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COMPARATIVE ASSESSMENT OF DETACHED LEAFLET AND TUBER DISC ASSAYS FOR STUDYING THE AGGRESSIVENESS OF DIFFERENT ISOLATES OF *PHYTOPHTHORA INFESTANS*

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Abstract

Study of the aggressiveness potentials of a pathogen has wide applications in plant pathology for determination of its population composition and consequent disease control methods in a given environment. Both, laboratory and field experiments are in general use to study the aggressiveness characteristics of plant pathogens. Comparative efficacy and reliability of a particular method employed for such studies could greatly reduce labor hurdles, time and associated costs. In this experiment, we evaluated and compared detached leaflet and tuber disc methods for the reliability of results of the aggressiveness characteristics (percent disease severity, latent infection period and lesion area and area under lesion expansion curve) of 15 isolates of the late blight pathogen (*Phytophthora infestans*) which were collected from different locations. In general, both methods revealed similar and consistent results for the aggressiveness rankings of different isolates; however, numerical values for aggressiveness components significantly varied among the tested isolates in each method. Results suggested that detached leaflets and tuber discs assay could be used as reliable and efficient methods for studying the aggressiveness characteristics of *P. infestans*.

Key words: Detached leaflet, Disease severity, Intact leaves, Late blight, Lesion area, Virulence.

Introduction

Potato (*Solanum tuberosum* L.) is an adaptable crop cultivated on more than 19 million hectares of land throughout the world (Anon., 2017). Total area under cultivation for the crop in Pakistan was recorded as 13×10^5 hectares which yielded 13.8 million tonnes potato during 2014 (Majeed & Muhammad, 2018). Climatic conditions of plains as well as hilly areas of the country are suitable for the propagation of potato and correspond to a significant share of production. There are many biotic and abiotic threats which have profound effects on growth, yield, and production of potato. Viral, bacterial and fungal diseases along with nematodal atrocities are major limiting biotic factors which cause drastic implications on potato productivity in the country. Among pathogenic infections, late blight of *Solanum* species is a challenging disease of family Solanaceae which has major impacts on the two most important crops –potato and tomato (Majeed *et al.,* 2018). Late blight caused by *Phytophthora infestans* has drastic yield reducing affect particularly on potato which serves as its primary host along with tomato (Flier *et al*., 2003; Majeed *et al*., 2017). The first cataclysmic effects of late blight were observed in Ireland during 1845 and 1846 when *P. infestans* infected the most popular and staple potato crop of the Irish peoples and subsequently caused hunger and starvation by eliminating potatoes from the fields (Scholthof, 2007). Since the Irish catastrophe, *P. infestans* has been remained of continuous focus because of its damage causing potentials towards potato and tomatoes. Evolutionary mechanisms and the occurrence of sexual recombination of this heterothallic fungus have resulted in the emergence of more aggressive and virulent strains which has further posed challenges for late blight control methods in recent decades (Fry, 2008; Gisi *et al*., 2011).

Study of the aggressiveness of the new races of *P. infestans* generally perceived to have occurred as a result of evolutionary events, mutation and sexual reproduction in the diverse isolates of the pathogen (Gisi *et al*., 2011) is significantly important in the determination of the pathogen's population composition and employment of appropriate disease control methods in a particular environment. Different experimental procedures, both under natural and artificial conditions, are carried out to determine the aggressiveness capacity, late blight reaction and host resistance studies of *P. infestans* (Vleeshouwers *et al*., 1999; Brooks, 2008) with each method having its own significance in the result outcomes and the aim of assessment. Variable results for aggressiveness screening have been obtained in earlier studies. Vleeshouwers *et al*., (1999) has reported the efficacy of detached leaflet assay as a reliable method for virulence tests of *P. infestans*. Brooks (2008) has documented consistent results in lab and field studies for host resistance to *P. colocasiae*. Nowakowska *et al*., (2014) demonstrated the high correlation in lab and field studies for aggressiveness results. On the other hand, Dorrance & Inglis (1997) recommended field experiments over lab assay as suitable methods due to high reliability for host resistance results.

