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Selective Isolation and Characterization of *Phytophthora infestans* from Potatoes Using Rye Agar Media in India

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Phytophthora infestans is a pathogen that causes late blight, a major disease of potatoes. The isolation of P. infestans from infected potato plants using agar media has been challenging. This study investigated the use of Rye A and Rye B agar media for the isolation of *P. infestans* from infected potato tubers collected from the Nilgiris district of Tamil Nadu during 2022. The media were evaluated for hyphal growth, sporangial production, oospore formation, and long-term storage of *P. infestans.* Phenotypic diagnosis based on cultural and morphological characteristics confirmed the identity of *P. infestans*. The results were confirmed by a molecular identification test using primers specific to *P. infestans*. Pathogenicity tests were carried out to assess the virulence of the isolates. This study provides a useful protocol for the selective isolation and characterization of *P. infestans*, the potato late blight pathogen.

Keywords: Phytophthora infestans; late blight disease; rye agar; potato; isolation.

1. INTRODUCTION

The oomycetes, *Phytophthora infestans* which causes potato late blight remains a major problem in India despite the widespread use of many strategies for its control [1]. Small, water-soaked spots and sometimes a V-shaped appearance were seen along the leaf margins when late blight was present. White mycelial growth was observed on the underside of leaves under moist and humid conditions; the spots can spread rapidly under ideal conditions [2]. High sporulation and the ability to spread by wind and water are critical for disease survival. For *P. infestans* to thrive throughout its life cycle, an environment with temperatures between 15°C and 25°C and relative humidity above 90% is ideal [3]. In subtropical and temperate climates, the presence of favorable environmental conditions or poor management practices can allow the disease to spread and cause total crop losses of up to 100% [4]. All stages of potato development are susceptible to *P. infestans* [5]. In addition to planting resistant or tolerant potato clones, culturing *P. infestans*, determining their growth rates, virulence of the isolates through pathogenicity test, and other characteristics of *P. infestans* are useful in controlling this disease in the field. *P. infestans* is notoriously difficult to culture in standard media [6]. Fungal growth, sporulation, and long-term preservation have all been supported by the development of several semi-synthetic and/or organic media [7]. Soybean and carrot [8], field corn [9], bean meal [10], chickpea, oat meal [11], cereal grains and V8 juice [6], and agar are just a few of the many substrates that have been used.

The effects of various media on mycelial growth, sporangial and oospore production, and long-term survival of *P. infestans* were compared with rye agar. Rye agar has been used as excellent media for mycelial growth [12]. Rye A agar supplemented with Beta-sitosterol significantly stimulated sporangial and oospore production in comparison to clarified V8 juice and carrot agar [6]. In rye-based media, *P. infestans* was shown to survive for almost a year [6]. High amounts of sporangia were produced when grown on ground rye agar [13,14]. Anand *et al*. [15] and others report that V8 is the most commonly used medium for culturing *P. infestans*. V8 agar medium is an essential component, but is difficult to obtain and somewhat expensive. While all *Phytophthora* species grow well on oatmeal agar, the medium is dense and opaque [16]. Most *Phytophthora* strains are unable to produce sporangia or develop oospores on potato dextrose agar (PDA) [17,18]. In a preliminary screen [6], the components of locally accessible rye agar media for *P. infestans* culture were investigated. The current study set out to learn more about *P. infestans* by isolating it from infected potato tubers using rye agar media and then studying its growth, sporangia, and oospore production, as well as confirming its identity by PCR amplification and identifying a virulent isolate through a pathogenicity assay.

2. MATERIALS AND METHODS

2.1 Pathogen Source

During the late blight season of 2022, multiple locations in the Nilgiris district were visited to collect diseased potato samples. Leaves and tubers exhibiting recognizable symptoms were collected from the field [10], labelled, and transported to the laboratory for isolation and characterization. To confirm the mating type of *P. infestans*, a culture of the A1 mating type (BDN22-2) was acquired from the ICAR-Central Potato Research Institute (CPRI) in Shimla (HP).

2.2 Media Preparation for *P. infestans* Isolation

Rye A and B agar were selected for their ability to support the growth and storage of *P. infestans*, while being somewhat impermeable to environmental contaminants.

