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Sampling and testing for plant pathogens

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Introduction

There are many methods used in the laboratory to determine whether *Phytophthora* or other fungi are present in growing media, water supply and diseased plants. These include:

- filtering water for spores,
- baiting for fungi in growing media and water samples,
- examining diseased plant tissue microscopically, and
- direct culturing from roots and other plant parts to isolate the fungal pathogen in pure culture.

This bulletin outlines some of the procedures involved. Growers may be able to do some basic baiting of soil or water samples themselves to alert them of a problem but the procedures involved in isolating and identifying pathogens are complex and best left to specialists trained in plant pathology. In addition, some of the chemicals used in some media may only be purchased under licence.

Laboratory certification

Many laboratories may offer disease diagnosis. Before using a laboratory, check the qualifications of staff. Key staff should be trained in plant pathology. Laboratories may also have NATA (National Association of Testing Authorities) certification. NATA is the peak authority in Australia for the accreditation of laboratories. It is independently evaluated on a regular basis by its international counterparts to ensure that its operations remain in-line with international practices. More information on NATA can be found at www.nata.asn.au.

Common terms

Primary (plant) pathogen. This is the organism that causes the disease. For example *Phytophthora.*

Secondary (plant) pathogen. These are other organism(s) which either MAY cause disease, or may simply infest and feed on dead plant tissue resulting from pest, disease or other injury. Secondary pathogens may function as a parasite, killing living tissue, or as a saprophyte.

Saprophyte. An organism that uses non-living/dead organic matter as food.

There tends to be a sliding scale of pathogenicity or ability to cause disease, amongst fungi and bacteria. Some pathogens are capable of infecting plants which are quite healthy, others require a weakened or stressed host before they will be able to infect tissue and cause disease. That stress may be physiological eg drought, waterlogging, salinity, frost damage, poor nutrition or physical damage from sand blasting or insect feeding.

Correct sampling and handling of material to be checked for the presence of fungi is essential.

- Samples should be collected from mildly to moderately affected plants, not from plants too advanced in decline, dead, or recently treated with fungicides.
- They should be despatched on the same day they are collected and protected from drying out and extremes of temperature.
- For root samples, take small quantities of affected roots with a small quantity (at least 200 g) of moist mix or soil from around the affected plant, seal in a plastic bag, and preferably store and despatch in an esky. Alternatively, whole plants can be dug up with the root ball and some surrounding soil and placed in a plastic bag for transport. For large plants it may be possible to cut off the top a few centimetres above ground level and send the root ball and attached soil together with a few representative branches. Nursery plants may be sent intact in the pot.
- Information on the name of the affected plant, total number of plants affected, distribution of damage both in the plant and within the crop, recent treatments and symptoms observed, should always be included with specimens submitted for disease diagnosis. Other information such as recent weather history, what is the water source (scheme, stream or dam), changes in routine procedures and speed of disease onset is also a useful aid to diagnosis.
- It is generally preferable to send samples so they arrive early in the week, rather than on a Friday.

Sampling precautions to prevent spread of disease or cross contamination – lower heading level

- Use clean plastic gloves or bags over hands to collect samples and use new ones for each new area sampled.
- Collect into over-sized, strong plastic bags which allow for easy mixing and prevent breakages.
- Avoid a direct contact with growing media and diseased plants as much as is possible. Wash hands thoroughly with soap and water if contamination is suspected and before moving to new sites.
- Ensure footwear is clean before entering new sites. Disposable plastic overwear covering shoes could be worn.
- Where sampling equipment such as trowels or shovels need to be used, preferably use clean on-site equipment. Alternatively, use equipment that is cleaned thoroughly and sterilised after each use. After equipment is cleaned it can be sterilised by several different methods. These are, in order of preference and probable effectiveness:
  - Dipping and flaming in 95 % alcohol.
  - Immersing in, or swabbing with a 1 % sodium hypochlorite solution, then rinsing with clean water (stainless steel equipment which will not corrode is preferred).
  - Immersing or swabbing with 40-70 % alcohol, allowing at least three minutes contact time.