Validity and reliability of results are important components of *P. infestans* inoculation procedure which needs consideration for adopting appropriate methods while testing host resistance and aggressiveness parameters of the pathogen (Vleeshouwers *et al*., 1999). Thus, selection of an appropriate method for the aggressiveness evaluation of *Phytophthora* spp. could greatly help with spatial, time, labor and cost management

(Brooks, 2008). The objective of this study was to make a comparative assessment of two lab bioassay (detached leaflets and tuber disc method) for the aggressiveness characteristic of different isolates of *P. infestans*.

Materials and Methods

During 2011, naturally infected potato leaf samples from cultivated fields in Mansehra (Man), Bata Kundi (Bat), Kaghan (Kg), Nathya Gali (Nat), Shinkiari (Shn), Bara Gali (Bar), Kalam (Kal), Sharan (Sha), Ayyubia (Ayy), Shangla (Shg), Naran (Nar), Shabqadar (Shb), Shougran (Sho), Balakot (Bal) and Mahaban (Mah) were collected. These regions are located in northern parts of Pakistan with an inter-regional distance of at least 25 km. High prevalence of rainfall and low temperature are characteristics of these areas, thus, providing a suitable environment for natural late blight occurrence. The diseased samples were used for culturing of isolates of *P. infestans* at Plant Pathology laboratory, Arid University Rawalpindi. 5 mm pieces of diseased leaves from each region were placed on rye agar B which contained antibiotics (rifamycin, ampicillin, vancomycin) following the methods as listed on http://www.plantpath.cornell.edu/. To stimulate sporangial development, Petri plates were incubated at 18°C in dark for 4 days. Zoospores of pure cultures of isolates were obtained and from re-incubation of filtered sporangia on freshly prepared growth medium which lacked antibiotics as reported by Pliakhnevich and Ivaniuk (2008), and which were kept for two hours at 4°C.

Detached leaflet (DL) assay was conducted by plucking 8-week old leaflets from potato plant (cv Desiree). 5 leaflets, cleaned with distilled water, were placed adaxial sides up in Petri dishes lined with twice folded moist filter paper. A single Petri dish lined with twice folded moist filter paper containing 5 leaflets (cleaned with distilled water and adaxial sides up) was used for zoospore inoculation. Experiment was repeated five times. For tuber disc (TD) assay, healthy potato tubers (cv Desiree) were cleaned and surface sterilized with sodium hypochlorite, water, and ethanol as reported by Dorrance & Inglis (1997). 25 mm × 5 mm pieces of tuber discs were cut with a sterile cork-borer and placed on filter papers in a similar manner as proceeded in detached leaflet method. 20µl of zoospores representing each of the sample-collected regions were dropped on each leaflet in the center of midrib and in the center of tuber discs respectively. Petri dishes both for leaflet and tuber slices were incubated at 18°C (12 h photoperiod).

Data for disease severity (DS) were calculated as percentage of infected areas on leaflets and tubers on a scale 0-100%; where DS = 0% - single leaflet and tuber disc infected, DS = 60% - three leaflets and tuber discs infection, DS = 80% - four leaflets and slices infected and, DS = 100% -five leaflets and tuber discs infected (Carlisle *et al*., 2002). On detached leaflets and tuber discs, time (days) from the initial appearance of symptoms till sporangial development was calculated as latent infection period (LIP) as reported by Chacon *et al*., (2007). Lesion area was measured for each inoculation in DL and TD by the relation $1/4\pi \times$ length × width of lesions following the method of Vleeshouwers *et al*., (1999). In order to find the area under lesion expansion curve for each isolate on both detached leaflets and tuber discs, method of Chacon *et al*., (2007) was followed with slight alteration.

Statistical analysis

The experimental design was completely randomized. Data were compiled for analysis of variance and Duncan's Multiple Range Test for comparing differences in results.

Results

Data for the aggressiveness attributes of *P. infestans* in two tested assays are presented in Tables 1 and 2. Detached leaflet and tuber disc assays employed in the current study revealed that isolates of *P. infestans* had a similar tendency of the aggressiveness. Diseases severity, latent period, lesion area and AULEC for the studied isolates were almost similar to both tested methods. Numerical values obtained for the studied parameters were helpful enough to arrange different isolates of the pathogen under study in a sequential order to present their aggressiveness capacity. In detached leaflet and tuber disc assay, highest disease severity, lesion area, and AULEC were recorded for Sha, Bat, Nat, Ayy and Bar while lowest parameters were observed for Shb and Man isolates (Tables 1 and 2). Conversely, latent periods were maximum for Shb and Man but minimum for Sha, Bat, Nat, Ayy, and Bar isolates.

