***Original Research Article***

# Selective isolation of *Phytophthora infestans* from potatoes using rye agar media


## ABSTRACT

*Phytophthora infestans* (Mont.) de Bary the pathogen that causes late blight has long been a major problem in many parts of the world where potatoes are grown. The isolation of *P. infestans* from diseased potato plants using agar media has always been a challenge for scientists. Studies were carried out to isolate *P. infestans* from infected potato tubers collected from the Nilgiris district of Tamil Nadu during 2022 using Rye A and Rye B agar media. The media were evaluated for hyphal growth, sporangial production, oospore formation in Rye B media, and long-term storage of *P. infestans in Rye A agar media*. Phenotypic diagnosis based on cultural and morphological characteristics allowed the pathogen to be identified as *P. infestans*. The results of this study 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 will be useful for the selective isolation and characterization of the potato late blight pathogen, *P. infestans*.



***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 [11]. Small, water-soaked spots and sometimes a V-shaped appearance were seen along the leaf margins when late blight was present. White mycelial growth is observed on the underside of leaves under moist and humid conditions; the spots can spread rapidly under ideal conditions [15]. High sporulation and the ability to spread by wind and water are critical for disease survival. For *P. infestans*to thrive through-out its life cycle, an environment with temperatures between 15°C and 25°C and relative humidity above 90% is ideal [10]. In subtropical and temperate climates, the presence of favorable environ-mental conditions or poor management practices can allow the disease to spread and cause total crop losses of up to 100% [5]. All stages of potato development are susceptible to *P. infestans*[1]. 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 [21]. Fungal growth, sporulation, and long-term preservation have all been supported by the development of several semi-synthetic and/or organic media [9]. Soybean and carrot [2], field corn [17], bean meal [19], chickpea, oat meal [12], cereal grains and V8 juice [21], 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.[20]. Rye A agar supplemented with Beta-sitosterol significantly stimulated sporangial and oospore production in comparison to clarified V8 juice and carrot agar [21]. In rye-based media, *P. infestans* was shown to survive for almost a year [21]. High amounts of sporangia were produced when grown on ground rye agar [16,14]. Anand *et al*. (2020) [3] 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 [4]. Most *Phytophthora* strains are unable to produce sporangia or develop oospores on potato dextrose agar (PDA) [23]. In a preliminary screen [21], 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

Field visits were made to several locations in the Nilgiris district to collect samples of diseased potatoes during the late blight season of 2022. Leaves and tubers showing recognizable symptoms were collected from the field [19], labeled, and taken to the laboratory for isolation and characterization. A1 mating type culture (BDN22-2) was obtained from ICAR- Central Potato Research Institute (CPRI), Shimla to confirm the mating types of *P. infestans*.

### 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. Strain the fermentation water using the muslin into a new flask. Blend the swollen grains for approximately 2 min (can add $dH_2O$) and incubate for 3 h at 50°C in distilled water [9]. Mix the hot water strained from the blended grains through muslin cloth with water that was previously used to soak the grains. Make up the volume to a litre with distilled water, and add 18 grams of sugar and 65 milligrams of pentachloronitrobenzene, 0.05g of ß-Sitosterol. Add 18 grams of agar to the solution and boil it for 3-5 minutes, or until the agar has dissolved properly (up to soup consistency). Administer them for 15 to 20 minutes in an autoclave at 121°C at 15 psi. Antibiotics (vancomycin @ 90mg/l, polymyxin B @ 55mg/l, ampicillin @ 210mg/l, and rifampicin @ 30mg/l) and water should be mixed and added to the media via pipette just before pouring it vials. For Long-term storage *P. infestans* isolates TNAUPI-1, TNAUPI-2, TNAUPI-3, and TNAUPI-4 were selected for this study[19]. The 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 at 4° and 18°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, using five replications per month.

#### 2.2.2. Rye B media

Sixty-five grams of rye grains were soaked in distilled water and fermented for 38 hours. Strain the fermentation water using muslin in a new flask. Separate the soaked rye grains into individual conical flasks, and boiling requires the grains to be well-soaked. Then, they are boiled for 15 to 20 min in a microwave pressure cooker. Mix the hot water that has been strained from the grains through muslin cloth with water that was previously used to soak the grains. As Afanasenko et

al. (2022) [2] described, 18 g of sugar and 65 mg of pentachloronitrobenzene were added to a liter of distilled water. To the solution,18 g of agar was added and boiled for 3-5 min or until the agar had dissolved properly (up to soup consistency). Administer them for 15 to 20 minutes in an autoclave at 121°C at 15 psi. Antibiotics (vancomycin @ 90mg/l, polymyxin B @ 55mg/l, ampicillin @ 210mg/l, and rifampicin @ 30mg/l) and water should be mixed and added to the media via pipette just before pouring it onto plates.

