Research Article

Morphological Characterization of *Phytophthora infestans* and its Growth on Different Growth Media

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Abstract | *Phytophthora infestans* is a destructive pathogen in the tropical and the sub-tropical areas of the world. Identification of pathogens is very important to find successful disease control strategies. Here, we isolated and identified *P. infestans*, as well as, determined the morphological characteristics, on different media. The phenotypic characteristic of the isolates were observed as fluffy cottony growth with striated pattern. The average length of sporangia among the isolates collected during 2017 from potato growing areas of Punjab ranged from 14.94 to 47.89 µm, and mean breadth of sporangia varied from 10.44 0 to 23.67 µm. During 2018, among all districts, isolate Jhg-22 showed the highest length (46.64 µm) and breadth (22.17 µm) followed by Shl-25 (46.59 µm) and breadth (22.61 µm), Oka-24 (46.31 x 21.72 µm) while the lowest value measured by Cht-14 (16.05 x 13.39 µm). The best growth and sporulation of *P. infestans* revealed on Rye agar media compared with other media, while the minimus growth was observed on carrot agar media across most isolates. Therefore, the determination of morphological characteristics for plant-pathogen may add knowledge of the taxonomic behavior of pathogen. The usage of various growth media is providing a useful pattern of the growth that can be utilized in determining the best possible control of late blight disease.

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Introduction

Potato (*Solanum tuberosum* L.) is third significant crop followed by rice and wheat and rice which consumed by more than billions of people worldwide (Birch *et al*., 2012; Arora *et al*., 2014; Mahr, 2021). Several factors both of plant pathogens and pests are causing the lower in yield and tuber quality (Kiptoo *et al*., 2021; Mahr, 2021; Nisa *et al*., 2022). The plant pathogen *P. infestans* (Mont.) de Bary causes very interesting disease called potato late blight that is considered most significant disease economically (Cooke

et al., 2011; Zang *et al*., 2017). The *Phytophthora* genus was observed for the first time in 1876, and by 2012 had about 117 described pathogen's species (Martin *et al*., 2012). *P. infestans* was identified as *Botrytis infestans* (Montagne, 1845) previously, and then *Peronospora infestans* (Mont.) Casp. (Caspary, 1879) had distinctive conidiophore characteristics, which he observed diverse enough being assigned as new genus name. Recently, *Phytophthora* spps. were considered among the organisms like fungi group because they have many morphological characteristics same, such as filamentous hyphal growth, asexual reproduction, and nutrition by absorption (Adl *et al*., 2005; Fry, 2008; Jaimasit and Prakob, 2011).

The late blight symptoms are small spots, typical V shaped, circular to irregular water-soaked at margins of leaves. Under most favorable environmental conditions the spots can expand quickly and white mycelia growth is observed during wet and humid conditions on the lower surface of the leaves (Flier *et al*., 2001; Fry, 2008). The survival of pathogen is having the ability to high sporulation and dispersed by wind and water. The epidemic progress is robustly dependent on optimum temperature range between 15-25°C and percentage relative humidity (100%) during pathogen's life cycle (Ho, 2018). Meanwhile, higher temperature, above 30°C, would be responsible to stop the pathogen growth but it can start to sporulate again with respect to favorable conditions (Ribeiro *et al*., 2013). The asexual reproduction of *P. infestans* is by producing sporangia and zoospores while oospores in the sexual reproduction (Tyler, 2002). The exchange of genetic material through three mechanisms might be involved in the phenotypic character of *P. infestans* including sensitivity to fungicide, aggressiveness, ability of sporulation, latent period, morphological change, *etc*.

However, the phenotypic distinction in the population of *P. infestans* needs further investigations in the wide world. Changes in the *P. infestans* population in the early 1980s (Gotoh *et al*., 2005), as well as frequent physiological studies of this pathogen across the world, have been documented by many researchers (Jaimasit and Prakob, 2011). The characterizations of macro-morphology include the variation in colony type and growth rate but micromorphology comprised the change in the size of the sporangium, and zoospores (Bower *et al*., 2007). Micromorphology have been used as a varying factor for *P. infestans* while the investigation of morphological, cultural and physiological variability within pathogen population is the most helpful in understanding host-pathogen interaction, epidemiology and developing strategies for disease management (Bower *et al*., 2007).

Various synthetic media and different substrates include sweet corn, pea seeds, rye seeds, carrot media, bean meal, field corn, oatmeal, chickpea, V8 juice, cereal grains, and lima bean (Caten and Jinks, 1968; Goth, 1981; Peters *et al*., 1998; Medina and Platt, 1999; Sanyong *et al*., 1993). That is helpful in developing the sporulation and long-term storage of *P. infestans* (Erwin and Ribeiro, 1996) because pathogen is hard to isolate and purify on general media. Among all media Rye Agar media is the most frequently available organic substrate based medium (Hartman and Huang, 1995) but it is not commonly available in Pakistan and is precious to import. Knowledge of variability in morphological, cultural parameters of *P. infestans* is still scarce. Therefore, the current study is aimed at exhibiting the morphological characteristics and to determine the growth and sporangia production on different media of the pathogen *P. infestans* which causes potato late blight.

Materials and Methods

The research work was planned in the Departmental laboratory of Plant Pathology, University of Sargodha during 2017-18 to determine morphological characteristics of the pathogen (*P. infestans*) isolates collected from different potato growing areas of the Punjab, Pakistan.

Sample collection

Survey for assessment of late blight was conducted in the major growing areas of potato (Figure 1) in Punjab viz. Khushab, Okara, Jhang, Sargodha, Sahiwal, and Chiniot during the cropping seasons of 2017 and 2018. Each district was represented by three locations and each location was further divided into three sites. Five plants were randomly selected from four corners and centre of the plot representing each site. Diseased samples were collected in plastic polythene bags from the fields and brought to laboratory for further processing.