Disease severity: Disease severity (DS) caused by the fifteen isolates on DL and TD were compared and it was found that both methods had consistent and non-significant differences in results; however, inter-isolates variation for DS were significant (Fig. 1). In general, 5 isolates (Sha, Bat, Ayy, Bar, and Nat) revealed greater DS which ranged between 88 and 97% on DL and 94-95% on TD respectively. Nine isolates (Kag, Nar, Sho, Bal, Mah, Shn, Shg and Kal) caused DS in the range 71-78% on DL and 80-85% on TD respectively. Remaining two isolates (Shb and Man) showed lower disease severity which ranged between 59 and 63% on DL and 70% on TD respectively. Data indicated DS values on TD were comparatively larger than those on DL; however, they were consistent for tested isolates on each method.

Latent infection period: Calculated latent infection period (LIP) for fifteen isolates revealed similar aggressiveness pattern on the two methods with no significant variation (Fig. 2). Those isolates which had lower LIP values on DL showed lower values on TD as well. On DL method, lowest range of LIP 5.3-5.8 days was recorded for isolates Sha, Bat, Ayy, Bar, and Nat followed by 7.0-7.8 days for Kag, Nar, Sho, Bal, Mah, Shn, Shg and Kal which were relatively moderate while highest values 9.4-9.6 days were shown by Shb and Man isolates. Similar and consistent results were obtained on TD method.

Table 1. Aggressiveness attributes of different isolates of *P. infestans* on detached leaflet assay.

Isolates	Aggressiveness attributes			
	Disease severity (%)	Latent period (days)	Lesion area (mm^2)	Area under lesion curve
Sharan	97^{+}	5.6^{+}	9.5^{+}	26^{+}
Batakundi	92^{+}	5.8^{+}	9.4^{+}	24.8^{+}
Ayyubia	96^{+}	5.8^{+}	9.5^{+}	21.1^{+}
Baragali	88^{+}	5.8^{+}	9.7^{+}	22.9^{+}
Nathyagali	96^{+}	5.3^{+}	9.9^{+}	19.1^{+}
Kaghan	$74^{\S}$	$7^{\S}$	$7.8^{\S}$	$15.4^{\S}$
Naran	$75^{\S}$	$7.8^{\S}$	$8^{\S}$	$15.7^{\S}$
Shoughran	$79^{\S}$	$7.4^{\S}$	$7.9^{\S}$	$15.9^{\S}$
Balakot	$71^{\S}$	$7.5^{\S}$	$8^{\S}$	$15.9^{\S}$
Mahaban	$78^{\S}$	$7.5^{\S}$	$7.8^{\S}$	$16^{\S}$
Shinkyari	$74^{\S}$	$7.5^{\S}$	$7.5^{\S}$	$16^{\S}$
Shangla	$77^{\S}$	$7.5^{\S}$	$7.4^{\S}$	$15.9^{\S}$
Kalam	$77^{\S}$	$7.5^{\S}$	$7.8^{\S}$	$16.2^{\S}$
Shabqadar	63^{-}	9.6^{-}	6.3^{-}	10.3^{-}
Mansehra	59^{-}	9.4^{-}	6^{-}	8.2^{-}

Table 2. Aggressiveness attributes of different isolates of *P. infestans* on tuber disc assay.

Isolates	Aggressiveness attributes			
	Disease severity (%)	Latent period (days)	Lesion area (mm^2)	Area under lesion curve
Sharan	95^{+}	6.1^{+}	15.1^{+}	37.4^{+}
Batakundi	95^{+}	6.3^{+}	15.5^{+}	38.7^{+}
Ayyubia	94^{+}	5.9^{+}	15.6^{+}	38^{+}
Baragali	95^{+}	5.9^{+}	15.9^{+}	33.3^{+}
Nathyagali	95^{+}	6.2^{+}	15.7^{+}	37^{+}
Kaghan	$83^{\S}$	$8.2^{\S}$	$9.7^{\S}$	$29^{\S}$
Naran	$84^{\S}$	$7.9^{\S}$	$9.6^{\S}$	$27^{\S}$
Shoughran	$85^{\S}$	$7.9^{\S}$	$10^{\S}$	$27.9^{\S}$
Balakot	$80^{\S}$	$8.1^{\S}$	$10.3^{\S}$	$28.3^{\S}$
Mahaban	$84^{\S}$	$8.2^{\S}$	$10^{\S}$	$25.8^{\S}$
Shinkyari	$80^{\S}$	$8.2^{\S}$	$9.6^{\S}$	$29^{\S}$
Shangla	$82^{\S}$	$8.3^{\S}$	$9.6^{\S}$	$28.4^{\S}$
Kalam	$85^{\S}$	$8^{\S}$	$10^{\S}$	$26^{\S}$
Shabqadar	70^{-}	9.8^{-}	6.1^{-}	18^{-}
Mansehra	70^{-}	10^{-}	5.9^{-}	19.5^{-}