Collecting samples

- From a future planting site. When soil sampling a plantation or future site for *Phytophthora,* target seepage and low lying areas. Also sample near remnant bush and all 'run-off' slopes. Look for "hot spots" where native vegetation is present and sample soil around any noticeably unthrifty vegetation. Keep samples separate and mark locations on a site map for future reference.
- From an existing planting. Collect soil samples from a number of plants in each representative area or

from plants that appear unhealthy. Small quantities of roots and soil from within the top 10 cm of the root zone should accompany the sample. Keep samples (maximum 500 g) moist in a sealed plastic bag. Leaf and stem samples should be taken from the basal part of the plant.

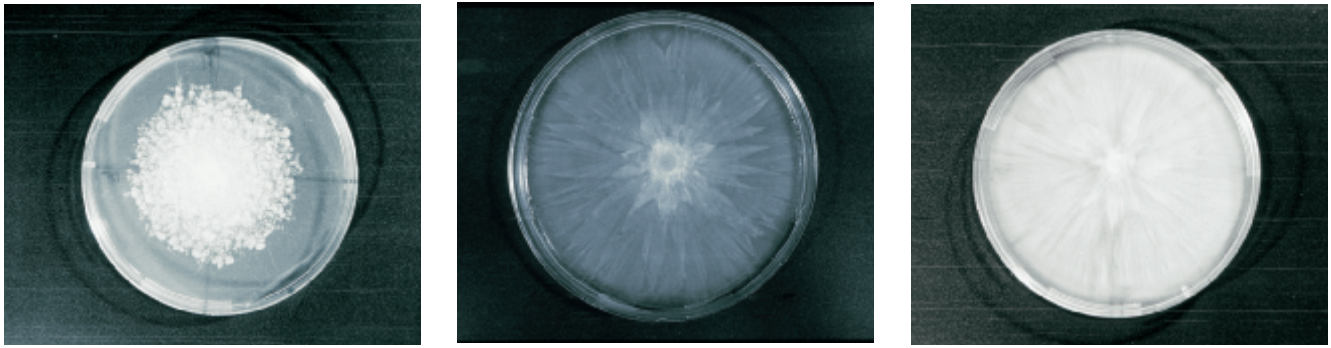
- From a nursery. Inspect root systems for disease systematically by tapping plants gently from the pots. If no disease symptoms are obvious, collect samples randomly from known hosts. Take a small quantity of roots and growing medium from the base of these pots. Collect from 20 different plants and place samples in a large strong plastic bag, mix thoroughly and label with a waterproof marker. A final sample should weigh about 500 g. Mix the bulk samples again before setting up baits.
- All samples must be kept cool and stored in an esky for transport to a laboratory.

Figure 1. Tools used in sampling for dieback. Trowels, alcohol in a spray bottle for disinfecting trowels between sites, plastic bags for sample collection and stakes to mark sampling points (for example when grid sampling a potential new site)

Direct isolations

Direct isolation of the plant pathogen from roots or other plant parts is the most reliable way to detect many fungal pathogens. With the aid of selective media, fungi such as *Phytophthora* are easily and rapidly cultured from freshly collected samples taken from recently infected plants. It is more difficult to isolate from old infections or dried out samples because of the presence of secondary pathogens, whose growth often overtakes that of the primary (disease causing) pathogen and makes a correct diagnosis difficult.

In the laboratory, root, stem or leaf material showing symptoms is washed several times under running tap water. Some laboratories may surface sterilise leaf material by dipping in a 1% sodium hypochlorite solution prior to rinsing in sterile water. Roots may also be surface sterilised by immersing in a 50% ethanol solution for 30 seconds, then rinsing in sterile water. The material is then dried between sheets of sterile blotting paper. Small segments of rotted roots or other parts from margins of rotted areas are placed onto a media selective for *Phytophthora*. However, when samples arrive it is



Figures 2a-c. Species of Phytophthora, *when cultured on agar, show variability. From left to right:* P. nicotiane, P. citricola, P. cinnamomi

