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CROP PROTECTION

A simple, fast and cheap isolation and multiplication technique of *Phytophthora infestans*

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ABSTRACT: In this study, we present a method for isolation and propagation of *Phytophthora infestans*, a challenging-to-isolate phytopathogenic oomycete. The procedure initiated with naturally infected potato leaves, which underwent a 30-minute sanitization under running water. Subsequently, healthy potato tubers were meticulously washed with a neutral detergent, sterilized using alcohol, and then flamed. Slices 4 mm thick were carefully cut out of the potato tubers. Slices were used to cover 2 x 4 mm sterilized leaf pieces cut from the border of two-day old young *P. infestans* lesions, within sterilized empty Petri dishes. The sealed plates were then transferred to BOD growth chambers set at 18 ºC in complete darkness for 5 days. At the end of this incubation period, the development of a sparse, white mycelium was observed on top of the potato slices. Using a Drigalski loop, the visible mycelium was carefully transferred to rye agar medium in Petri dishes. To avoid contamination, care was taken not no touch potato slices. After mycelium reaching the Petri dish border, and with aid of a Neubauer chamber, the inoculum density per plate was of 1.79 x 10^4 sporangia mL^{-1}. Sporangia germination rate ranged from 69 to 78%. This isolation technique simplify *in vitro* production of *P. infestans*, enhancing the possibility for research with this important pathogen.
Key words: hemibiotrophic, late blight, massive inoculum production.

Técnica simples, rápida e barata de isolamento e multiplicação de *Phytophthora infestans*

RESUMO: Neste estudo, apresentamos técnica de isolamento e multiplicação de *Phytophthora infestans*, oomiceto fitopatogênico de isolamento desafiador. O procedimento teve início com folhas de batata naturalmente infectadas, as quais foram higienizadas em água corrente por 30 minutos. Posteriormente, tubérculos de batata saudáveis foram lavados com detergente neutro, esterilizados em álcool e flambados. Em seguida, foram cortadas rodelas de 4 mm do tubérculo. As rodelas foram usadas para cobrir pedaços de 2 x 4 mm da borda de lesões foliares de *P. infestans* com dois dias, em placas de Petri vazias e esterilizadas. As placas foram seladas e transferidas para câmaras de crescimento do tipo BOD, mantidas a 18 ºC no escuro por cinco dias. Após esse período, observou-se o crescimento de micélio ralo de cor branca sobre as rodelas de batata. Utilizando alça de Drigalski, o micélio visível foi transferido para meio de centeio e ágar. Após o preenchimento da placa por *P. infestans*, estimou-se com câmara de Neubauer a concentração de 1,79 x 10^4 esporângios mL^{-1}, com taxa de germinação variando entre 69 e 78%. Essa técnica de isolamento simplifica o processo de obtenção *in vitro* de *P. infestans*, contribuindo significativamente para pesquisas relacionadas a essa importante doença.
Palavras-chave: hemibiotrófico, produção massiva de inóculo, requeima.

The oomycete *Phytophthora infestans* (Mont.) de Bary stands as the causative agent of potato late blight, a globally pervasive affliction on the crop (DONG & ZHOU, 2022), particularly pronounced in southern Brazil (CASA-COILA et al., 2020). Historically, late blight gained notoriety for its indirect role in causing widespread fatalities and societal upheaval in Ireland during the mid-nineteenth century when the staple food, potatoes, succumbed to the disease, leading to a significant population decline (ZADOKS, 2008). Today, late blight remains a persistent challenge for potato cultivators worldwide (AL HARETHI et al., 2023).

Oomycetes, though morphologically resembling true fungi, trace their origins to marine environments, with parasitism representing a pivotal evolutionary adaptation (MENDOZA & VILELA, 2013). These eukaryotic organisms, equipped with flagella, are currently classified within the Straminopila kingdom alongside algae (BEAKES et al., 2012). In natural agricultural settings, *P. infestans* behaves as a hemibiotrophic pathogen, existing solely within living plant tissue (FRY, 2008). Initially, it engages in a biotrophic phase, colonizing living plants before transitioning to necrotrophy, leading to cell death (STEVENSON, 2001). Upon

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penetrating the leaf cuticle, the mycelium infiltrates cells, forming haustoria in mesophyll cells (MEIJER et al., 2019). Asexual reproduction structures, namely sporangiophores, proliferate in infected tissue, producing up to 300,000 sporangia per lesion (FRY, 2008).