+, Aggressive; §, Intermediate; -, Mild. Isolates were arranged in descending order for disease severity, lesion area and AULEC while in ascending order for latent period

Lesion area: Isolates Sha, Bat, Ayy, Bar, and Nat showed higher lesion area (LA) which ranged between 9.4 and 9.9 mm2 on detached leaflets and 15.1 and 15.9 mm2 on tuber disc methods respectively (Fig. 3). Genotypes Kag, Nar, Sho, Bal, Mah, Shn, Shg, and Kal exhibited relatively intermediate LAs which ranged between 7.4 and 8 mm2 on DL while 9.6-10.3 mm2 on TD respectively. Conversely, isolates Man and Shb revealed lowest LA which ranged between 6 and 6.3 mm2 on DL and 5.9 and 6.1 mm2 on TD respectively. Results also showed that there was no significant variation between the two methods tested which indicated consistency for the LA but variation among different isolates on each method was significant.

Area under lesion expansion curve: Data presented in Fig. 4 exhibit different values of area under lesion expansion curve (ALEC) for different isolates of *P. infestans*. It was evident that isolates collected from Sharan, Batakundi, Ayyubia, Baragali and Nathyagali resulted in higher ALEC values on the detached leaflet and tuber disc assay than the rest of the isolates. In both assays, minimum ALEC values were observed for isolates Shb and Man. Intermediate values were obtained for isolates Kag, Nar, Sho, Bal, Mah, Shn, Shg, and Kal. ALEC values for isolates Sha, Bat, Ayy, Bar and Nat ranged between 19.1 and 26 on detached leaves while 33 and 38.7 on tuber disc methods. Data clearly indicated highest aggressiveness potency of these isolates. For isolates Kag, Nar, Sho, Bal, Mah, Shn, Shg and Kal somewhat intermediate aggressiveness effects were recorded and their ALEC values were found in the range of 15.4-15.9 on detached leaves whereas 25.8-29 on tuber disc method. Isolates Shb and Man exhibited mild aggressive characters by causing lowest ALEC on detached leaflets (8.2-10.3) and tuber disc method (18-19.5) respectively. Like other aggressive characters in the study, isolates and experimental models varied to different extent; however, each method for a particular isolate revealed consistent results.

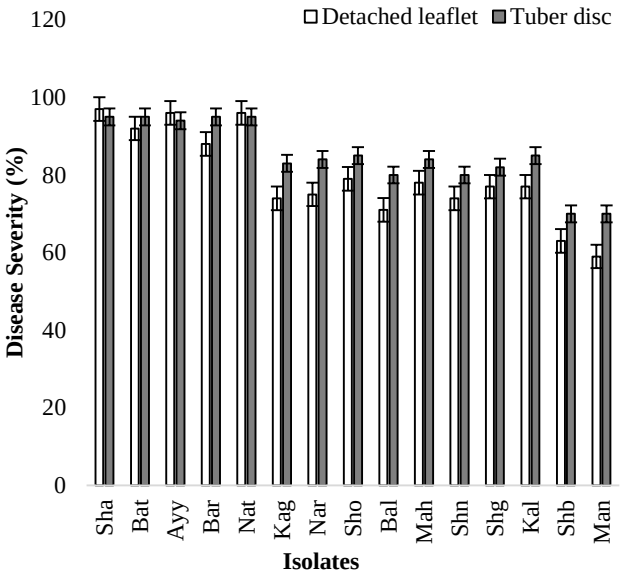

Fig. 1. Percent disease severity of *Phytophthora infestans'* isolates on detached leaves and tuber slices.