2.2.1 Rye A media

Sixty-five grams of rye grains were soaked in distilled water and fermented for 38 hours. The fermentation water was strained using muslin and transferred to a new flask.The swollen grains were blended for approximately 2 minutes (distilled water can be added) and incubated for 3 hours at 50°C in distilled water.The hot water strained from the blended grains was mixed with water previously used to soak the grains. The volume was then brought to 1 liter with distilled water, 18 grams of sugar, 65 milligrams of pentachloronitrobenzene, and 0.05 grams of ß-sitosterol.Agar was added to the solution and boiled for 3-5 minutes, or until the agar dissolved properly. The solution was then autoclaved for 15-20 minutes at 121°C and 15 psi.Antibiotics (vancomycin at 90 mg/l, polymyxin B at 55 mg/l, ampicillin at 210 mg/l, and rifampicin at 30 mg/l) and water were mixed and added to the medium via pipette immediately before pouring the vials.For long-term storage, P. infestans isolates TNAUPI-1, TNAUPI-2, TNAUPI-3, and TNAUPI-4 were selected [10]. Glass vials containing 5 ml of Rye A agar medium were prepared.An agar plug of the isolate was obtained from the colony margin after 14 days of incubation and transferred to vials of the medium. The vials were maintained in the dark at18°C.At one-month intervals, the fungus was transferred from the vial to Petri dishes containing RB agar to determine its viability (colonial growth) and sporangial production, with five replications per month.

2.2.2 Rye B media

Sixty-five grams of rye grains were soaked in distilled water for 38 hours and fermented. The fermentation water was strained using muslin and transferred to a new flask. The swollen grains were then incubated for 3 hours at 50°C in distilled water. The hot water strained from the incubated grains was mixed with water previously used to soak the grains. The volume was then brought to 1 liter with distilled water, 18 grams of sugar, and 65 milligrams of pentachloronitrobenzene [8]. Agar was added to the solution and boiled for 3-5 minutes, or until the agar dissolved properly. The solution was then autoclaved for 15-20 minutes at 121°C and 15 psi. Antibiotics (vancomycin at 90 mg/l, polymyxin B at 55 mg/l, ampicillin at 210 mg/l, and rifampicin at 30 mg/l) and water were mixed and added to the medium via pipette immediately before pouring the vials.

2.2.3 Pea broth

Pea seeds (130 g) were boiled for 20-30 minutes, or until the peas reached the meshing level. The pea water was then strained using muslin cloth. Sufficient water was added to make up the volume to 1000 mL. The solution was then autoclaved for 15-20 minutes at 121°C and 15 psi [19]. After autoclaving, a two- to three-centimeter mycelial bit was added to the broth and allowed to grow for 10 days before being used for molecular analysis.

2.3 Isolation of *P. infestans* from Infected Tubers

Infected potato tubers collected from Nilgiris were washed under running water to remove dirt and debris. Then, a sterile knife was used to cut 0.5-0.8 cm cubes of tuber flesh from the dividing line between healthy and infected tissue (60% healthy tissue:40% infected tissue). The cubes were soaked in 1% NaOH for two to three minutes, and then rinsed three times with distilled water [20]. After washing the cubes with water, they were aerated by placing them on sterile tissue paper. The cubes containing antibiotic-supplemented rye B medium were stored in the dark at 18°C in an incubator for 10 days.

2.4 Sporangial Production

To remove mycelia and other debris, sporangia were rinsed from the top of a sporulation lesion on a potato slice or culture plate with distilled water. The filtrate was then filtered through a 35-micron mesh filter [21]. Sporangia were removed from the filtrate by passing it through a 15-micron mesh filter. They were recovered from the filter by contacting it with distilled water after several rinses with freshwater. To stimulate zoospore discharge, the sporangial suspensions were incubated for two hours at 6°C. After the zoospores were discharged, the solution was filtered again through a 10-micron mesh filter to isolate the zoospores from the sporangia.

2.5 Oospore Production

In vitro oospore production requires at least one known A1 (or) A2 culture plate, as has been well documented [12]. In this study, a culture of the

known mating type (A1) was placed in one corner of a Petri dish, and the test isolate were placed opposite to each other. If an oospore was formed, the mating was opposite; otherwise, it was identical. The culture plate was incubated for 20 days for oospore production.