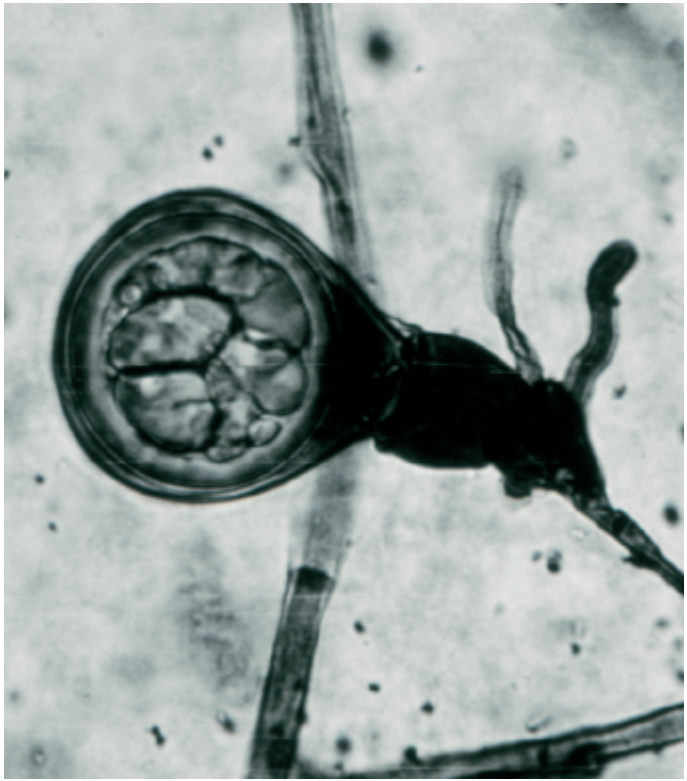


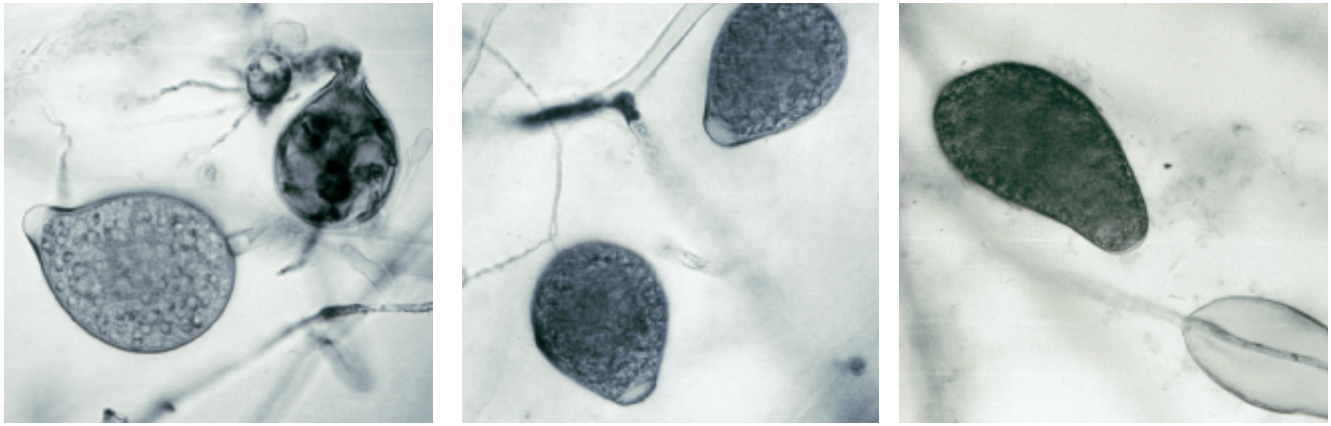
Figure 3. Sexual fruiting body (oogonium), containing oospores, of Phytophthora cinnamomi

essential to also screen for other pathogens such as *Rhizoctonia* or *Fusarium.* Therefore, a more general medium is also used when isolating from rotted roots or other diseased material. Antibiotics such as streptomycin sulphate may be added to inhibit growth of unwanted bacteria.

Depending on what fungi grow from the sample, sub-cultures may be taken and placed on other media for sporulation and identification. Again, specialist knowledge is required to know which fungi may be causing the actual disease and which are those that grow primarily on dead plant tissue.

Slow growing, coarse hyphae of *Phytophthora* can usually be seen growing out from material on selective media within 24-72 hours (Figs 2a-c). *Phytophthora* cultures can be distinguished from *Pythium* by their comparatively slow growing, stout and stiffly branched hyphae, often with distinct and obvious hyphal swellings.