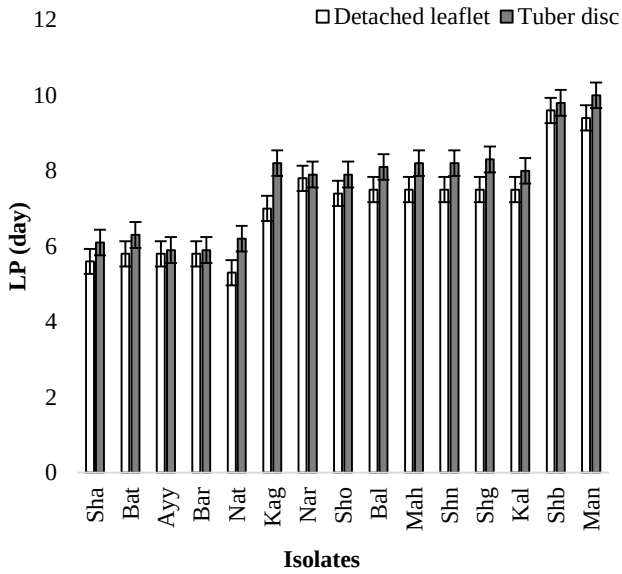

Fig. 2. Latent period of different isolates of *P. infestans* on after inoculation on detached leaves and tuber discs.

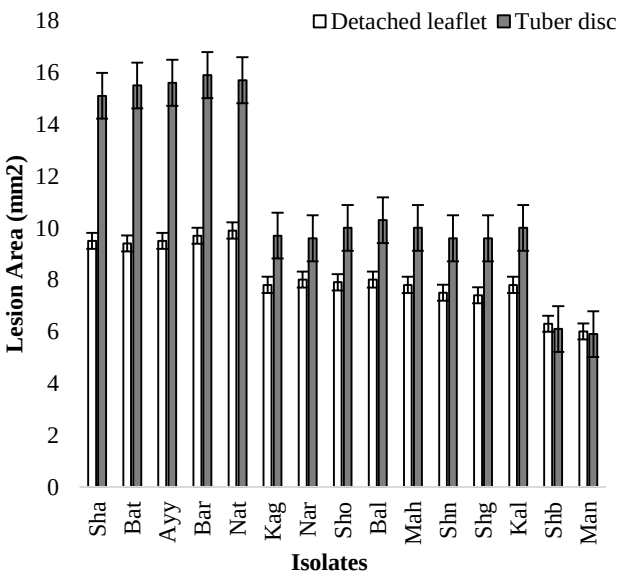

Fig. 3. Lesion area of different isolates on detached leaves and tuber discs.

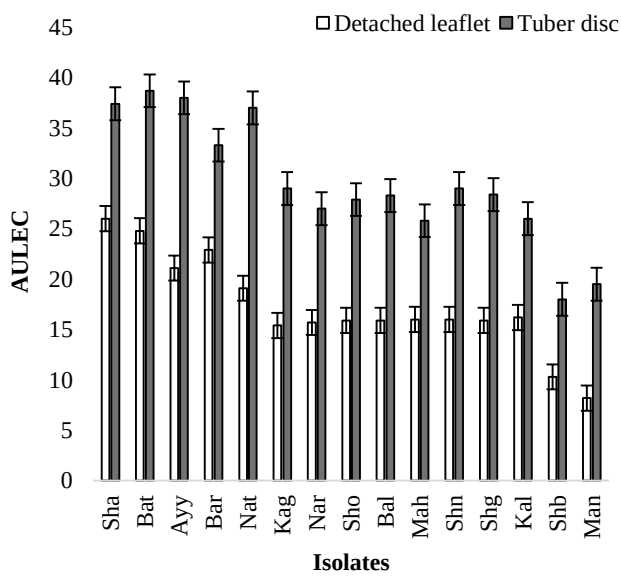

Fig. 4. Area under lesion expansion curve of different isolates of *P. infestans* on detached leaves and tuber discs. Sha –Sahran; Bat –Bata Kundi; Ayy –Ayyubia; Bar –Bara Gali; Nat –Nathya Gali; Kag –Kaghan; Nar –Naran; Sho –Shougran; Bal –Balakot; Mah –Mahaban; Shn –Shinkiari; Shg –Shangla; Kal –Kalam; Shb –Shabqadar; Man –Mansehra.

