outgrowth in week 3.
- Ruler, week 2, 3, and 4.
- Camera (only to be handled without gloves).
- Bunsen Burner to keep areas in the lab clean and aseptic.
- Things to sterilise scissors, scalpels, and forceps between handling different treatment groups.
- Light box to take pictures in week 2, 3, and 4.

SAFETY WARNINGS

This protocol requires the following hazardous substances:

- 100% ethanol.
- 80% ethanol.

In addition, greatest care needs to be giving when using scissors and scalpels.

You need to wear safety equipment at all times including lab coats, gloves, and safety goggles when handling chemicals and biological agents. While the major biological agents used for the wheat infection are pathogens commonly found in Australia, you must treat them as they were infective agents of general concern. Treat them with care. Do not remove them from the laboratory. Do not spread them via clothing. Use a dedicated notebook and pen to make notes during the mini-research project. Do not put anything into your mouth while in the laboratory. Wash your hands each time you leave the laboratory.

BEFORE START INSTRUCTIONS

You must study the protocol carefully before you start. If anything is unclear post questions directly here on protocols.io.

Week 2: Selection of representative leaf samples, surface s..

6d 0h 1m 30s

1 You will receive five pots with wheat plants as detailed in the "Description" section. Please label each pot with your group name, the date, and the treatment group. 5m

2 Carefully, study the plants in each pot and select two leaves with symptoms of infected plants (if possible) and control leaves of uninfected plants. Select two leaves for each treatment group. 5m

3 Perform the following steps [3.1 to 3.3] for each treatment group [#1-5] independently and consecutively. This will reduce the risk of cross contamination. Sterilise your equipment after each step. Start with the uninfected control samples treatment group #5 [TG#5].

3.1 Clearly, label the provided falcon tube with 1% hypochlorite solution with the treatment group number [eg TG#5] and your group identifier.

3.2 Using the provided scissors, Cut the leaves into about 1 cm long pieces and place the pieces into the labelled falcon tube with 1% hypochlorite solution. 2m

This step aims to perform a surface sterilisation of the leaf to avoid contamination of fungi and bacteria that are attached to the leaf surface. Fungi and bacteria within the leaf will not be killed with this treatment.

3.3 Incubate the leaf pieces for about 00:01:00 1 min with slight movement tipping the falcon tube upside down multiple times. 1m

3.4 Discard the 1% hypochlorite solution into the provided beaker while making sure the leaves stay within the falcon tube. 1m

3.5 Wash the leaves within the falcon tube with about 10 ml sterilised water for about 00:01:00 1min each. The water is provided to you on the bench in a flask. Discard the water into the provided beaker while making sure the leaves stay within the falcon tube.. 1m

3.6 Repeat step 3.5 two more times. 3.5

4 Now make sure that you work under the clean area close to the bunsen burner.

Safety information

Take care close to the open flame. Do not bring highly flammable chemicals close to the open flame in an uncontrolled manner.

The following steps will place the leaf pieces on the water agar plates (TWA) to try to culture the infective agent from the leaf material, if at all possible with this specific culture approach.

4.1 Obtain one TWA plate and label it with the treatment group name, your research group name, and the date.

4.2 Using sterile forceps, place 4-5 leaf pieces equally distributed around the plate on the surface of the water agar.

4.3 Carefully close the plate and wrap it in clingfilm.

4.4 Sterilise your forceps as indicated by the demonstrator before moving to the next treatment group in go to step #4.1 .

5 Incubate the plate at 16-20^{o}C for 7 days 168:00:00 Incubate till the next practical session. . 1w

Week 3: Subculture of microbial outgrowth from leaves.

6 We would expect that there is filamentous or bacterial growth at the edges of the leaf if the infectious agent is culturable on agar and under the tested conditions. You will now aim to subculture one representative microbial outgrowth per treatment group on rich PDA media.

7 Perform the following steps [7.1 to 7.8] for each treatment group [TG#1-5] independently and consecutively. This will reduce the risk of cross contamination. Sterilise your equipment after

each treatment group. Start with the uninfected control samples TG#5.

Safety information

Take extreme care when handling the scalpel. This is the most dangerous step of the whole practical. If unsure how to handle the scalpel consult with the demonstrator before using it.

7.1 Unwrap the plate but keep the lid on.

7.2 Close to the sterile bunsen burner area, remove any condensation that might have formed on the lid and place the lid back on top of the plate.

7.3 Take a picture of the plate on a white background or a light box. Place a ruler and a label that clearly identifies the treatment group next to the plate before taking a picture. This will allow you to clearly identify the plate later on and add a scale bar to the picture digitally if required.

7.4 For each treatment group, identify one representative out growth you intend to subculture.

7.5 Obtain a PDA plate and label it with the treatment group name, your research group name, and the date.

7.6 Close to the sterile bunsen burner area, cut out the representative outgrowth with a sterile scalpel.

7.7 Place the cut out piece in the center of the PDA plate using sterile forceps.

7.8 Close the plate and seal it with a mircotape.

7.9 Discard the old TWA plate in the appropriate waste bin.

7.10 Sterilise your forceps as indicated by the demonstrator before moving to the next treatment group i go to step #7 .

8 Incubate the new PDA plate at 16-20^{o}C for 7 days 1w

168:00:00 Incubate till the next practical session.

Week 4: Carefully assess and record representative microb...

9 We would expect extensive growth of the subcultured microbes on the rich PDA media. You will have to assess and record the microbial growth. This will include taking pictures of each plate.

10 Perform the following steps [9.1 to x] for each treatment group [#1-5] independently and consecutively. Start with the uninfected control samples TG#5.

10.1 Unwrap the plate but keep the lid on.

10.2 Remove any condensation that might have formed on the lid and place the lid back on top of the plate. You can perform this step on your bench as you will discard the plate after this weeks prac.

10.3 Take a picture of the plate on a white background or a light box. Place a ruler and a label that

clearly identifies the treatment group next to the plate before taking a picture. This will allow you to clearly identify the plate later on and add a scale bar to the picture digitally if required.

10.4 Carefully assess the type of microbial growth on the plate and describe the characteristics in your lab notebook.

10.5 Discard the old PDA plate in the appropriate waste bin.

11 This represents the end of the lab part of this protocol.

12 To do.