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Pathotype determination of *Phytophthora infestans* isolates on detached potato leaves under laboratory conditions

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Abstract
Potato late blight (*Phytophthora infestans*) is an important disease causing severe damage in potato crop. One hundred forty nine single lesion isolates of *P. infestans* collected from naturally different potato growing areas of Punjab province during 2017-18 were characterized for pathotype determination based on their lesion area and infected area percentage after their inoculation onto detached leaves of potato. The results showed that large variations in virulence were present among isolates for each regional *P. infestans* population studied. Those isolates collected during 2017-18 exhibited 69.44% and 70.12% virulence percentage respectively. The highest lesion length and infected percentage area of isolate Oka-24 recorded 44.33 mm (63.33%) while lowest was 1 mm (1.43%) shown by isolate Srg-5 during both years. This is the first comprehensive study to determine pathotype of isolates in Pakistan. The experimental findings indicated that population of *P. infestans* in the Punjab province comprises diverse isolates with low to high virulence potential as observed.

Keywords: potato, disease, pathotype, lesion, virulence

1. Introduction
Potato (*Solanum tuberosum* L.) is the most significant food crop after cereals such as wheat, rice and maize [1] and produces more calories as compared to rice and wheat [21, 22]. Though it was originated in the Andes region and from there it reached to subcontinent by Portuguese traders and in Pakistan it has becomes an integral part of almost every dish. Pakistan having the most favorable weather [30] which is suitable for the production of potato crop [5]. For the past few decades, the production has been increased in the region of Asia like Pakistan and become a major source of income for the small land holding farmers. Potato crop plays a significant role in guaranteeing food security among four major global food crops [19]. Potato crop is prone to several biotic factors. Among them, late blight of potato cause by *P. infestans* is the most severe biotic constraint to potato production and key threat to food safety both quality and quantitative in developing counties [2, 16]. The pathogen is diploid, heterothallic and infects leaves, stems and tubers and cause very devastating yield losses [13]. The pathogen reproduces both sexually as well as asexually [17]. The asexual life cycle is the predominant mode of reproduction worldwide. A large number of spores are produced and spread through wind, water and rain splashes on other host tissues and release sporangia through host stomata [17]. The whole plant may wilt so quickly within several weeks [29]. The overwintering survival of the plant pathogen done by the production of oospores formation which lead towards the change in population structure [17]. The pathogen, *P. infestans* act as an obligate biotroph in nature [20] although it can be isolated and preserve on synthetic media [3]. Different techniques are available to analyze populations for aggressiveness nature of selected pathogen [15, 4].
Those isolates which are more aggressive in nature are not necessarily the fittest when a several potato growing seasons are considered. Thus it is possible that highly virulent isolates due to their incapability to survive in absence of living host might be the under negative selection pressure during winter season [14].
The moderately virulent isolates those do not infect all infected potato tubers in potato growing seasons, would tend to survive fitter than highly aggressive virulent strains that significantly infect tubers. The transmission of pathogen from one state to other often through movement of potato tubers have shared genotypes found across the Asian, Russian and European populations [8]. Additionally, the migrations might increase the level of variations genetically in pathogen populations and sub populations by bringing compatible strains. The worldwide situation has been changed from North America, Mexico to Europe in 1976 after import potato seed tubers [24]. These perceptions coupled with fact that *P. infestans* populations were dramatically diversed and were the important component in life history in these locations.
Different plant pathologists and researches in recent years have studied the phenotypic characterization of the pathogen (*P. infestans)* populations across the world as well as pathotype determination [6, 15, 17, 25]. More studies constantly shown that population of pathogen in various regions across world have become increasingly diverse and became highly complexed in the number of pathotypes lacking a virulence components found on the standard set of differentials and on detached leaves [18].
Due to importance of tuber different management strategies have been adopted by the farmers [10]. In accordance to importance of this disease, before knowing the actual cause the management strategies are not so enough to combat with the challenges of farmers, Therefore, pathotype development is the more reliable tool for accessing the disease causing ability of the different isolates which are the present in the different fields [15]. A potential drawback of widespread of potato verities which are more resistant in nature might be the increased instability of host resistance that is associated with the more aggressiveness population

across the world [13]. This finding might be helpful for commercial potato growers who are suffering from late blight disease incidence in their areas leading to serious yield losses. Although, the increase in yield losses in all organic crops has raised our concerns in pathogenicity and the development of pathotypes for further management strategies [10]. The information about the pathotype of different isolates collected from different locations may also help us to access the increase or decrease in virulence of pathogen.