2.6 Molecular Characterization of *P. infestans*

2.6.1 DNA isolation

Mycelium was harvested from an 8-10 day old P. infestans culture grown in pea broth. It was then air-dried until the mycelia were no longer wet (26). In a mortar, 5 g of mycelia were mixed with 3 ml of 10% CTAB buffer and pounded for approximately 3 minutes. The resulting mixture was placed in a water bath at 65°C with intermittent stirring for 45 minutes, and then cooled to 37 °C for 15 minutes. A mixture of phenol, chloroform, and isoamyl alcohol (25:24:1, v/v) was then added to the mixture. After gentle stirring for 10 minutes at room temperature, the mixture was centrifuged at 12,000 rpm for 10 minutes. The clear upper layer was removed by gradually inverting the tube, and the remaining liquid was mixed with 650 µL of isopropanol. The nucleic acids were extracted by centrifuging the samples at 12,000 rpm for 10 minutes. The nucleic acid pellets were stored at -20 °C overnight.The following day, the tubes were centrifuged at 12,000 rpm for 10 minutes. The supernatant was discarded, and 300 µL of ethanol was added to the pellet. The ethanol was then centrifuged at 12,000 rpm for 10 minutes. The ethanol was discarded, and the pellet were dried until no more ethanol remained. The resulting nucleic acid pellet were dissolved in 30 µL of milli-Q water. The concentration of extracted DNA was determined using a NanoDrop 2000 UV-vis spectrophotometer.

2.6.2 PCR assay

The total volume of each reaction mixture was 40 µL. It consisted of 20 µL of TaqPCR MasterMix, 1 µL of each primer (PINF:5’ -TCGCTACAAT AGGAGGGTC3’ and ITS5:5’GGA AGTAAA AGTCGTAACAAGG3’), 1 µL of template DNA, and sterilized double-distilled water [18]. All reactions were performed in a thermocycler, which was set for an initial denaturation step at 96°C for 2 minutes, followed by 35 cycles of denaturation at 96°C for 1 minute, annealing for 1 minute at 55°C, extension for 1 minute at 72°C, and a final extension temperature of 72°C for 10 minutes.

The PCR products were subjected to agarose gel electrophoresis (1%). A 100 bp DNA molecular ladder was used to estimate the size of the amplicon (580 bp). After electrophoretic separation, the gel was read using a Gel Doc 2000 Bio-Rad system. The amplified PCR products were sequenced at M/s BioServe Biotech-nologies (India) Pvt. Ltd, Hyderabad, for double-pass DNA sequencing using the *P. infestans*-specific primers described above. The BLAST program was used to identify the related sequences available from the NCBI GenBank database.

2.7 Pathogenicity Assay

The pathogenicity of the isolates was tested in vitro. Potato leaves were detached from 6-week-old plants grown at ICAR-CPRS, Ooty. They were washed with distilled water for 10 minutes and air-dried to remove moisture. A plug of mycelial bits was cut with a cork borer and placed in the middle of the leaves using a needle [22]. The inoculated leaflets were then placed adaxially side up in Petri plates at 18±2°C in the dark for 4 days with 14 hours of light and 10 hours of darkness, respectively, to assess pathogenicity.

3. RESULTS AND DISCUSSION

In total, fifty late blight-infected potato samples were collected from different locations in the Nilgiris district, and the disease incidence ranged from 20.0 to 48.0% across the surveyed areas (Table 1). The primary objective of this research was to examine the substrates that are essential for the successful isolation of *P*. *infestans*. Rye grain and V8 juice were used as a standard growing medium for this Oomycetes-like fungus. For the most part, V8 agar media is either unavailable or prohibitively expensive [23]. Researchers found that the pathogen *P. infestans* grew well in rye grain culture media and produced numerous sporangia [6]. Rye A agar was successfully employed as a sporulation medium in a study by [19,22]. In this study, we tried rye agar media for this purpose. Cubed potatoes infected with *P. infestans* were placed in Petri dishes with 20 ml of rye B media and then incubated at 18°C in the dark. Mycelial threads, which began to emerge on day 4 of incubation (Fig. 1) were morphologically confirmed to be *P. infestans* after being evaluated visually on day 12.

Table 1. Isolation of *P. infestans* from late blight-infected potato leaves

S. No.	Isolate code	Place of collection	Latitude/ Longitude	Disease incidence (%)	NCBI Accession number
1	TNAUPI 1	Nanjanad, The Nilgiris	11.366948, 76.64591	30.0-42.0	OR145849
2	TNAUPI 2	Ithalar, The Nilgiris	11.373863, 76.658017	25.0-38.0	OR253053
3	TNAUPI 3	Kagguchi, The Nilgiris	11.325963, 76.633097	29.0-48.0	OR253485
4	TNAUPI 4	Thuneri, The Nilgiris	11.387068, 76.718913	20.0-30.0	OR253590