Isolating a pathogen serves the purpose of obtaining a pure culture for a range of studies, including morphology, taxonomy, fungicide resistance tests, inoculations, physiology, and reproduction (AKINO et al., 2014). Traditionally, two primary techniques for isolating phytopathogenic fungi are direct and indirect isolation. Direct isolation involves gently scraping pathogenic structures, such as sporangia or hyphae, from the infected tissue onto a Petri dish with a suitable culture medium (IVORS, 2015). In cases where the tissue lacks active sporulation or visible mycelium, it is placed in a damp chamber to induce the formation of reproductive structures before being transferred to the culture medium (GAMBOA et al., 2019). However, direct isolation of *P. infestans* proves challenging due to its sensitivity to leaf disinfecting agents, resulting in plate contamination and the growth of other microorganisms in the culture medium.

Conversely, indirect isolation requires more effort and involves cutting small fragments (approximately 4 mm) of leaf tissue between infected and healthy areas. These fragments undergo sanitation with sodium hypochloride, alcohol, and autoclaved distilled water before being transferred to a culture medium (GAMBOA et al., 2019). However, *P. infestans* is very sensitive to this kind of disinfectants, being usually killed during this procedure, and rendering growth of other microorganisms on the culture media.

The choice of culture medium is crucial, as it not only provides nutrients for the pathogen but must also meet specific pH, osmotic pressure, humidity, temperature, and atmospheric conditions conducive to pathogen development (SOBKOWIAK & ŚLIWKA, 2017). Variations in culture media depend on the targeted pathogen, encompassing diverse formulas, liquid or solid states, synthetic or natural compositions, and container types such as test tubes or plates for pathogen repotting (TAKOOREE et al., 2022).

Described as a quasi-obligate or ecologically obligate parasite, *P. infestans* relies on living plant parts for sustenance (O'CONNELL & PANSTRUGA, 2006). This characteristic poses challenges for *in vitro* and *in vivo* testing and research. Despite the significance of *P. infestans*, scant information is available in the literature regarding isolation techniques under tropical and subtropical conditions (GÓMEZ-GONZÁLEZ et al., 2020).

This study disseminated a technique for the isolation and *in vitro* multiplication of *P. infestans*.

The presented technique was tested in the laboratory, greenhouse and field conditions, at Unicentro, between December 2020 and January 2021, utilizing plants exhibiting initial symptoms (circa 2-day old lesions) of late blight. These plants were gathered in the Entre Rios district, municipality of Guarapuava-PR, Brazil (25°23' S and 51°29' W), from an organic potato crop of the susceptible Agata cultivar.

Autoclaved instruments (20 min, 120 °C), including knives, 70 mm Petri dishes, 600 mL beakers, tweezers, and scalpels, were employed in the procedure. Late blight symptomatic leaves were isolated, placed within gauze covered beakers, and subjected to a preliminary cleaning and disinfestation under running tap water for 30 minutes (Figure 1A). This initial step aimed to facilitate coarse disinfestation, crucial for potato leaves with *P. infestans* inoculum, given the organism's sensitivity to conventional disinfectants like sodium hypochlorite. The washed leaves were then cut into 2 x 4 mm pieces from young lesion edge, and three to four fragments were positioned within sterilized plates.

Large commercial potato tubers, free from visible skin lesions, were thoroughly washed with a neutral detergent and carefully dried using paper towels. Subsequently, the tubers underwent treatment with 96% alcohol, and using tweezers, they were gently flamed for a duration of approximately 3 seconds. Slices, approximately 4 mm thick, were cut from the tubers with an autoclaved saw knife (Figure 1B). These potato slices were used to cover the leaf pieces inside the plates (Figure 1C), with the process continuing until all plates were completed (Figure 1D). The plates were sealed with plastic film to ensure high humidity and stored in a BOD growth chamber at 18 °C in the dark, providing optimal conditions for *P. infestans* development.

After approximately five days, *P. infestans* colonized the potato tissue, which turned into a brownish color, like that of naturally infected potato tubers. White sparse mycelium began growing on the tuber slices (Figure 1E), and under a stereoscopic microscope, shiny specks on the mycelium surface, identified as sporangia, were observed.