Therefore, by keeping in mind of the above concerns, the present study was planned to develop the pathotype of pathogen isolates collected from different potato growing areas of the Punjab, Pakistan for further evaluation of aggressiveness behavior.

2. Materials and Methods

The research work was conducted in the laboratory of Plant Pathology, College of Agriculture, University of Sargodha, Sargodha, Punjab, Pakistan during 2017-18 to determine pathotypes of the pathogen (*P. infestans*) isolates collected from potato growing areas of Punjab, Pakistan.

2.1 Sample Collection

Diseased samples during occurrence of late blight of potato were collected during field visit to different areas of Punjab, Pakistan including districts of Khushab, Sargodha, Sahiwal, Okara, Jhang and Chiniot during year 2017-18.

The leaves with distinctive symptoms (lesions was small and white mildew was visible at the lower sides of the leaves) were selected from the field. The infected samples collected in zipper bags and brought to Plant Pathology laboratory, College of Agriculture, Sargodha for culturing *P. infestans* isolates to determine virulence. After those diseased samples was stored at -4°C until further action as described [23].

2.2 Isolation of *P. infestans*

Blighted leaves and potato tubers taken from the field was incubated overnight in humid boxes at 18±2°C to encourage sporulation. The pathogen was isolated from infected leaves of potato and the isolation was made by cutting a small section of infected margins along with healthy areas, which was surface sterilized with 0.1% bleach solution and rinsed twice in double distilled water. It was then air dried and placed into the sterilized Rye agar media plates and incubated at 18±2ºC.

2.3 Preparation of Inoculation

Newly formed sporangia were collected by needle and add 10mL of distilled water to each petri dish and spore suspensions were filtered through cheese cloth by making concentration of 60000 sporangia/ml through haemocytometer. These collected spores were chilled at 4°c for ≈2 hours to release of zoospores for inoculation [27].

2.4 Pathotype determination

Pathotype of isolates (72 during 2017 and 77 from 2018) were tested in the laboratory of Plant Pathology, University of Sargodha. Potato leaves were detached from 6 weeks plants grown in departmental greenhouse, washed with distilled water ≈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 18±2ºC in the dark for 4 days with 14h illumination and 10 h dark photoperiod respectively (Fig 1) as described [13]. 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. The spore suspensions were also produced from lesions which were excised and placed into polypropylene culture tubes (14ml) with 3 ml of preservative solution (0.2 M sodium acetate, acetic acid and 0.04 M copper sulfate, pH 5.4. These tubes were then vortexed for 15 seconds suspend sporangia and then counted with a haemocytometer. These counted spores/sporangia were incubated at 4°C for 1h to encourage germination. Consequently, these sporangial suspensions and plug of mycelium from pure colony were applied on detached leaves and later, these leaflets were assessed for infected leaf area percentage and lesion length as described [16, 25]. The isolates were considered more virulent on detached leaves when mean sporulating lesion was ≥1 cm in length and based on these findings the pathotypes were developed [13, 25].