#### 2.2.3. Pea broth

Pea seeds of 130 g were boiled for 20-30 minutes, or until the peas reached the meshing level. Pea water should be strained using muslin cloth after boiling. Then, 4 g of sucrose and sufficient water were added to make up the volume to 1000 mL. They were added for 15–20 min in an autoclave at 121°C at 15 psi [24]. Following autoclaving under sterile circumstances, 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 the Nilgiris were washed in running water to remove dirt and debris. Using a sterile knife, 0.5-0.8 cm cubes of tuber flesh were taken 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 before being rinsed three times in distilled water. NaOH was rinsed for at least one to two minutes after each water rinse, as described by [8]. Cubes were aerated by placing them on sterile tissue paper after water washing. 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 mycelium and other debris, sporangia are rinsed from the top of a sporulation lesion on a potato slice or culture plate with distilled water and then filtered through a 35µm mesh filter [13]. Sporangia were removed from the filtrate by passing it through a 15-micron mesh filter. They were recovered from the filter by contact with distilled water after several rinses with fresh water. To stimulate zoospore discharge, 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 that is well documented [20]. In the present study, a culture of the known mating type (A1) was placed in one corner of a Petri dish and our test isolates were placed directly opposite. If an oospore was formed, the mating was sexually opposite, otherwise it was identical. The culture plate was incubated for 20 days for oospore production. The shelf life/ storability of Rye A agar for maintaining *P. infestans* culture was then evaluated for up to 8 months.

### 2.6. Molecular characterization of *P. infestans*

#### 2.6.1. DNA isolation

Mycelium was harvested from 8-10-day-old *P. infestans*culture grown on pea broth. They were then air-dried until the mycelium was no longer wet [25]. In a mortar, 5 g of mycelia was mixed with 3 ml of 10% CTAB buffer and pounded for approximately 3 min. The resulting mixture was heated to 65° C with intermittent stirring for 45 min and then cooled to 37° C for 15 min. A mixture of phenol, chloroform, and isoamyl alcohol 200ul (25:24:1) was added to the mixture. After gentle stirring for 10 min at room temperature, the mixture was centrifuged at 12,000 rpm for another 10 min. After removing the clear upper layer with gradual inversion, mix it well with 650ul of isopropanol. Nucleic

acids were extracted by centrifuging the sample at 12,000 rpm for 10 min. At -20 °C, the nucleic acid pellets were stored overnight. The following day, the tubes were centrifuged at 12,000 rpm for 10 min, the supernatant was discarded, and 300 µL ethanol was added to the pellet. After centrifugation at 12,000 rpm for 10 min, the ethanol was discarded and the particles were dried until no more ethanol remained [25]. The resulting nucleic acid pellets were Dissolved in 30µl milli-Q water. Extracted DNA concentration was checked using a NanoDrop 2000 UV-vis spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

#### 2.6.2. PCR assay

The total volume of each reaction mixture was 40 µl. It was composed of 20 µL of TaqPCRMasterMix, 1 µL of each primer (PINF: 5'CTCGCTACAATAGGAGGGTC3' and ITS5: 5'GGAAGTAAAAGTCGTAACAAGG3'), 1ul of template DNA, and sterilized double-distilled water [25]. All reactions were performed in a Thermocycler (Eppendorf Germany), which was set for an initial denaturation step at 96° C for 2min, followed by 35 cycles of denaturation at 96° C for 1 min, annealing for 1 min at 55° C, and extension for 1 min at 72° C, with a final extension temperature of 72° C for 10 min as described by Lindqvist *et al*. (2020).

The PCR products were subjected to agarose (1%) gel electrophoresis. A 100 bp DNA molecular ladder was used to estimate the size of the amplicon (580 bp). After electrophoretic separation, the gel was read under the Gel Doc 2000 Bio-Rad system (Bio-Rad Laboratories, Hercules, California, USA) for a more detailed analysis. The amplified PCR products were sequenced at M/s BioServe Biotechnologies (India) Pvt. Ltd, Hyderabad for double-pass DNA sequencing using the *P. infestans-specific* primers mentioned above. The BLAST program was used to identify 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 RS, Ooty, washed with distilled water for 10 min, and air dried to remove moisture. A plug of mycelial bits was cut with a cork borer and the mycelial bits were placed in the middle part of the leaves using a needle [19]. The inoculated leaflets were then placed adaxial side up in Petri plates at 18±2° C in the dark for 4 days with 14h light and 10h dark photoperiods, 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 are used as a standard growing medium for this Oomycetes-like fungus. For the most part, V8 agar media is either unavailable or prohibitively expensive [18]. Researchers found that the pathogen *P. infestans* grew well in rye grain culture media and produced numerous sporangia [21]. Rye A agar was successfully employed as a sporulation medium in a study by [24]. 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, | 11.366948, | 30.0-42.0 | OR145849 |

| | | | | | |
|---|---|---|---|---|---|
| | | The Nilgiris | 76.64591 | | |
| 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 |