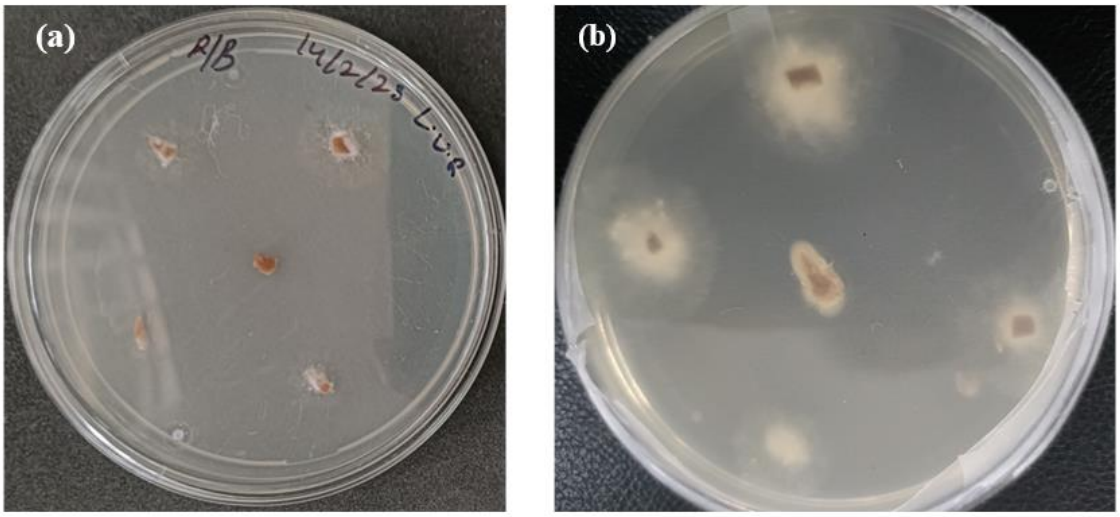

Fig. 1. (a) Mycelial threads that started emerging from 4th day and (b) 12th day of incubation

P. infestans was successfully recovered after 8 months in storage at 18°C from vials containing rye agar A (Table 2). Even after being frozen for 8 months, isolates maintained their ability to produce a high number of sporangia (Table 2). Fungal cultures kept in vials containing rye agar A incubated at 18°C for 1 month produced abundant sporangia. Fungal isolates evaluated after 3 and 5 months of storage showed a high sporangial production compared to those tested after 8 months of storage at 18°C (Table 2). The sporangia were oval, ellipsoid to limoniform, tapered at the base, caducous, and semi-papillate (Fig. 2 a,b), with sizes ranging from 19.259.2-1235.6 m for TNAUPI-1, TNAUPI-2, and TNAUPI-3 to 18.40-14.20.8 m for isolate TNAUPI-3 (Table 3). Their sporangiophores were compound sympodial, with a little bulge directly beneath the sporangium. On rye B agar plates, all four potato-derived *P. infestans* isolates (TNAUPI 1, TNAUPI 2, TNAUPI 3, and TNAUPI 4) examined grew between 10 and 25° C, but not at or above 26° C. Oospore formation by *P. infestans* was also stimulated by the addition of ß-sitosterol to Rye A agar. Rye agar supplemented with ß-sitosterol increased oospore generation as reported [7].

Table 2. Recovery (%) and sporulation of *P. infestans* stored at 18°C on Rye A agar media

Month interval	Mean no. of sporangia/cm^2
1 month	7.3×10^4
3 month	6.4×10^4
5 month	5.0×10^4
8 month	4.4×10^4