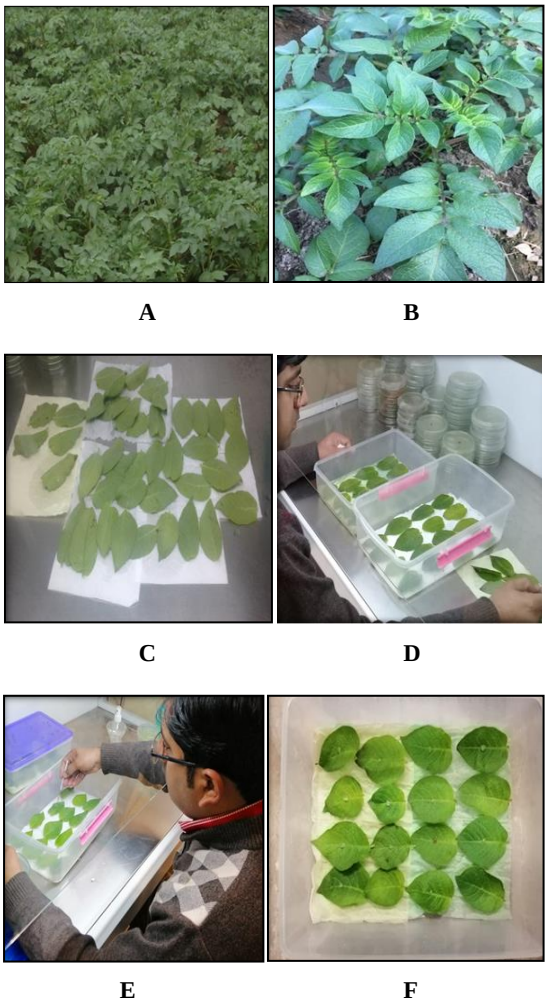


Fig 1: (A) Potato crop grown in greenhouse **(B)** Selected healthy potato plant **(C)** Healthy leaves brought in laboratory and remove excess moisture on tissue paper **(D)** Placed detached leaves in moist box **(E)** Inoculation of pathogen plugs from pure culture of *P. infestans* **(F)** Representative inoculated detached leaves

3. Results

3.1 Isolation and purification

Isolates were obtained from inoculated infected potato leaves showing typical colony growth of pathogen. Inoculated leaves and potato tubers also showed white mycelia growth (sporulation) on their surface which was pick up by cutting small cubes of antibiotic Rye agar media (approx. 2 x 2 mm) using a sterile scalpel by lowering them gently (using a needle or scalpel) onto the sporulation trying to avoid directly touching the infected tissue for obtaining more pure cultures for further study.

3.2 Pathotype determination

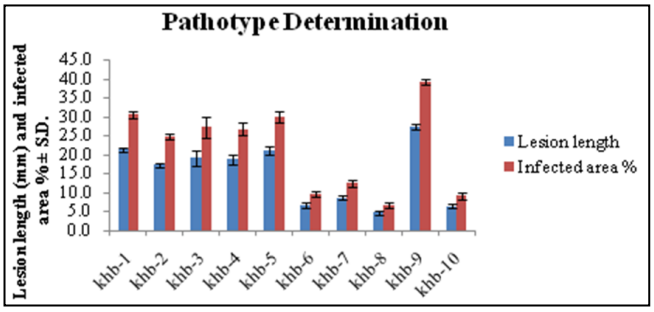
The lesion size and percentage infected area per lesion were measured after inoculation. Lesion size was measured by taking two measurements (width and length) initial from diameter through the use of ruler.

The lesion size and amount of leaf damage percentage varied significantly with the isolates. All the inoculated isolates caused disease on potato detached leaves. The results clearly showed that the isolates collected from different potato growing areas during 2017, the highest lesion length and infected percentage area recorded 42.33 mm and 60.48% respectively of isolate Cht-6, while lowest was 1mm and 1.43% shown by isolate Srg-5 (Fig 2).

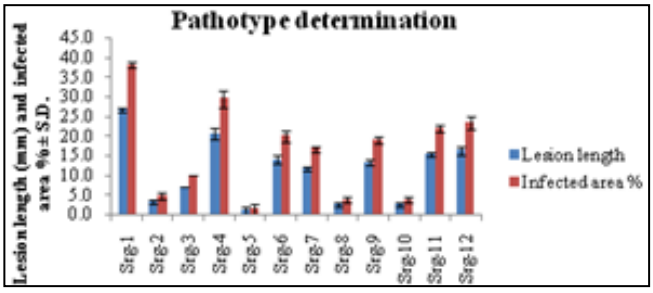
For the 2018 collection, the isolates that caused highest lesion length and infected area percentage among six districts included Khb-21 (31.33 mm-44.76%), Srg-14 (27.33 mm-39.05%), Shl-25 (37.67 mm-53.81%), Oka-24 (44.33 mm-63.33%), Jhg-22 (41.67 mm-59.52%) and Cht-18 (43.67 mm-62.38%). The overall lowest lesion length recorded 1.33 mm for isolate Srg-20 and the percentage area affected was only 1.90%. The isolates Oka-24 and Cht-18 caused significantly more disease on the detached potato leaves among 2018 isolate collection (Fig 2).