Pythium spp. are generally fast growing, with finer, flexuous hyphae. *Phytophthora* and *Pythium* cultures may be sub-cultured, and identification



Figures 4a, 4b, 4c. Examples of variability in the structure of sporangia (asexual) of Phytophthora *species*

confirmed by inducing and examining sexual (Fig 3) and asexual spores (Figs 4a-c). This additional process is time consuming and therefore adds extra expense which is not generally required. For the purpose of treatment, most growers only need identification to genus level, for example, *Phytophthora* or *Rhizoctonia*.

Isolation of most root rot pathogens is relatively easy from freshly infected plants. Non-selective culture media is successful for many pathogens including *Phytophthora*. However, the use of selective media for *Phytophthora* and *Pythium,* gives rapid results and allows for quick identification of these common and serious pathogens.

Direct microscopic examination

Small segments of rotted plant tissue can be teased out, stained and examined under a microscope. Some fungi have particular morphological characteristics that

enable identification in this manner. For example, spores can sometimes be detected on the surface of diseased above-ground plant parts. Downy mildew, powdery mildew, grey mould, and leaf spots caused by *Bipolaris* and *Alternaria* are fungal diseases that can often be identified this way.

Incubating affected material on trays in moist plastic bags may stimulate sporangial production, fungal growth or spore production allowing direct identification by microscope. Although a few *Phytophthora* species infect leaves and can be detected this way, most of the species we commonly encounter in Western Australia cannot, and other methods, such as direct isolation, must be used to determine their presence.

Baiting for *Phytophthora*

Baiting refers to a technique used to detect *Phytophthora* in growing media or water. Baits consisting of plant material are floated on the surface of growing media flooded with distilled water in cups, or in irrigation supplies. Zoospores are produced and these infect and rot the baits, which are then removed, examined microscopically and, if necessary, the fungus cultured and identified further.

Bait materials that have been used successfully include seedlings of *Eucalyptus sieberi* and the roots of freshly germinated lupin seedlings. Pears, apples, avocadoes, pine needles (Fig. 5), rose petals and leaf or leaf pieces of African violets or umbrella tree may also be used.

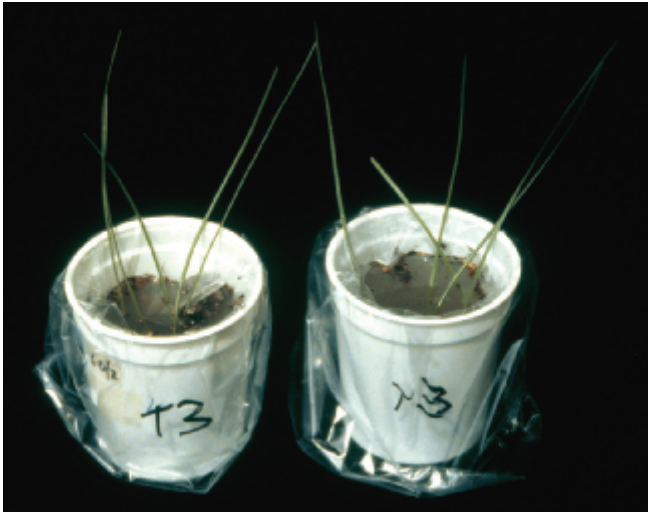


Figure 5. Baiting for Phytophthora *using pine needles*

Baiting water supplies

If drawing water from any source in contact with the ground, it is wise to test for the presence of *Phytophthora.* If found, the water source can then be treated to eliminate this pathogen. In some cases, when growing difficult species, it may also be desirable to exclude *Pythium.*

Unripe pears or apples are commonly used to test water sources such as dams or streams. These are secured in some way (eg in an old stocking) and floated in the water for 5-7 days. If pathogens are present, rots develop on the fruit, and fungi are subcultured for further identification. This is necessary because bacteria and *Pythium* spp. can also infect baits, causing confusion. Early isolation from such baits can confirm the presence of *Phytophthora.* The method works well because the fruit are exposed to a large volume of water, effectively sampling a much greater volume than other methods would permit.

Microfiltration (4 micron) can also be used to detect zoospores of *Phytophthora* and *Pythium* in water supplies. Microfiltration is sometimes used to exclude these fungi in circumstances where low volumes of water are being used (such as propagation). After a few days use, the residue from the filter is plated onto selective growth media.