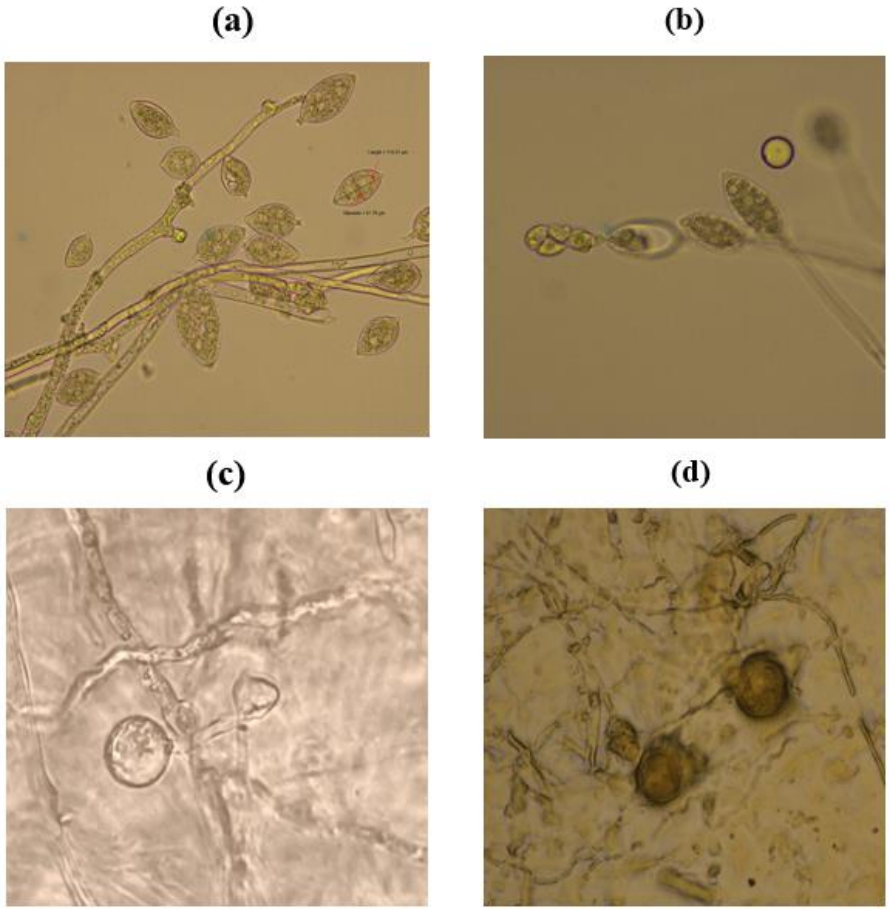

Fig. 2. (a) Sporangiophores and sporangia of *P. infestans*, (b) Sporangia releasing zoospores, (c) Chlamydospore, (d) Oospores

Table 3. Sporangial characteristics of *P. infestans* isolates

Isolate	Size of sporangia (µm)			Length of pedicel (um)
	Length	Width	Length/width	
TNAUPI 1	24.8-59.2 (38.2)	16.0-35.4 (24.5)	1.21-2.16(1.56)	2.0-6.7 (3.56)
TNAUPI 2	25.9-48.0 (36.0)	16.0-26.0 (20.6)	1.31-2.30 (1.76)	0.8-4.8 (3.10)
TNAUPI 3	19.8-52.0 (31.6)	12.0-24.0 (19.8)	1.10-2.50 (1.60)	1.2-4.6 (3.25)
TNAUPI 4	18.0-41.0 (28.0)	14.0-20.8 (18.1)	1.22-2.0 (1.54)	2.0-4.0 (2.89)

In a mating test with BDN22-2 isolate, all four *P. infestans* isolates collected from the Nilgiris were able to yield oospores. Isolates TNAUPI 1, TNAUPI 2, TNAUPI 3, and TNAUPI 4 produced oospores, hence they belong to A2 mating type (Fig. 2d). At the same time, chlamydospores were observed on Rye B agar medium in relatively excess humidity (Fig. 2c). For molecular characterization, *P. infestans* grown in pea broth were grounded and followed PCR assay (Fig. 3). The nucleotide sequences obtained from this study showed more than 99% sequence similarity to GenBank sequences *(P. infestans)* deposited with the NCBI. Accession numbers obtained from the NCBI GenBank for each isolate are listed in (Table 1). It has been shown that, depending on the genotype, *P. infestans* cultures can be kept alive for at least a year [17].

Rye A agar was employed for numerous purposes, including long-term storage, maintaining viability and asexual reproduction for 8 months, and other applications. It was found that this media may safely hold isolates for up to 8 months. Long-term storage media for