In general, isolates were arranged in descending order for disease severity, lesion area and area under lesion expansion curve. While in ascending order for LIP which depicted three categories of the isolates on the basis of aggressive outputs, a) aggressive those with higher DS, LA, and ALEC but lower LIP, b) intermediate intermediate DS, LA, ALEC and LIP values and c) mild – lower DS, LA and ALEC but higher LIP values (Fig. 5).

Discussion

Identification of suitable methods for testing the aggressiveness potential of different genotypes of *P. infestans*, the resistance of cultivars and disease reactions are necessary for time, labor and cost management (Dorrance & Inglis, 1997; Brooks, 2008). Laboratory studies for such tests seem to be ideal because of the easy availability of plant materials for inoculation during off-seasons, rapid assay and comparatively less labor input as compared to field trials. Our results agreed with previous studies (Vleeshouwers *et al*., 1999; Huang *et al*., 2005; Foolad *et al*., 2015) which had described detached leaflet methods as accurate, reliable, repeatable and consistent to field screening for the aggressiveness, partial resistance studies and virulence testing of *P. infestans*. In other similar studies, results of detached leaflets and whole plants encompassing the resistance attributes of P. infestans were compared with field conditions which demonstrated close consistency (Nowakowska *et al.*, 2014). Nath *et al*., (2016) have also shown that modified

detached leaflet yielded consistent results to field assessment for separating susceptible and resistant varieties of Taro to leaf blight caused by *P. colocasiae*. Recently, Majeed *et al*., (2018) demonstrated the variation in the leaf and stems of potato, tomato and eggplant in response to *P. infestans* infection. Dorrance & Inglis (1997) preferred greenhouse experiments over laboratory methods for evaluation of the aggressiveness and cultivar resistance tests because greenhouse methods could be used for a large number of diseases components while lab methods could be partially effective in the assessment of specific disease components. These studies do not support our results which may be due to different experimental conditions, cultivars and races of *P. infestans.*

In our study, leaflets and tuber of the same cultivar (Desiree) were used under uniform experimental conditions using the same amount of spore inoculation while isolates of *P. infestans* were variable. Aggressiveness ranking on the isolates were similar to both methods which indicated that leaflets and tubers of cv Desiree responded similarly in terms of resistance or susceptibility irrespective of the methods used; however, isolates of *P. infestans* varied considerably in their aggressiveness which might be due to different aggressiveness capacity and population composition in the locations from where isolates were collected. Tuber discs revealed higher values of the disease components than detached leaflets although that elevation did not affect the consistency of results which could be attributed to relatively soft tissues of tubers, stimulating the spread of disease more rapidly than detached leaflets.

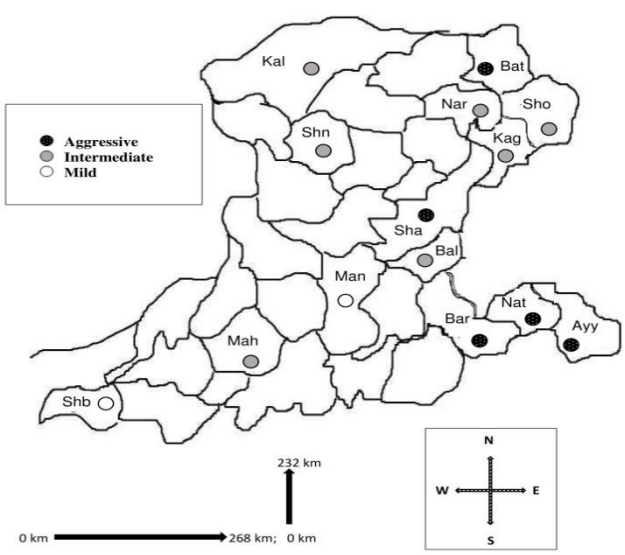

Fig. 5. Degree of aggressiveness of different isolates of *Phytopthora infestans* on the basis of aggressiveness components.

Conclusion

Detached leaflet and tuber disc methods we used for testing the aggressiveness potential of 15 different genotypes of *P. infestans*. The results were reliable and consistent and could be used for rapid evaluation of disease assessment. In respect to other studies which widely use detached leaflet method, we report tuber disc method as reliable, efficient and suitable particularly during the off-season when plant leaves are not available.

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