Infected potato growing areas were significantly (P=0.05) different in different potato growing areas of the Punjab during sample collection both the years. Maximum infected area was recorded during 2017-18 in Okara district (44.31%) and Chiniot district (42.38%), respectively. Similarly, lesion length was significantly different (P=0.05) with maximum lesion length was noticed/recorded in Okara district (31.02mm) and Chiniot district (29.66mm) respectively Fig 3.

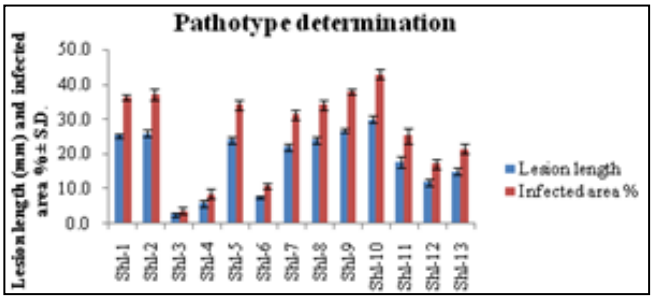
At the end of the period of observation the rating results were used to determine the intensity of disease. The isolates (22 from 2017 and 23 from 2018) caused less than 1cm lesion on detached leaf assay were marked and excluded for further experiments while the best picture concerning the differences between the behaviors of isolates shown in Fig 2. The tendency is the same as the isolate those caused more lesion length caused highest infected percentage area. Therefore, it can be concluded from experimental results that the higher the infected area caused by *Phytophthora infestans* isolates on detached leaves collected from different potato growing areas of Punjab, the more severe was the infection.



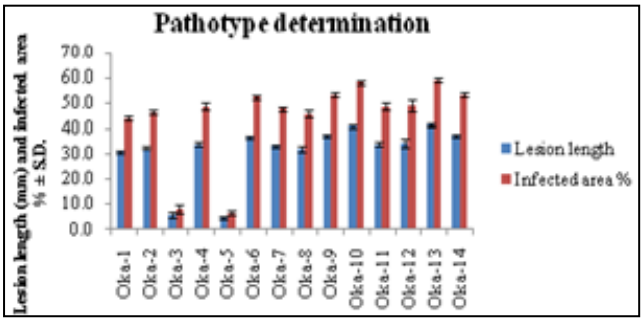
(A) Isolates collected from khushab district during 2017



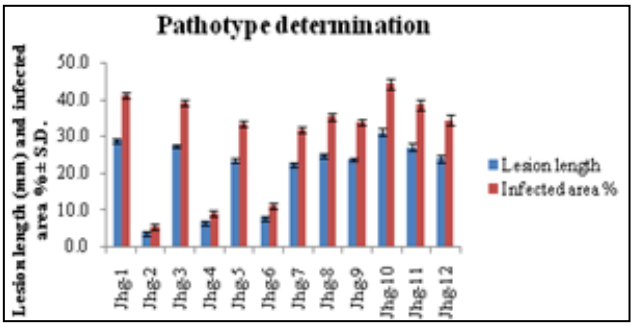
(B) Isolates collected from Sargodha district during 2017



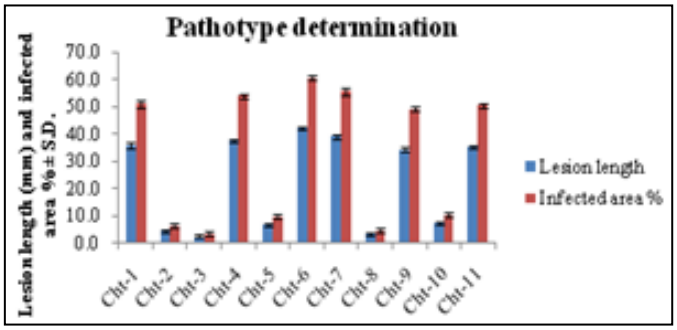
(C) Isolates collected from Sahiwal district during 2017



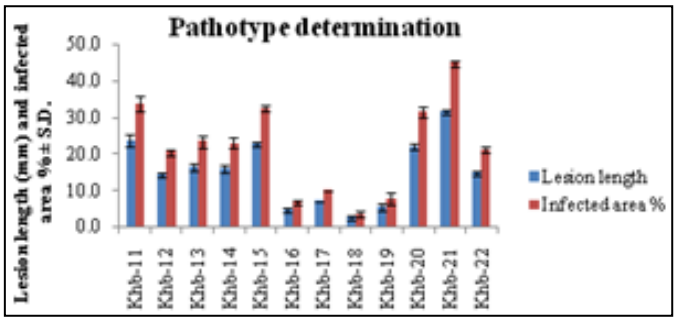
(D) Isolates collected from Okara district during 2017