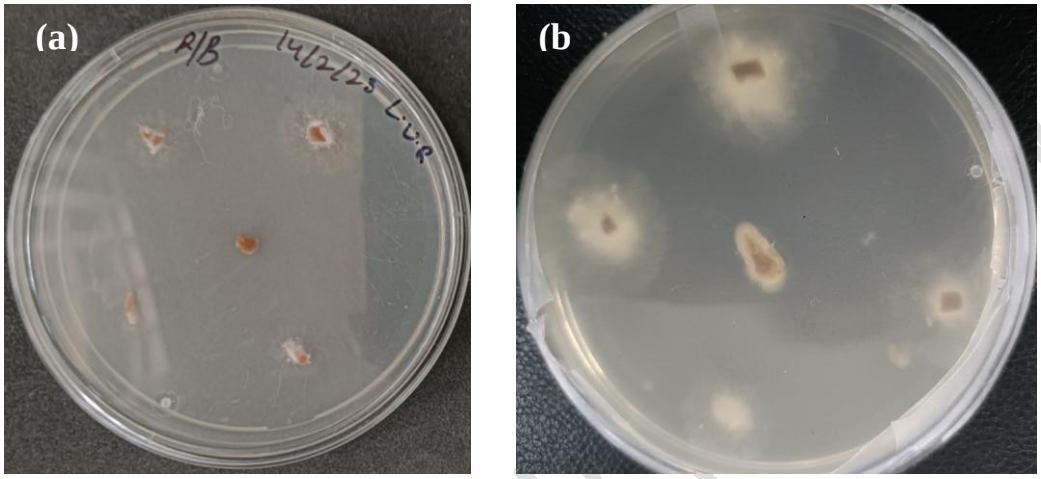


Fig.1. (a) Mycelial threads that started emerging from 4th day and (b) 12th day of incubation

*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 kept 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 the growth medium of Rye A agar. Rye agar supplemented with ß-sitosterol increased oospore generation as reported by [9].



**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 | $7.3\times10^4$ |
| 3 month | $6.4\times10^4$ |

| | |
|---|---|
| 5 month | $5.0\times10^4$ |
| 8 month | $4.4\times10^4$ |

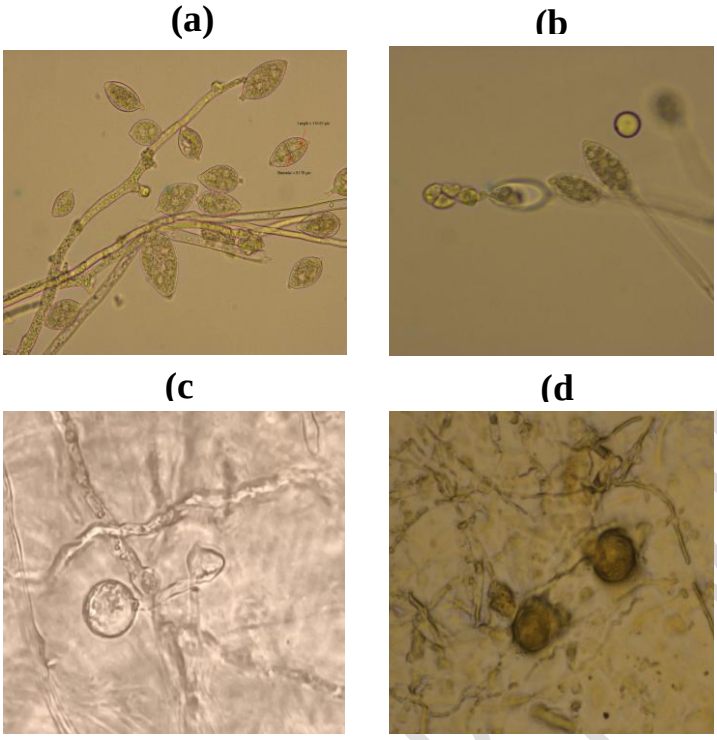


Fig.2. (a) Sporangiophores and sporangia of *P. infestans*, (b) Sporangia releasing zoospores, (c) Chlamydospore, (d) Oospores

**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 can produce oospores, hence they belong to the 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 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 [23].