The infected leaf samples took during the occurrence of late blight from potato growing areas of Punjab. Each sample was labeled with the place, date of collection. The single lesion leaves with the most obvious symptoms (white mildew was visible) selected. The lesion was cut into small pieces (2 - 5 mm^2) at the ad

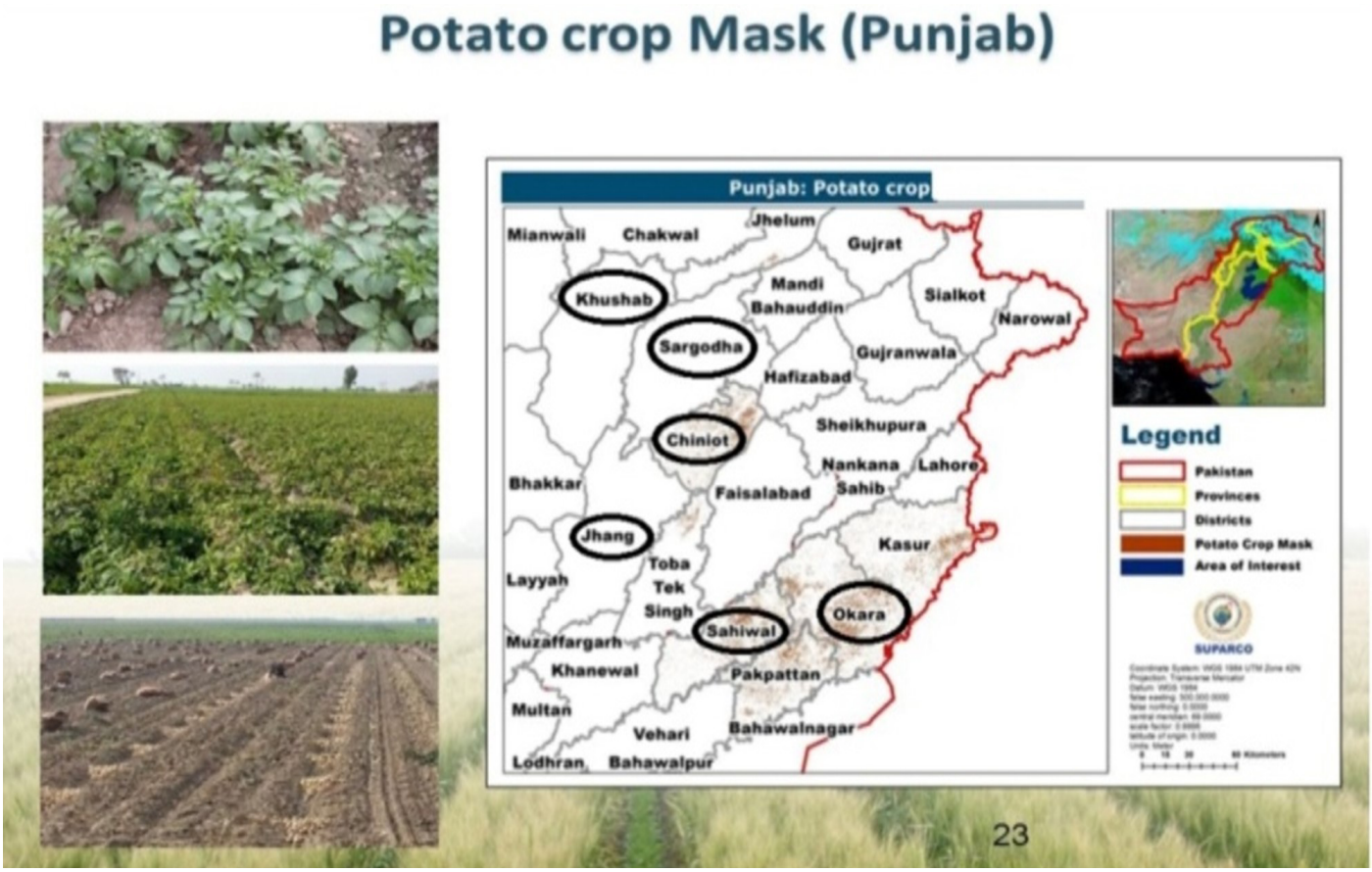

Figure 1: *Potato Crop Mask of Punjab, Pakistan.*

vancing margin of the lesion and then inoculated on-petri plates containing Rye agar media as well as potato tubers for sporulation. The Petri plates were then incubated at 17-18°C. Mycelia grew of samples for all isolates between 4-7 days depending on the virulence. The mycelia were transferred aseptically onto specific media plates for isolating purification in the next step.

Isolation and purification of Phytophthora infestans

The white colony from tuber from inoculated plates was removed and inoculated on plate medium antibiotics (rifampicin 20 mg. mL^{-1} or ampicillin 200 mg. mL^{-1}) and stored for 8-10 days at 18-20°C. The pure colony of *P. infestans* was transferred to slant further for long-term storage at 10-12°C and maintenance as described by Raza and Ghazanfar, 2019. Pure cultures were observed to maintain for periods of up to six months on 9 cm Petri plates (25 ml agar per plate) using Rye agar A media (Young, 2007).

Pathogenicity test

Pathogenicity test was carried out to confirm the pathogen, *P. infestans*. For this purpose, healthy potato leaves were detached from six weeks old plants grown in green house, washed with distilled water for ≈10 minutes, and air dried to remove moisture. Leaves were covered to reduce leaf desiccation with pieces of moist cotton. The inoculated leaflets were then placed adaxial side up into moist box placed in incubator at 16-18°C in the dark for 4 days with 14 h illumination and 10 h dark photoperiod respectively (Figure 2) as described (Flier *et al.*, 2007). The sporangial suspension was produced from 7 day fresh cultures by lightly washing the mycelium with distilled water and adjusted to about 60,000 sporangia/ml with the help of haemacytometer (Pliakhnevich and Ivaniuk, 2008). The spore suspensions were also produced from lesions which were placed into polypropylene culture tubes (14 ml) with 3-4 ml of preservative solution (0.2 M sodium acetate, acetic acid and 0.04 M copper sulfate, pH 5.4. Then these tubes were vortexed for 15-20 seconds to suspend sporangia and then counted with a haemocytometer. These counted spores/sporangia of the pathogen were incubated at 4°C for 1h to push germination. Consequently, these sporangial suspensions and plug of mycelium from pure colony were applied on detached leaves and later, these leaflets were assessed (Frobes *et al.*, 2012; Odilbekov *et al.*, 2014). The isolates were considered more virulent on detached leaves when mean sporulating lesion was ≥1 cm in length when seen with naked eye (Mukalazi *et al.*, 2001; Flier *et al.*, 2007).

