PLANT BREEDING AND SEED SCIENCE
Volume 76 2017

DOI: 10.1515/plass-2017-00015

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PHYTOPHTHORA INFESTANS: ISOLATION OF PURE CULTURES, STORAGE AND INOCULUM PREPARATION

ABSTRACT

Phytophthora infestans causes potato and tomato late blight, economically the most important disease of these plant species. The Oomycete pathogen is frequently sampled, isolated to pure cultures, stored, and characterized. The knowledge of its diversity, migrations and evolution is essential for breeding resistant plants and for designing appropriate control strategies. The article presents methods for collection, storage and preparation of *P. infestans* isolates for inoculation of plant tissues, based on the publication by Zarzycka (2001), later updated and modified.

Key words: cryopreservation, isolate, late blight, potato, rye B agar medium

INTRODUCTION

Late blight caused by *Phytophthora infestans* (Mont.) de Bary is one of the most devastating diseases of potato and tomato. The pathogen is a fungus-like Oomycete originating from Toluca Valley in Mexico. This fast-evolving organism followed its hosts to all parts of the world. It is airborne and heterothallic, and while in some regions sexual reproduction generates high diversity of strains, other populations are dominated by highly aggressive and well-adapted clonal lineages (Fry *et al.*, 2015). The knowledge on the local population structure, migrations and evolution of *P. infestans* is essential for the understanding of late blight epidemiology, appropriate chemical control and resistance breeding. Since 1985, population of *P. infestans* in Poland, has been monitored in terms of virulence towards

Communicated by Ewa Zimnoch-Guzowska

Black's differential set (Sujkowski *et al.*, 1996) and later more traits were studied (Chmielarz *et al.*, 2014, Brylińska *et al.*, 2016).

The presented methods for collection, storage, and preparation of *P. infestans* isolates used both in studies of the pathogen population and of host resistance are based on the publication by Zarzycka (2001) but since them they have been modified and improved.

The procedure for isolation of *P. infestans* pure cultures was changed as a result of the European Concerted Action on Blight (EUCABLIGHT: A Late Blight Network for Europe, www.euroblight.net). Following the protocol developed within this project, the medium for pure culture isolation was enriched with antibiotics: antibacterial rifamycin and antifungal pimaricin. This amendment improves efficiency and speeds up the process of *P. infestans* isolation from the other microorganisms.

The method of storage of *P. infestans* cultures in liquid nitrogen was developed in the 1960s (Hwang, 1966). It was optimized and its effect on the survival and the pathogenicity of the *P. infestans* isolates was satisfactory according to Sobkowiak *et al.* (2012). This method, as cheaper, less labour-consuming and more reliable, is now used to preserve IHAR-PIB's long-term collection instead of storage on agar slants under paraffin oil (Zarzycka, 2001).

Artificial media and storage affect the ability of *P. infestans* isolates to cause infection and there is big variation in the downstream traits such as virulence (Andrivon *et al.*, 2011). The passages through potato tissue enhance the aggressiveness and the virulence of *P. infestans* isolates after storage and at least two or three passages are recommended to recover the before-storage qualities of the isolates (Zarzycka, 1996; Sobkowiak *et al.*, 2012).

MATERIALS

1) Rye grains (use grains that are not protected by fungicides, well stored and not moldy)
2) Distilled water
3) Ethanol 70%
4) Paraffin oil
5) Liquid nitrogen
6) Sodium hypochlorite 1%
7) Tubers of cultivars susceptible to late blight (e.g. Bintje, Irga, Irys)
8) Petri dishes
9) Cryovials 2 ml
10) Test tubes
11) Scalpels
12) Pipette tips
13) Rifamycin, (Sigma-Aldrich, cat. No R8626-5G, CAS-No 14897-39-3)
14) Pimaricin (natamycin), (Sigma-Aldrich, cat. No 32417-50MG, CAS -No 7681-93-8)

15) Sucrose, (e.g. Chempur, Poland, cat. No 427720906, CAS-No 57-50-1)
16) Agar, (Sigma-Aldrich, cat. No 05040, CAS-No 9002-18-0)
17) Dimethyl sulfoxide (DMSO), (Sigma-Aldrich, cat. No D4540, CAS-No 67-68-5)

EQUIPMENT

1) Autoclave
2) Laminar flow cabinet
3) Microscope
4) Hemocytometer
5) Fridge 4-7°C
6) Freezer -70°C
7) Incubator 16°C
8) Liquid nitrogen storage container
9) Freezing device: NALGENE® Cryo Freezing Container (Thermo Fisher Scientific, Waltham, MA, USA) or CoolCell™ LX Freezing Container (BioCision LLC, San Rafael, CA, USA)

PROCEDURES

Rye B agar medium according to the Caten and Jinks (1968)

Compositions [g × l]:

1) 60 g rye grain,
2) 20 g sucrose or glucose,
3) 15 g agar*.