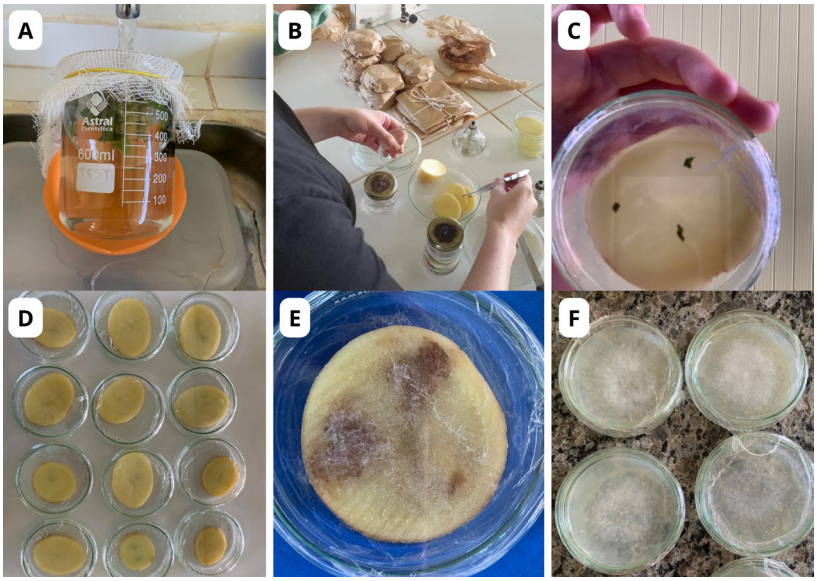

Figure 1 - Isolation procedure of *Phytophthora infestans*. Infected leaves being sanitized under continuous flow of running water (A); potato tuber slices cut, externally disinfected (B); disinfested diseased leaf pieces ready to be covered with tuber slices for incubation (C); plates ready to go to growth chamber (D); *P. infestans* mycelium invading (brown color) the tuber tissue and growing out on top (E); mycelial growth on plates with rye agar medium (F). Guarapuava, Unicentro, 2021.

Despite attempts with other isolation methods, the direct approach yielded no *P. infestans* growth, resulting in the proliferation of opportunistic microorganisms. In contrast, the indirect method, described in this study, was successful.

The most commonly utilized culture media for *P. infestans*, as extensively documented in the literature, include V-8 or tomato juice, rye medium, rye agar medium, and pea broth (TUMWINE et al., 2000). Notably, the V-8 culture medium finds widespread application in various *in vitro* methodologies and techniques for *P. infestans* research (AL HARETHI et al., 2023). FRY LAB (2014) advocates the use of two culture media: one incorporating 10% uncleared V-8 juice, and another employing rye agar medium. The rye agar medium is widely recognized for its efficacy in sustaining and propagating the oomycete (BERMEJO et al., 2020). In this study, the choice of the rye agar medium was influenced by its cost-effectiveness when compared to V-8. Additionally, the medium was subjected to modification through the incorporation of rye flour to enhance its suitability for our specific research purposes.

Mycelium from 5-day-old tuber slices was carefully scraped off onto plates containing rye agar medium, using either a needle or Drigalski loop. The collected mycelium was then gently deposited at the center of each plate. While scraping off the mycelium, extremely care was taken to avoid contact with tuber slices to prevent contamination. Mycelia of the oomycete is characterized by its initial sparse and weak growth, appearing white in color (Figure 1F). For optimal multiplication, it is advisable to pick segments of mycelia from the border of the colony where high metabolic activity occurs. This area is the youngest and most sporulating part of the plate (GAMBOA et al., 2019).

Over the course of 8 days, the mycelium of *P. infestans* exhibited vigorous growth, completely filling the plate. The quantification of sporulation was conducted on 8-day-old mycelial growth plates. The plates were treated with 5 mL of distilled and autoclaved water, and a Drigalski loop was used to smear over the mycelium on the plate for the release of sporangia. This sporangial suspension was filtered through gauze, and the number of sporangia was quantified using a Neubauer chamber. Sporangial germination followed the methodology adopted by GARCIA et

al. (2018) with some modifications. This method involved the preparation of a standard suspension of $1x10^4$ sporangia mL^{-1}. Aliquots of 40 µL of this suspension were placed on ELISA plates kept in a growth chamber at 18 ºC in the dark for 24 hours. After this period, lactophenol blue dye was added to halt germination, and the germination of 100 sporangia was counted using an optical microscope. The pathogen sporulation reached a concentration of 1.79 x 10^4 sporangia mL^{-1}, with a germination rate of 73.1% on average among the total sporangia produced (n = 8). It is noteworthy that the observed germination percentage was slightly lower than the findings reported by DORN et al. (2007), who documented an 85% germination rate in their control treatment (distilled water).

Small fragments of rye agar medium containing *P. infestans* were positioned at the center of sterilized plates, and hygienized potato slices were then overlaid on these fragments (Figure 2A). After approximately 5 days, colonization of the tuber tissue became evident, forming a conspicuous white mycelial mass (Figure 2B). Subsequently, 5 mL of distilled and autoclaved water were added to each plate and gently rubbed with a Drigalski loop to facilitate the release of sporangia. The resulting liquid was filtered, and the solution was calibrated using a Neubauer chamber, yielding a suspension containing $1x10^4$ sporangia mL^{-1}. This prepared suspension was subsequently employed for inoculations on healthy plants.