Figure 2: *Pathogenicity determination assay (A) Potato crop grown in greenhouse (B) Selected healthy potato plant (C) Healthy leaves brought in laboratory and remove excess moisture on tissue paper (D) Placed detached leaves in moist box (E) Inoculation of pathogen plugs from pure culture of P. infestans (F) Representative inoculated detached leaves.*

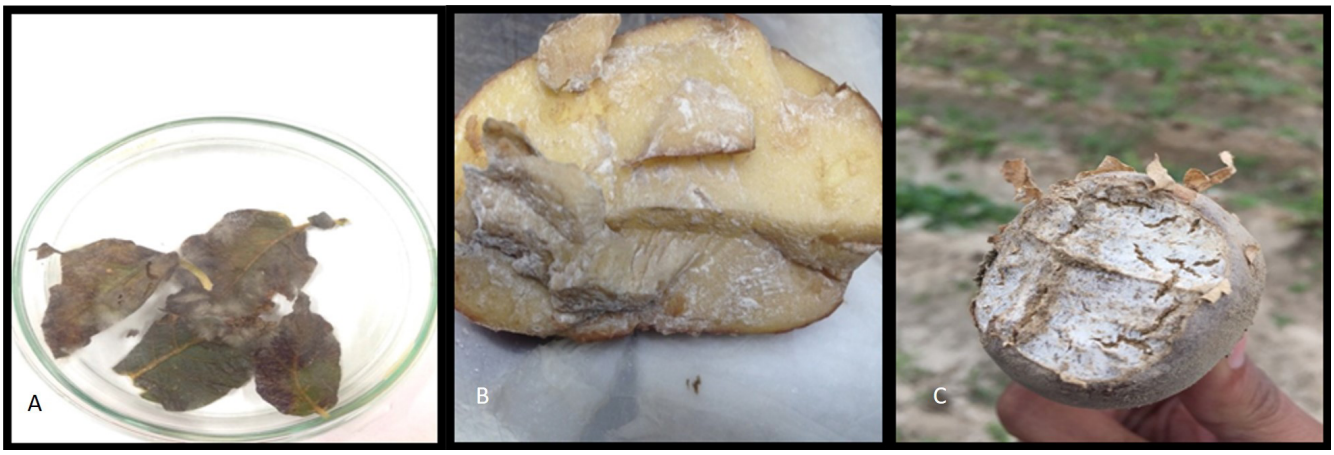

Figure 3: *Mycelium growth (sporulation) on leaves and tuber Sample (A, B, C).*

Morphological characterization

Mycelial growth: Agar plug (5 mm) taken from the pure culture and placed in the center of each petri plate had 20 ml of media including Rye Agar A, Corn Meal Agar and Carrot Meal Agar and three replicates for each medium and placed at 18-20°C in the incubator. The pathogen mycelial growth was assessed on 7th day of colony growth with the help of ruler. Then compared with tested media while three mycelial plugs (one from adjacent to original inoculum, 2nd plug among the original inoculum and colony margin, and 3rd plug of margin of colony) cut after 10 days of incubation from unlike positions of the inoculated media plates and gently shacked in 2 ml distilled water to get sporangia suspension. A heamacyto-meter was used to count the numbers of sporangia from three plugs (three petri dishes average used for each medium).

Microscopic observation of sporangia: The morphological characteristics evaluated by using seven-day-old cultures of *P. infestans*. The sporangia were dislodged in 1 ml of double distilled water added to each plate. The resulting suspension was recovered into a 1.5 ml microcentrifuge tube and dislodged sporangia fixed using 50 µl of acid fuchsin (10 mg acid fuchsin, 100 ml distilled water, 100 ml lactic acid, and the total volume brought to 1.0 ml with double