*The liquid rye B medium (without agar) is used to produce mycelium suitable for freeze-drying before nucleic acid extraction.

Preparation:

1) Soak the rye grains in distilled water, so that it covers the grains with a 2 cm layer, and place at room temperature for 36 hours.
2) Pour off (but keep) the supernatant and add fresh distilled water to the grains and heat in a water bath for 3 h at 50 °C.
3) Filter the mixture through a sieve, discard the grain.
4) Combine the liquid with the original supernatant (#2), and mix with glucose or sucrose and agar. Adjust the volume to 1 liter.
5) Autoclave for 20 min at 120 °C (Tumwine *et al.*, 2000).

Medium with antibiotics (www.eucablight.org)

Compositions:

1) Rifamycin – 1% stock in 70% ethanol,
2) Pimaricin – 1% stock in 70% ethanol.

Preparation:

Cool down the autoclaved rye B medium to 45°C, add 3 ml of the stock rifamycin (final concentration 30 µg × ml^{-1}) and 1 ml of the stock pimarycin (final concentration 10 µg × ml^{-1}).

ISOLATION OF *P. INFESTANS* CULTURES

The choice of material is essential for successful isolation of *P. infestans* cultures. Typical symptoms include shapeless dark brown/black spots, gradually expanding and first appearing on lower shaded leaves. A single rounded lesion usually develops from a single sporangium. Under conditions of high humidity, on the border between healthy and diseased tissue, a white mycelium can be visible on the lower side of the leaf. On stems, the mycelium is maintained even during periods of dry weather (Kapsa, 2001). On the surface of potato tubers, the large grey and later brown spots are symptomatic. Under these spots rusty brown discoloration of the tuber flesh is visible.

PROCEDURE:

1) Place fragments of leaves or stems of the potato with single lesions caused by *P. infestans* into the tubers of cultivars susceptible to late blight (e.g. Bintje, Irga, Irys) cut in halves and fastened back together with a rubber band.
2) Incubate at 16°C for 4-9 days. Isolation from infected tubers can be performed either directly or through a susceptible tuber.
3) After the appearance of typical symptoms of the disease cut out 0.5-0.8 cm cubes with a sterile scalpel from the border between healthy and infected by *P. infestans* tuber flesh.
4) Disinfect in 70% ethanol for 15-20 s and then de-aerate in 0.1% sublimate of $HgCl_2$ for 30-45 s. Alternatively, the cubes can be disinfected in 1% sodium hypochlorite for 180 s.
5) Rinse the cubes three times with sterile distilled water.
6) Place the cubes on the rye B medium containing antibiotics and maintain in dark at 16°C for 10 days.
7) Observe the colonies with a microscope at e.g. 400× magnification. Sporangia of *P. infestans* are in the shape of a lemon with the dimensions of 21-38 per 12-23 µm (Hartman and Huang, 1995) (Photo. 1).

The cells of *P. infestans* hyphae have many nuclei without transverse walls (Harder, 1967).