To validate the viability of the isolation technique and ensure the production of viable sporangia, a suspension of autoclaved distilled water containing sporangia at a concentration of $1x10^4$ mL^{-1} was prepared. This suspension was meticulously sprayed onto the aerial parts of potato plants of the Agata cultivar grown in greenhouse pots until reaching the runoff point (Figure 3A). Subsequently, the inoculated plants were placed in damp chamber for 12 hours, maintaining a microclimate conducive to late blight development. Two days post-inoculation, the first symptoms began to manifest (Figure 3B), providing conclusive evidence of the viability of the *in vitro* isolates of *P. infestans* as described by the technique (Figure 3C). This successful outcome fulfills Koch's Postulates, further validating the efficacy of the isolation approach.

In conclusion, the described technique offers a simple, cost-effective means of *in vitro P. infestans* isolation without contamination, facilitating subsequent inoculum multiplication for greenhouse and field studies. It is recommended for mass inoculum production.

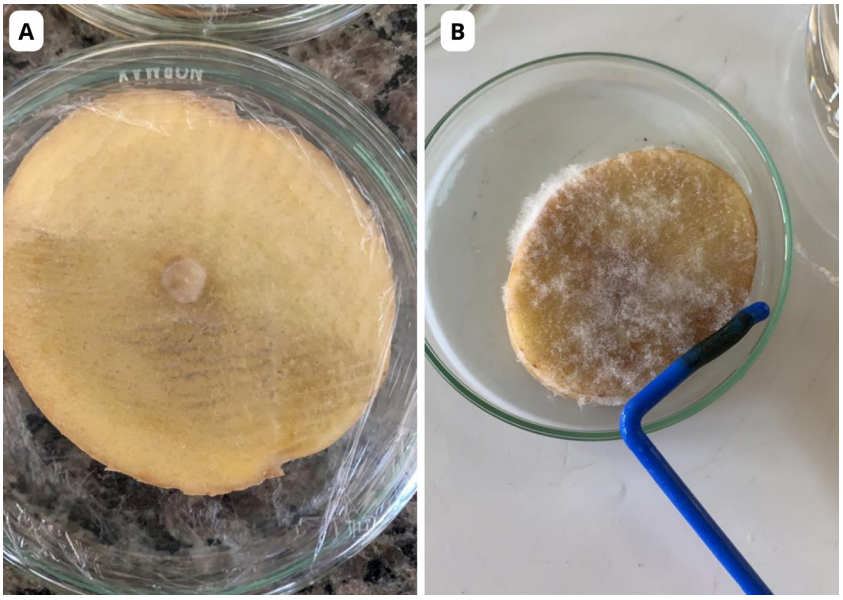

Figure 2 - Potato slice with a plug of culture medium containing mycelium of *Phytophthora infestans* (A); after 5 days of incubation at 18 °C in the dark, the mycelium of *P. infestans* invades the tissue and produces more inoculum (B). Guarapuava, Unicentro, 2021.

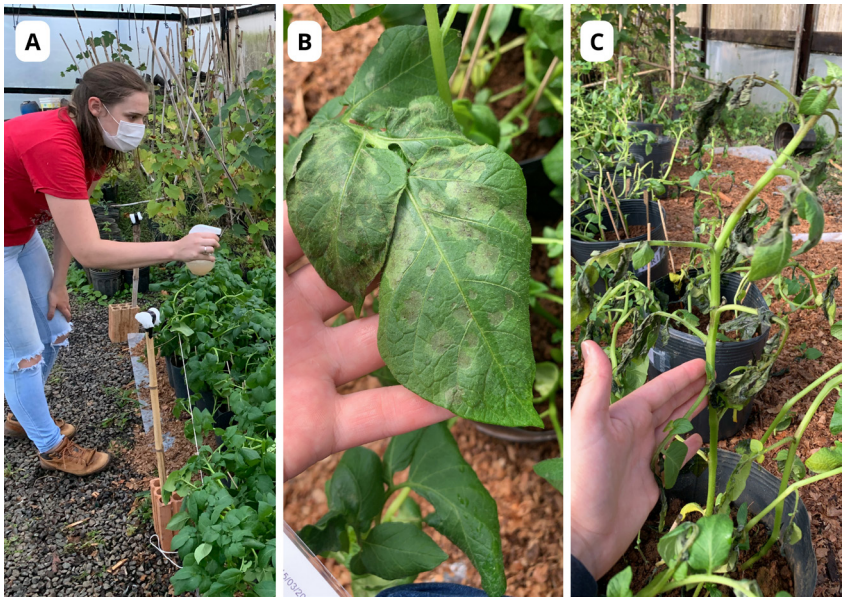

Figure 3 - Inoculation of *Phytophthora infestans* with sporangia suspension (A); the onset of the first symptoms occurred two days after inoculation (B); five days after inoculation, observable symptoms manifested (C). Guarapuava, Unicentro, 2021.

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DECLARATION OF CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS' CONTRIBUTION

All authors contributed equally to the conception and writing of the manuscript. All authors critically revised the manuscript and approved of the final version.

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