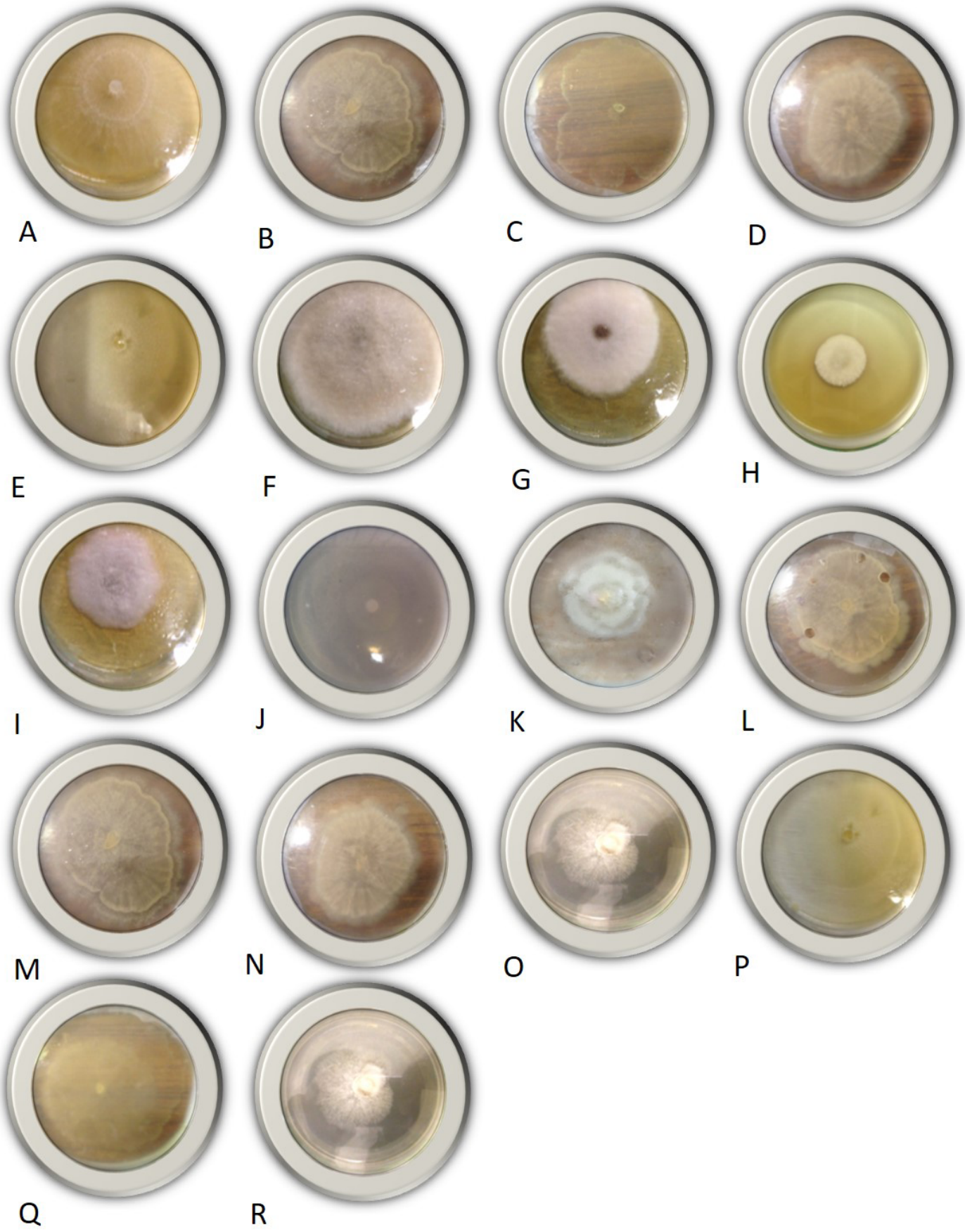

Figure 4: *(A-R): Represented pure cultures of P. infestans isolates (showing diversity in colony morphology) of 6 districts from 3 locations each showing different growth patterns (A, B, C) Sargodha. (D, E, F) Sahiwal. (G, H, I) Khushab. (J, K, L) Okara. (M, N, O) Jhang. (P, Q, R) Chiniot.*

Links Researchers

distilled water according to the procedure described by Granke and Hausbeck, 2011. The sporangia of the pathogen were counted through hemocytometer. The sporangia measurements (length, breadth, and pedicel length) were made visually at 200X magnification using a personalized macro. (Torres-Londono, 2016).

Statistical analysis

The morphological characteristics of the isolate compared for each isolate using least significant difference test (LSD) individually. The experimental data subjected to ANOVA using SPSS software v. 19.

Results and Discussion

Sample collection

Late blight identified after visual observing the disease symptoms under field conditions. The earliest symptoms were observed on lower leaves consist of small dark green spots that change from brown-black lesions typically "V" shaped (Hannukkala *et al.*, 2007; Fry, 2008). The samples collection was done from the infected potato fields having sporulation during survey (Figure 3). The phenotypic characteristics of isolates were fluffy cottony mycelia having slight striated patterns; and slow growth rate on different culture media (Figure 4). The culture of *P. infestans* on Rye agar A at 18°C in dark condition showed white-concentric ring to the cottony colony. A mycelium was hyaline, branched, and coenocyte.

Pathogenicity Assay.

The lesions on detached inoculated leaves were observed after inoculation. The lesion size and amount of leaf damage percentage varied significantly with the isolates. All the inoculated isolates caused disease with same symptoms (described in 3.1) on potato detached leaves. These new infected diseased leaves were again purified for further study.

At the end of the period of inspection, the isolates caused less than 1 cm lesion on detached leaf assay were marked while the best picture concerning the differences between the behaviors of isolates shown in Figure 5. The tendency was the same as the isolate those caused more lesion length caused highest infected percentage area. Therefore, it can be concluded from Pathogenicity assay that the higher the infected area caused by *P. infestans* isolates on detached leaves the more severe was the infection.

The level of pathogenicity diversity might be arisen as result of selection stress enforced by different cultivated potato cultivars (Blandón-Díaz *et al.*, 2012). High pathotype diversity in northern China has also been reported (Guo *et al.*, 2009).

Mycelial Growth

The mycelia of *P. infestans* characterized by lack of cross walls and have both asexual and sexual reproduction. The sporangiophores and sporangia of the pathogen appear at asexual reproduction stage. The pathogen's sporangia are the lemon shaped and developed at the end of these sporangiophores (Figure 6). All collected isolates fit the morphological description with deciduous and semi-papillate sporangia as reported in literature for *P. infestans* (Erwin *et al.*, 1996).

Sporangial morphology

All isolates produced papillate and deciduous sporangia and the average length of sporangia among the

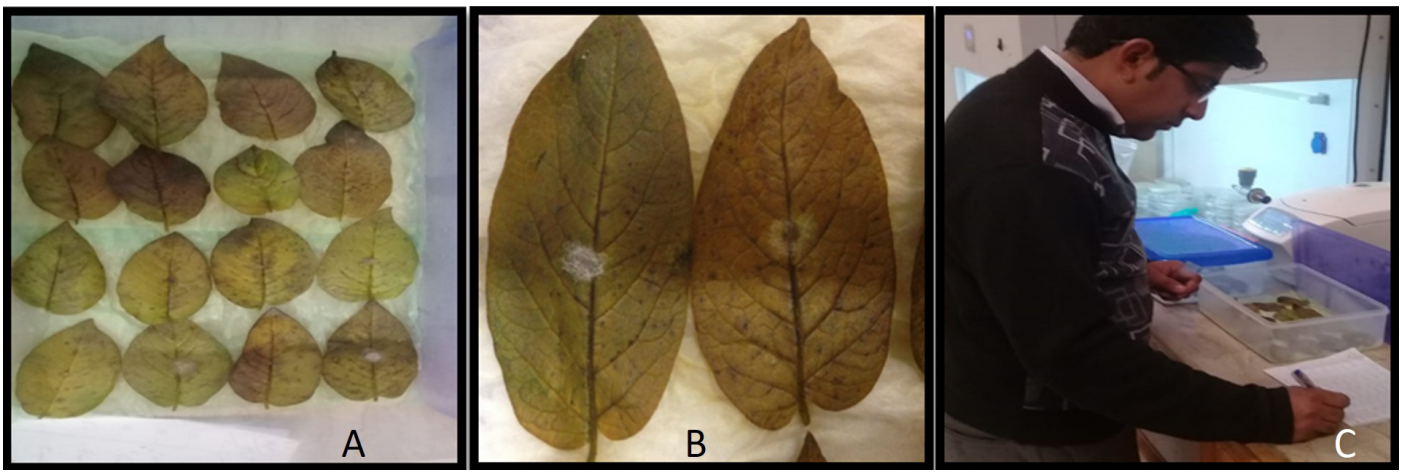

Figure 5: *Data recording for pathogencity (A) Leaves showing lesions on representative inoculated detached leaflets (B) Inoculated leaves showing sporulation (C) Data recorded.*