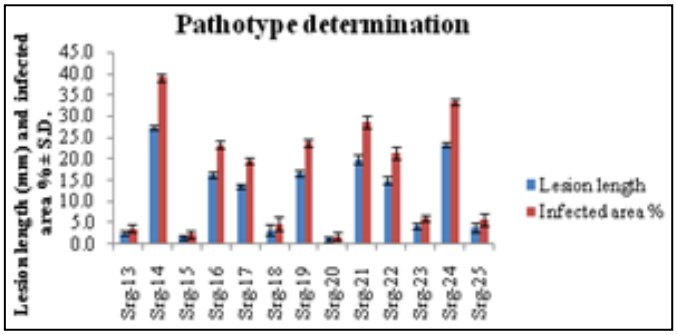
(E) Isolates collected from Jhang district during 2017



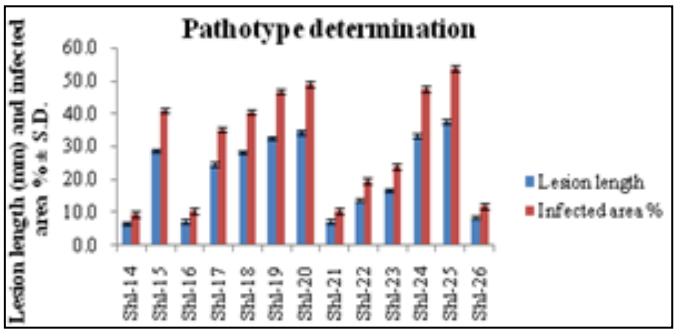
(F) Isolates collected from Chiniot district during 2017



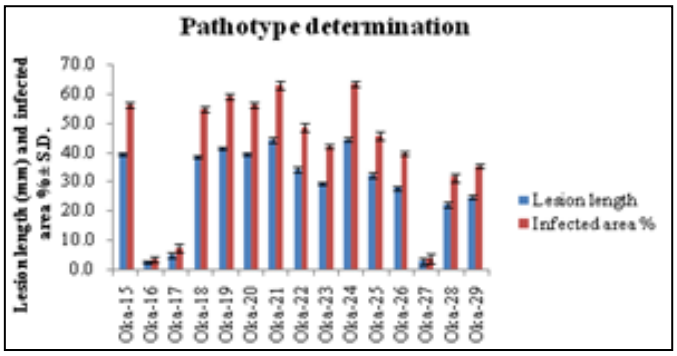
(G) Isolates collected from Khushab district during 2018



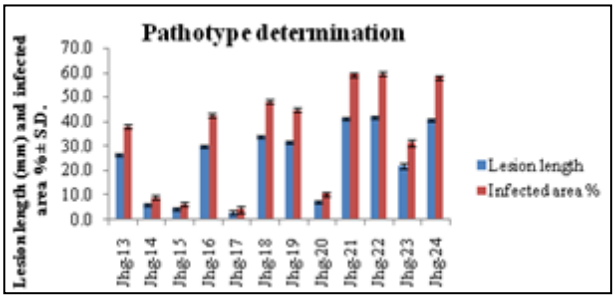
(H) Isolates collected from Sargodha district during 2018



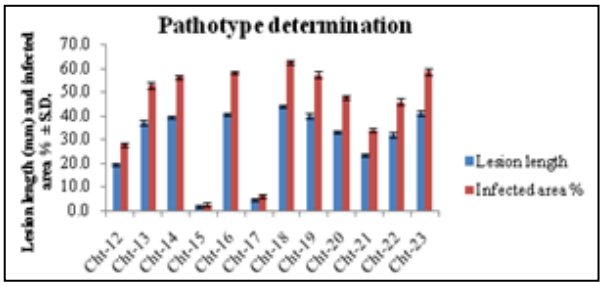
(I) Isolates collected from Sahiwal district during 2018