Lupin baiting

Lupin baiting is a cheap technique that can be performed by anyone, to at least give an initial indication of the possible presence of fungal pathogens (usually *Pythium* or *Phytophthora*). Initial baiting to identify problem areas, or to do more intense monitoring (particularly if plants highly susceptible to root rot are grown) may be very useful. This permits testing of many more samples than would normally be referred to diagnostic laboratories.

Lupin baiting technique

- New Zealand blue lupins *(Lupinus angustifolius)* are preferred but white lupins have also been used. Make sure the seeds are clean and healthy.
- Surface sterilise the lupin seed for 2 minutes in 1% sodium hypochlorite then rinse well with distilled

water. Do not use tap water as it may contain copper (from copper pipes) or chlorine, both of which will inhibit fungal growth.

- Soak seed in distilled water overnight, then plant in pre-moistened vermiculite (in sterile trays, punnets, or pots, loosely covered with plastic). Alternatively, soak a seed germination towel in distilled water, drain excess water, place lupins on surface at 1 cm spacings. Roll up towel with seed, place elastic bands around ends and seal in a plastic bag. It is preferable to use distilled water as there may be residual copper (from copper pipes) or chlorine, in tap water, both of which may inhibit oospore germination.

- Keep the seed at about 25°C. They are ready to use when the root is about 2-3 cm long, (within 2-3 days). Do not use seedlings much bigger than this as their susceptibility to *Phytophthora* may decrease as they get older.

Preparing baits

- Place media samples in sterile cups or beakers. Disposable 250 mL clear plastic drink cups with matching lids are ideal for this purpose. Lupin roots can be easily examined and the holes for the seedlings to sit in can be easily cut or melted into the lids. Place a maximum of one part of growing medium to five parts of distilled water (for example about 50 mL of medium in a 250 mL cup). Do not increase the volume of mix as correct dilution is important for the best zoospore production.

- Insert the germinated lupins (five per cup) through holes made in plastic lids or polystyrene floats, so that the roots are just immersed in water.

- Set up a bait cup with a growing medium unlikely to be contaminated (such as an unused potting mix), to act as a comparison.

- Leave the cups on a bench at about 25°C for up to 7 days. Suitable temperatures are between 22° and 28°C, for the majority of *Phytophthora* species.

In most cases *Phytophthora* severely rots the baits within 5 days, and infected, (obviously rotted) lupins do not grow. However, the severity of rotting may vary with different species and conditions. Therefore, apparently healthy baits (Fig 6a) are left for 7 days, before being removed, washed and examined for rotting.

If rotting is present (Fig 6b), growers can then track back to identify those specific samples and submit them to a laboratory for testing and identification.

If this procedure is carried out in a laboratory, rotted lupin roots are removed and the techniques described earlier for direct isolation, followed to identify the pathogen.

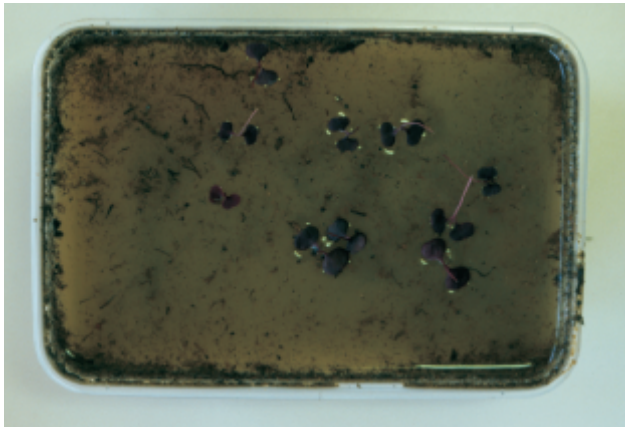
In some cases, lupins may be removed and placed in small petri dishes of water and examined microscopically for sporangia. Sporangia of several species of *Phytophthora* can be found on many positive baits, thus allowing confirmation of presence of a *Phytophthora* sp. before the fungus is isolated. However, in most cases isolation of