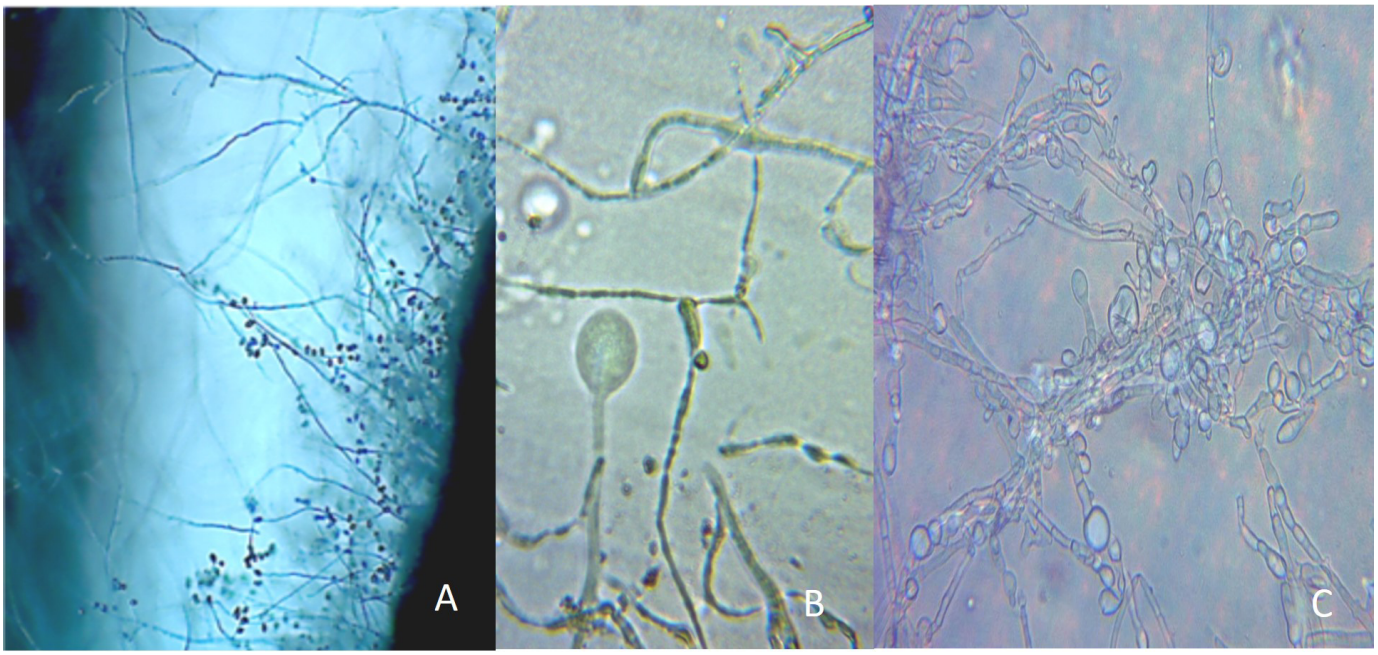

Figure 6: *(A-C): Microscopic view of sporangia and Sporangiophore of P. infestans in which zoospores are formed.*

isolates collected during 2017 from potato growing areas of Punjab ranged from 14.94 to 47.89 µm, and average breadth of sporangia ranged from 10.44 0 to 23.67 µm. The length/breadth ratio of sporangia ranged from 1.16 to 2.16 amongst the isolates as described by Islam *et al.* (2005). The isolates during 2017 showed highest length and breadth among six districts were Khb-9 (45.44 x 21.22 µm), Srg-1 (42.89 x 19.89 µm), Shl-10 (47.89 x 23.22 µm), Oka-13 (47.83 x 23.56 µm), Jhg-10 (47.56 x 23.57 µm) and Cht-6 (45.28 x 21.61 µm) (Figure 7). During 2018 tested isolates against morphological characteristics, among all districts, isolate Jhg-22 showed highest length (46.64 µm) and breadth (22.17 µm) followed by Shl-25 (46.59 µm) and breadth (22.61 µm), Oka-24 (46.31 x 21.72 µm) while lowest value measured by Cht-14 (16.05 x 13.39 µm). The isolate mean values compared, sorted within the district and presented in graphs (Figure 8).

The length and breadth (46.25 x 22.61 µm) was the highest value recorded in case of Khb-21 isolate from Khushab district while Srg-14, Shl-25, Oka-24, Jhg-22, Cht-18 (4.83 x 22.17 µm, 46.59 x 22.61 µm, 46.31 x 21.72 µm, 46.64 x 22.17 µm and 44.06 x 23.05 µm) from Sargodha, Sahiwal, Okara, Jhang and Chiniot respectively (Figure 8). It observed during both the years that isolates caused the highest values of length and breadth selected isolates from previous aggressiveness experiment by Raza *et al.* (2019). They observed that more aggressive isolates had the highest values of morphological characteristics as compared to other and the same trend observed in the current study.

Culture Media Evaluation

Variation in the colony color, the margin of the colony on three different solid media adds the vital information, which may help in taxonomic identification of *P. infestans*. It showed a difference in growth ability and sporulation ability on the selected media. In the present study, it was found that Rye agar media showed the best sporulation of the fungus, followed by Cornmeal media, and the least growth was recorded in case of carrot agar media.

The overall data of six districts revealed that during 2017 study, Rye agar medium had significantly the highest growth of Oka-13 (70.33 mm) followed by Jhang-10 (69.67 mm), Shl-10 (69.33 mm) while the least growth was observed by Oka-7 having 34.67 mm. The spores were produced more on Rye agar media by isolate Oka-13 (228.67 per square cm) as compared to other media used (Figure 9).