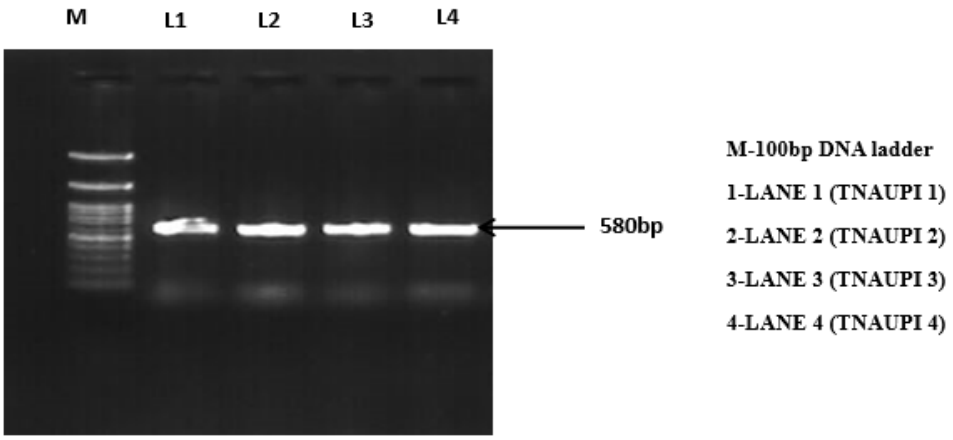

Fig. 3. PCR amplification of *P. infestans*

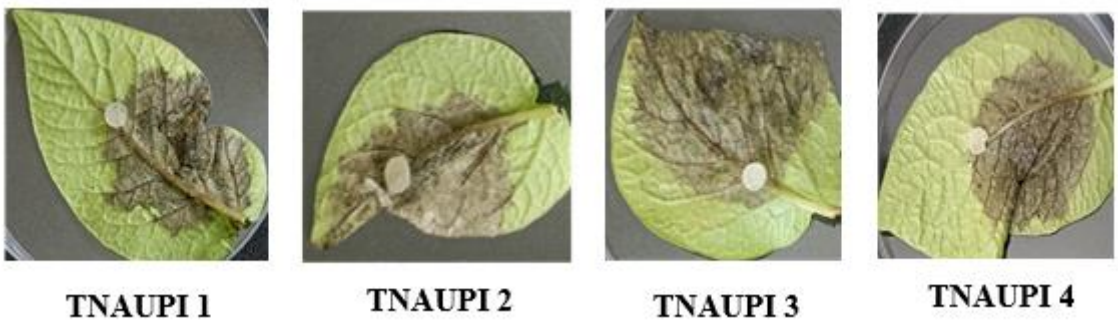

Fig. 4. Pathogenicity of *P. infestans*

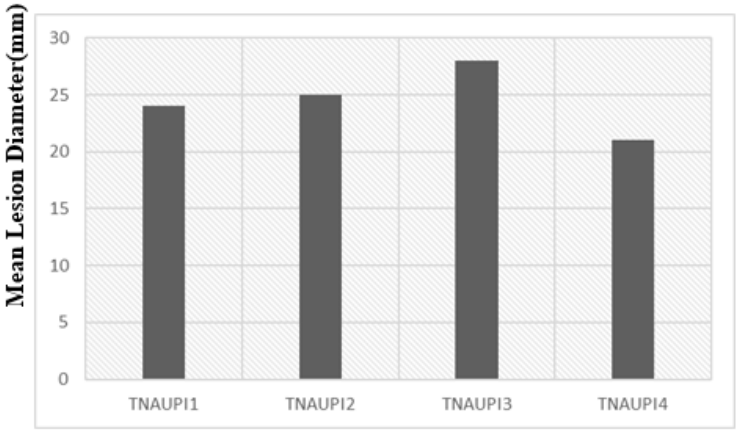

Fig. 5. Pathogenic ability of *P. infestans* isolates on potato leaves (Detached leaf assay)
The level of mean lesion diameter of each isolate is rated on a scale

P. infestans have been tested, including agar-based and whole-seed media made from materials such as corn and V8 [16]. Corn and rye-based media were shown to be the most successful at preserving *P. infestans* viability [24-27]. Our results also show that the *P. infestans* isolates can grow and sporulate on agar-based Rye agar medium, which can also be used for long-term storage. The detached leaf assay showed that the isolate TNAUPI 3 was found to

be most virulent with a mean lesion width of 28.2 mm (Fig. 4 and Fig. 5). This was followed by TNAUPI 2 (25.3 mm), TNAUPI 1 (24.1mm), and TNAUPI 4 (21.6 mm). Pathogenicity testing can be used to identify the most virulent isolate [15]. Pathogenicity testing in our study was performed using the detached leaf assay.

4. CONCLUSION

The pathogen *P. infestans* causes widespread damage throughout the tropical and subtropical regions of the world. Successful disease control techniques benefit greatly from the phenotypic identification of pathogens. In this paper, we describe a method for isolating *P. infestans* without exposing it to external contaminants. As a result, insight into the taxonomic behavior of infections through the assessment of morphological traits may contribute to their eventual management.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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