(J) Isolates collected from Okara district during 2018



(K) Isolates collected from Jhang district during 2018



(L) Isolates collected from Chiniot district during 2018

Fig 2: (A-L): Pathotype determination of isolates collected from six potato growing districts of Punjab, Pakistan during 2017- Khushab (A), Sargodha (B), Sahiwal (C), Okara (D), Jhang (E), Chiniot (F) & during 2018- Khushab (G), Sargodha (H), Sahiwal (I), Okara (J), Jhang (K), Chiniot (L)

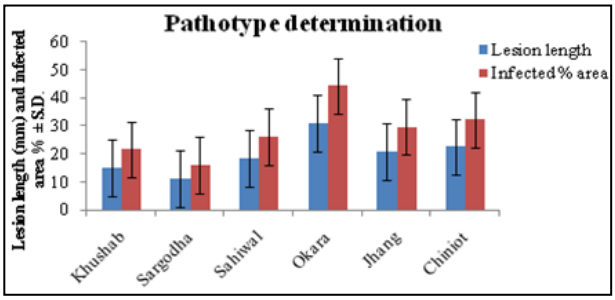


Fig 1: Isolates collected from six districts during 2017

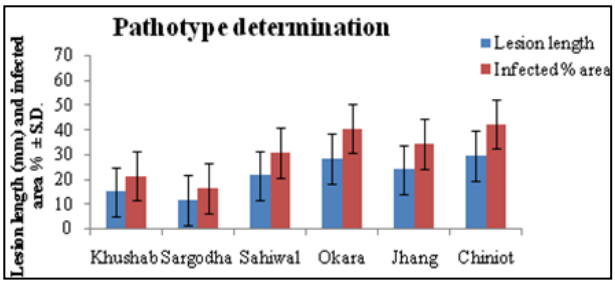


Fig 2: Isolates collected from six districts during 2018

Fig 3(1-2): Graphical presentation of different potato growing areas showing lesion length and infected percentage area

4. Discussion

It is a very important to develop pathotypes form the infected sample collected from main potato producing areas, because it is more feasible to select the more virulent isolates to check their aggressiveness and pathogen fitness on the basis of pathotypes later which ultimately lead towards disease development, oospores formation and sexual reproduction of the pathogen. Therefore, the present research work was conducted during 2017-18 in Agriculture College, Sargodha to develop pathotypes of the pathogen (*P. infestans)* in potato growing areas of Punjab, Pakistan.

The overall results revealed that the isolates collected from different potato growing areas during 2017, the highest lesion length and infected percentage area recorded 42.33 mm and 60.48% respectively of isolate Cht-6, while lowest was 1mm and 1.43% shown by isolate Srg-5 while the isolates, Khb-21, Srg-14, Shl-25, Oka-24, Jhg-22 and Cht-18 (44.76%, 39.05%, 53.81%, 63.33%, 59.52%, 62.38%) respectively caused more infected area percentage on detached leaves during 2018 collection.

Results from this study support the hypothesis that the phenomenon of pathogen *P. infestans* has the potential of continuous change in virulence during its reproduction [28, 9, 32] and variation in pathogenicity among pure cultures was noticed also in some of these studies. The inoculated leaflets increase symptoms development as reported [7] but in contrast to these findings the development of disease does not always need wounded leaves for severe infection [31]. The development of disease symptoms is the result of phytotxic metabolites produced by pathogen as observed [17]. However, it is also determined [26] that high level of infection was exhibited in detached leaf assay due to having less environmental pressure in controlled conditions.

In the South America, the authors hypothesized that most of the potato landraces are susceptible to potato late blight disease and the pathotype diversity might be due to the high mutation rates and no selection pressure [11]. The pathotype development of *P. infestans* population had been studied worldwide e.g United States, and Asia, South America [12, 13, 20, 33].

The pathotype diversity which observed in *P. infestans* isolates could have arisen as a result of selection pressure forced by different potato cultivars [6] which support our study. High pathotype diversity in northern China has also been reported which supported our present findings [18].

5. Conclusion

The results of experiment provided the information essential for the selection of virulent isolates for further experiments and ultimately for growth of affective disease management strategies. This is supported by the fact that more than 50% of *P. infestans* isolates were virulent as reported by many researchers. Therefore, concern should be taken for alternative strategies and to minimize use of chemicals at the same level at different regions. Definitely, to reduce the further progress of chemical, application of fungicides and resistant strains should be combined through use of host resistance.

6. Acknowledgement

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7. Conflict of interest

"The authors declare that there is no conflict of interest to publish the article"

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