Generally, the isolates showed a similar pattern of growth on the second year 2018 and here presented an overview of top three isolates of each district that Rye agar media was the best media for the radial growth e.g Khushab district isolates were Khb-21 (65 mm) followed by Khb-11 (57.67 mm) and Khb-15 (49.33 mm). Among Sargodha district isolates, Srg-14 (65 mm), Srg-24 (57.67 mm), Srg-21 (51 mm). In the case of Sahiwal district isolates, Shl-25 (67 mm),

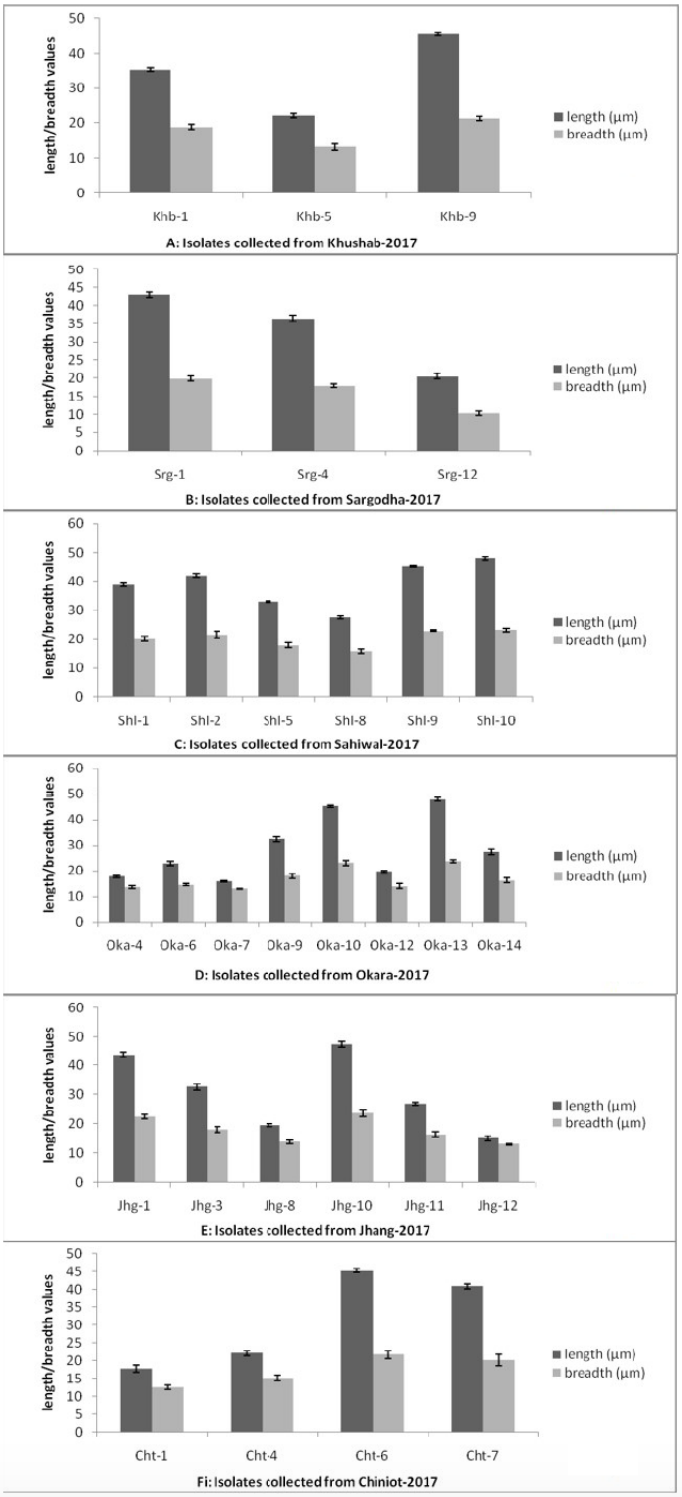

Figure 7: *(A-F): Morphological characterization of isolates collected from potato growing areas of Punjab during 2017 - Khushab (A), Sargodha (B), Sahiwal (C), Okara (D), Jhang (E), Chiniot (F).*

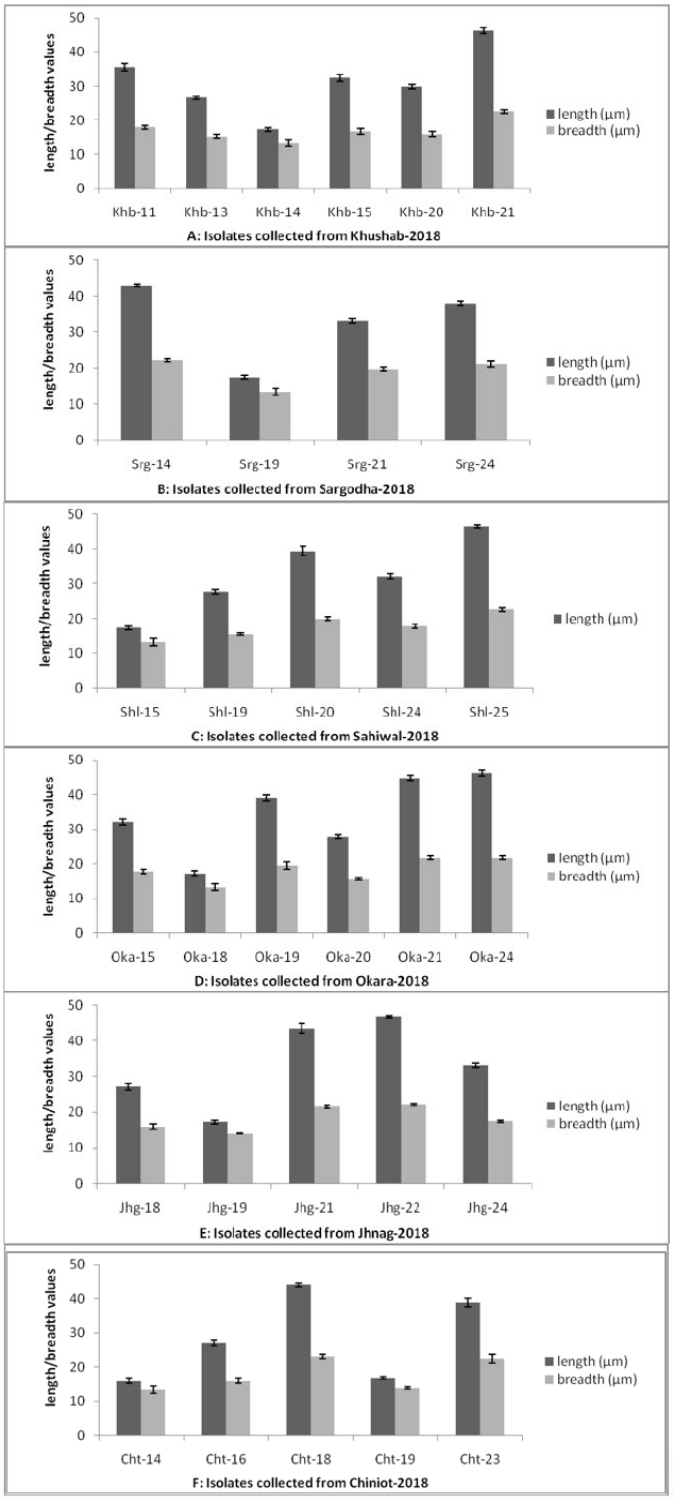

Figure 8: *(A-F): Morphological characterization of isolates collected from potato growing areas of Punjab during 2018 - Khushab (A), Sargodha (B), Sahiwal (C), Okara (D), Jhang (E), Chiniot (F).*