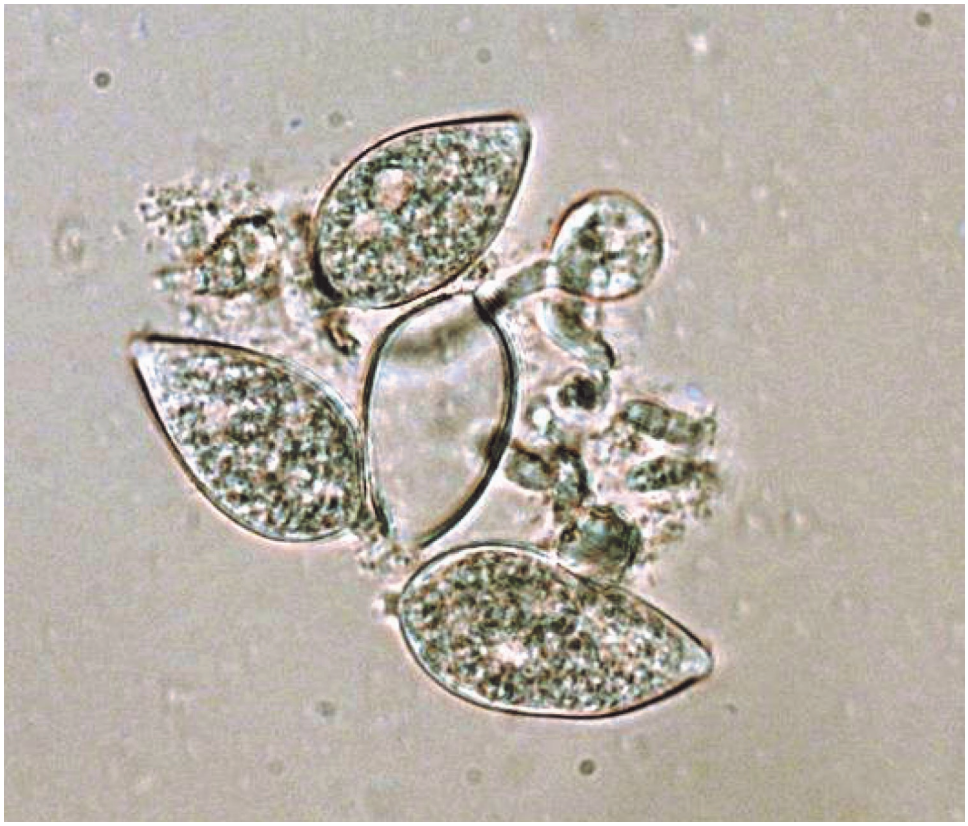

Photo 1. Sporangia of *Phytophthora infestans* – 400× magnification. An empty sporangium in the centre has released zoospores and is surrounded by three sporangia full of cytoplasm. A germinating cyst is visible to the right of the empty sporangium (photographed by E. Stefańczyk).

Storage of P. infestans isolates on agar slopes under paraffin oil or water

1) Grow the *P. infestans* cultures for two weeks on rye B agar slants.
2) Flood with sterile paraffin oil or water and store at 4-7°C.
3) Passage to a fresh medium at least once every three years.

Storage of P. infestans isolates in liquid nitrogen

1) Cut out medium discs covered with mycelium from 10-14-day-old *P. infestans* cultures on rye B medium in Petri dishes and place into 1.5 ml cryovials. Sterile cork-borers or 1 ml pipette tips can be used to cut discs of ca. 7 mm diameter.

2) Fill the cryovials with samples with a sterile 15% solution of dimethyl sulfoxide (DMSO) until the samples are immersed.
3) Lower the vials' temperature gradually -1°C/minute for at least 4 h at -70°C using a device such as NALGENE® Cryo Freezing Container or CoolCell™ LX Freezing Container. The latter one is an alcohol-free foam container while the first one requires use of propanol.
4) Transfer the frozen samples into liquid nitrogen (-196°C). DMSO has a toxic effect on the *P. infestans*, so when out of the liquid nitrogen, the samples should be quickly thawed and rinsed carefully and thoroughly with sterile distilled water.

Inoculum preparation

1) Place the mycelium of the *P. infestans* isolate developed on the medium between two 1 cm slices of potato tubers of a late blight susceptible cultivar. The two tuber slices are not completely cut off from each other, ca. 20% of the surface is left uncut to ensure close contact of the slices and humid environment for the development of the pathogen proliferation.
2) Incubate for a week at 16°C and high relative air humidity (80-100%) until thick mycelium appears on the upper surface of the top slice.
3) Collect the sporangia from the mycelium with a brush and wash to deionized water. Adjust the inoculum to the appropriate concentration using a hemocytometer, e.g. Thom's one. A standard concentration of inoculum used in our tests is 50 sporangia μl^{-1}.
4) Place the inoculum for 2 h at 7°C, and then for 0.5 h at room temperature, in order to increase liberation of zoospores from sporangia. While being used, the inoculum is to be constantly and gently mixed to prevent sporangia sedimentation and concentration of zoospores that show negative geotropism, on the solution surface.

Phytophthora infestans maintained on the artificial medium loses aggressiveness and virulence. Therefore, before assessing potato resistance to late blight or the isolate's virulence, the isolate should be passaged onto potato tissues (slices or leaves) at least two or three times (Zarzycka; 1996, Andrivon *et al.*, 2011).

ACKNOWLEDGMENTS

This work was financed by the Polish Ministry of Agriculture and Rural Development, Multiannual program "Creating the scientific basis for biological progress and the protection of plant genetic resources as source of innovation and support for sustainable agriculture and food security of the country" Task 3.1 Monitoring of changes in populations of harmful and quarantine organisms for potato.

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