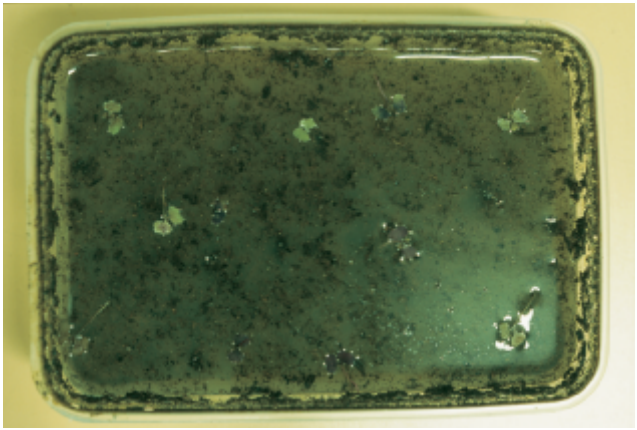
Figures 6a, 6b. Lupin baiting for root pathogens. Healthy lupin seedling left, roots of seedling on the right rotting. This sample will be plated onto agar for further examination

the fungus is necessary as sporangia are not present or when the fungus is to be identified further. In some cases, *Phytophthora* has been isolated where only minor rotting of the lupin root tip is apparent. It is possible that this results from direct infection from the mix at the bottom of the cup, rather than from zoospore infection. Samples which have been recently treated with fungicides can act in the same way.

Eucalyptus seiberi baiting

- Cut drainage holes in the base of 700 ml plastic or aluminium foil containers.
- Add about 5 cm of vermiculite and wet thoroughly with de-ionised water, draining the excess.
- Sprinkle about one spoonful of seed evenly over the surface and lightly scrape in with a fork until just covered.
- Rest a transparent lid over the top to reduce evaporation but to still allow some air flow.
- Place at 25°C under light. And add water every few days as required.
- Seeds are germinated to the cotyledon stage, which takes 10-14 days. Remove cotyledons with a pair of tweezers The detached cotyledons are then floated on the soil or growing media mixture (Fig 7a). there may be residual copper (from copper pipes) or chlorine, in tap water, both of which may inhibit oospore germination.
- Keep the seed at about 25°C. They are ready to use when the root is about 2-3 cm long, (within 2-3 days). Do not use seedlings much bigger than this as their susceptibility to *Phytophthora* may decrease as they get older.
- If the cotyledons become infected by *Pythium, Phytophthora* or some other fungi, the undersides of the cotyledons will fade from purple to green (Fig 7b).
- To further identify the pathogen, the bleached cotyledons are plated out onto selective agar (Fig. 8).





Figures 7a, 7b. Eucalyptus seiberi *cotyledons may be used to test for fungal pathogens. The picture on the right shows bleaching of the undersides of the cotyledons after a few days when in the presence of fungal pathogens such as* Phytophthora *and* Pythium

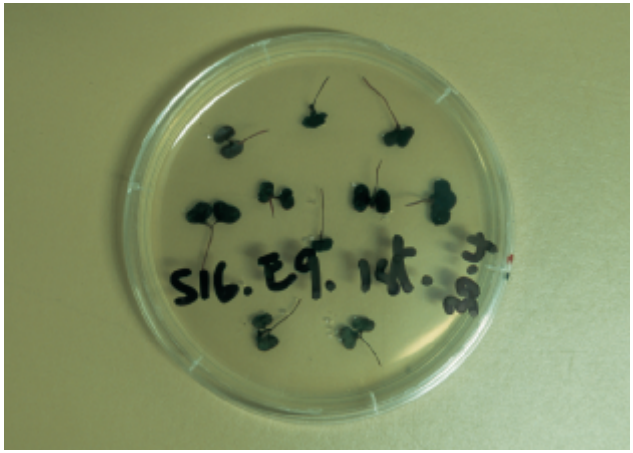


Figure 8. To further identify the pathogen, E. seiberii cotyledons *are plated out onto normal selective agar*

Summary

For effective prevention and management of plant disease, correct identification of the causal organism is necessary. Whilst growers are able to contribute to this process, the procedures involved are often complicated. Specialist training in plant pathology is essential to ensure correct diagnosis and advice. In Western Australia, the following laboratories offer plant disease diagnosis:

AGWEST Plant Laboratories

Department of Agriculture and Food Western Australia
3 Baron-Hay Court
South Perth WA 6151
Phone: 08 9368 3721
Fax: 08 9474 2658
Email: agwestplantlabs@agric.wa.gov.au

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