Shl-20 (62.33 mm), Shl-24 (56 mm). The greatest growth was recorded from Okara isolates was Oka-24 (68 mm), Oka-21 (60 mm) and Oka-19 (50 mm). Radial growth of Isolates collected from Jhang district ranged from 36.33 mm - 44.33 mm against Jhg-19 and Jhg-22 respectively on tested media at 18°C. Similar trends were also noted in district in isolate Cht-18 from Chiniot showed good colony growth (66.67 mm) followed by Cht-23 (57 mm) and Cht-16 (54.67 mm) (Figure 10). Indeed, all isolates among 6 districts studied showed almost similar response towards Rye agar media while somewhat less on media containing Corn meal agar and carrot agar media. The smallest radial growth obtained on Carrot agar medium may due to incompatibility with pathogen requirements.

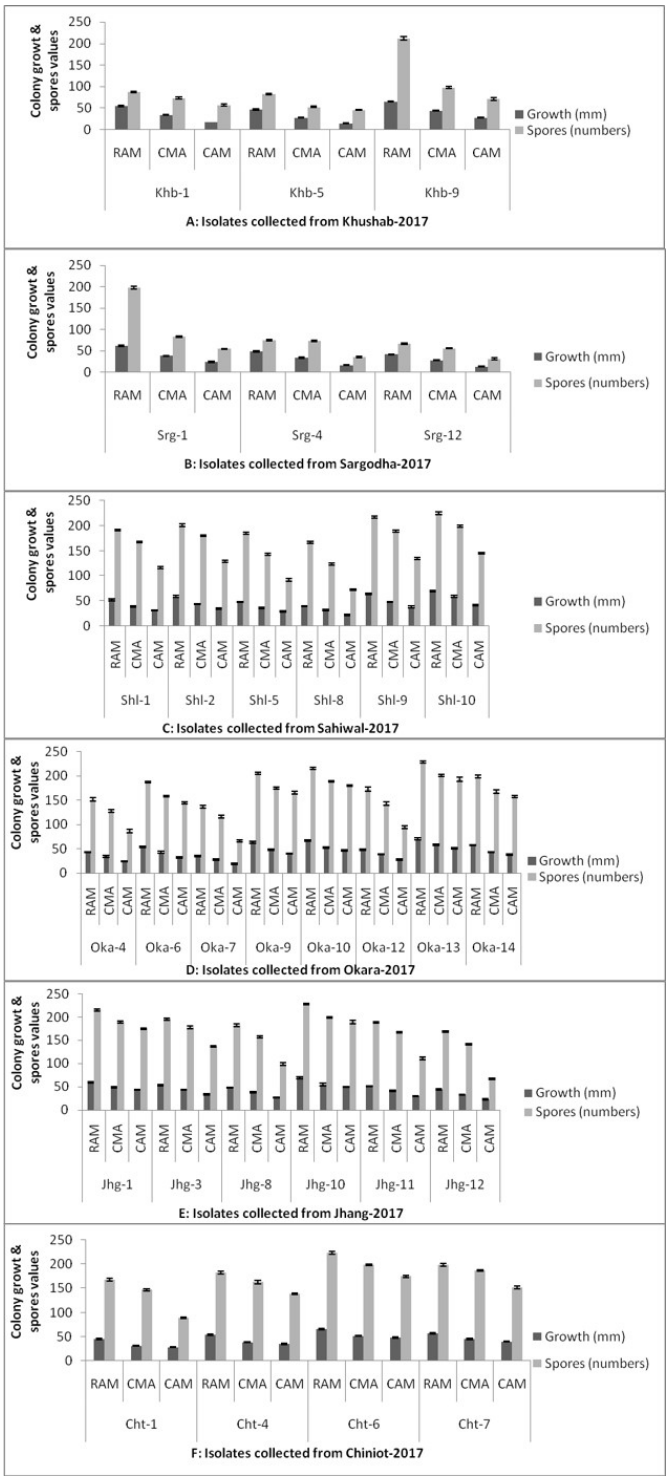

Figure 9: *(A-F): Colony growth of isolates on different growth media collected from potato growing areas of Punjab during 2017 - Khushab (A), Sargodha (B), Sahiwal (C), Okara (D), Jhang (E), Chiniot (F).*