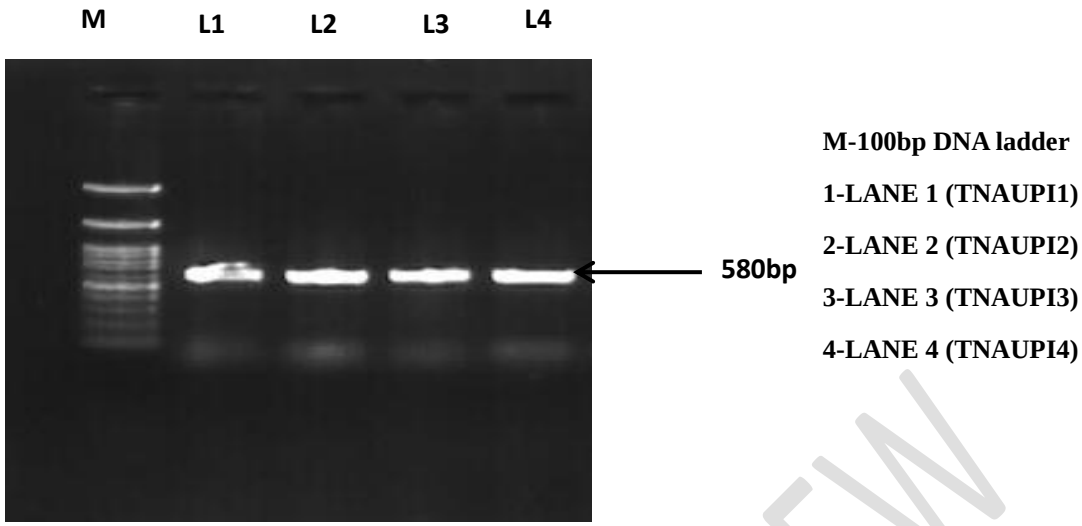


**Fig. 3. PCR amplification of *P. infestans***

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 *P. infestans* have been tested, including agar-based and whole-seed media made from materials such as corn and V8 [4]. Corn and rye-based media were shown to be the most successful at preserving *P. infestans* viability [6]. 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 [3]. Pathogenicity testing in our study was performed using the detached leaf assay.


**Fig.4. Pathogenicity of *P. infestans***

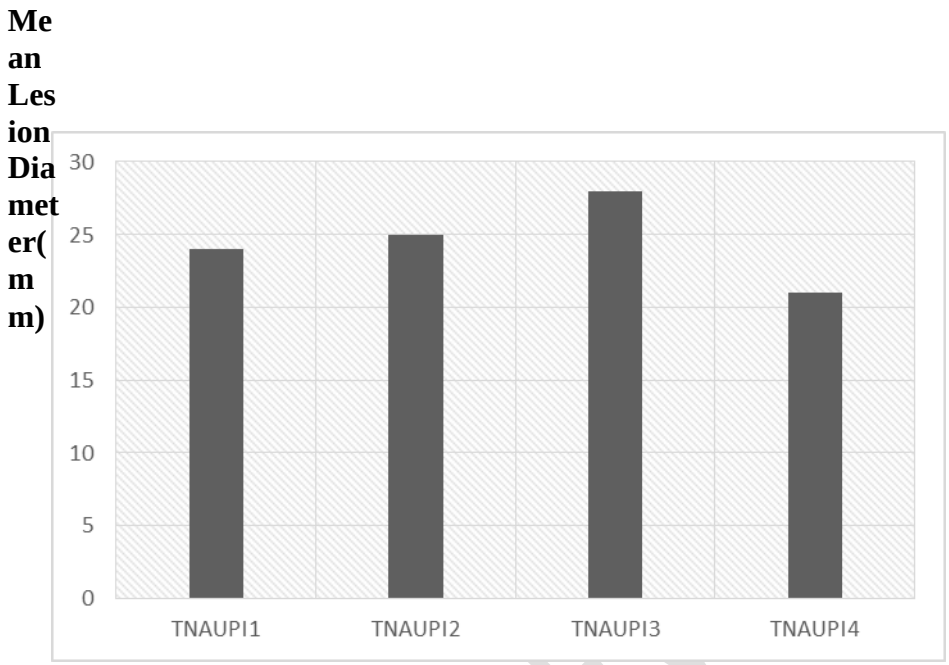


**Fig.5. Pathogenic ability of *P. infestans* isolates on potato leaves (Detached leaf assay)**

The level of mean lesion diameter of each isolate is rated on a scale

## CONCLUSION

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

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