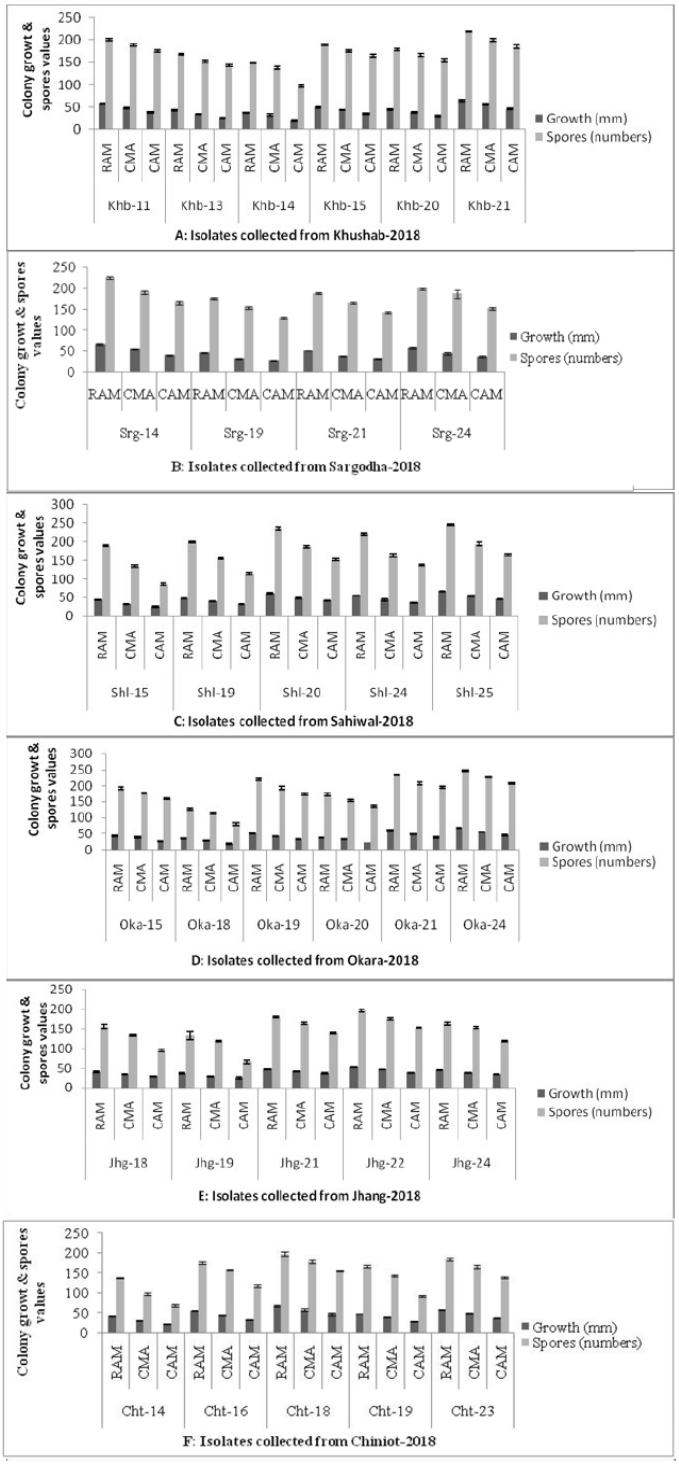

Figure 10: *(A-F): Colony growth of isolates on different growth media collected from potato growing areas of Punjab during 2018 - Khushab (A), Sargodha (B), Sahiwal (C), Okara (D), Jhang (E), Chiniot (F).*

Potato late blight is the most economical and significant plant disease worldwide. Morphological identification of this plant pathogen is an important subject that caused high destruction on potato. There are several factors that possible affecting the purified of the isolations such as the presence of contaminants and the environmental conditions. The *Phytophthora* genus can be isolated from a small proportion of the soil samples taken by the infected leaves (Vettraino *et al.*, 2001).

However, isolation of *P. infestans* from the infected sample requires substantial time with using the selective media (Yamak *et al.*, 2002). A major concern to get a pure culture of *Phytophthora* is possible the presence of fast-growing organisms such as *Pythium*

and other genera of fungi, which affected the growth of *Phytophthora* (Canaday and Schmitthenner, 1982). As well, the material of potato seed can affect the fungal co mmunity in the fields (Runno-Paurson *et al*., 2010b, Drenth *et al*., 2001; Lal *et al*., 2015). In the present study, morphological characters of sporangial size was undistinguished which was found to be high values of aggressive isolates. Sopee *et al*. (2011a) revealed a variation on size of the pedicel, sporangium and oospore of *P. infestans* isolates and analyzed on the relationship of the size of these propagules with metalaxyl sensitivity.

Interestingly, the difference in the culture nutrient for the plant fungal pathogens is showing some variability among races and isolates of the same pathogen (Lehtinen *et al*., 2007). Different techniques are often used for the revealing of *Phytophthora* spp. within the fungal population (Gallegly and Hong, 2008). The primary obstacle of contamination led to the use of selective medium to enhance the growth of the desired pathogens (Kumbar, 2017; Fry, 2008**;** Ristaino, 2012; Grünwald *et al*., 2011; Tian *et al*., 2016; Ho, 2018). In the current study, variation in the growth pattern of the races of *Phytophthora* in different culture media was obtained. The variations of the isolates were based on morphological characteristics, which were detected in this study. The characteristic of morphological may be relevant with changing in the temperatures that possible degradation the components of culture media (Cooke and Lees, 2004).

Several growth media such as Rye Agar media, Corn meal agar and Carrot Agar media are very important for the growth of the *P. infestans* (Peters *et al*., 1998). Rye Agar media used to prepare *P. infestans* culture media (Peters *et al*., 1998). Other cultural media used to grow and sporulate of *P. infestans* but inappropriate for many isolates (Savage *et al*., 1968). It is using a specific media for getting a typical growth of *P. infestans* that can help to study this organism and capability to cause high damage for the potato fields.

On the other hand, carrot agar media does not support abundant sporangial production as compared to other media (Erwin and Ribeiro, 1996**).** Lower growth of the fungus in carrot meal and cornmeal agar medium may return into three reasons, first, not contain the substances supporting the growth of pathogens, second, the substances may be destroyed during autoclaving and when they integrated into the medium, and third, the substances complexed. Two cultural media such as carrot meal and cornmeal agar may be lacking the suitable nutrient content or not ready (Complex nutrient) for growing and sporulation of *P. infestans* that caused poor growth. This species as *P. infestans* may be lacking some enzymes that are degrading the nutrient from complex to be more simple nutrients. For the highest growth and appearance, nutrient contents are very significant. Based on observations by different researchers, carbohydrates are much necessary for fungal growth and its sporulation (Kumbar, 2017). This present investigation revealed that Rye Agar medium is considered as the best medium to grow of *P. infestans* and the next medium is a cornmeal agar. While carrot agar medium supports the sporulation of *P. infestans* causes of potato late blight. This detected a suitable medium to grow and reproduction of *P. infestans* that will utilize in studying of different sides of this species including the molecular study, and management strategies of the blight late disease.

Novelty Statement

Phytophthora infestans is a destructive plant pathogen in the tropical and the sub-tropical areas of worldwide. Phenotypic identification of pathogens is very helpful to find successful disease control strategies. Knowledge of variability in morphological, cultural parameters of *P. infestans* is still scarce. Therefore, the determination of morphological characteristics may add knowledge of the taxonomic behavior of pathogen which ultimately leads towards its control.

Author's Contribution

Waqas Raza: Executed the field visits, wrote and finalized the manuscript.
Muhammad Usman Ghazanfar: Conceived the idea and facilitated guided and supervised the experiment.
Muhammad Asif: Guided for experiments.
Ikram Ul Haq: Help in data analysis.
Muhammad Zakria: Co-supervised the experiments.
Laith Khalil Tawfeeq Al-Ani: Guided and help for data analysis.

Conflict of Interest
The authors declare that there is no conflict of interest to publish the article.

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