The Development of Simple Methods for the Maintenance and Quantification of *Polymyxa graminis*

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Abstract *Polymyxa graminis*, a root endoparasite of several cereal species, is considered to be non-pathogenic but serves as a vector of various plant viruses belonging to the genera *Bymovirus*, *Furovirus*, and *Pecluvirus*. Specifically, it reduces barley productivity by transmitting the *Barley Yellow Mosaic Virus* (BaYMV). To date, due to its obligate biotrophic property, no artificial culturing of *P. graminis* was reported and its quantification was also technically challenging. Here, we developed a novel and simple method to infect *P. graminis* within sterile barley roots in contamination free by preparing nearly pure zoospore inoculum. Such artificial maintenance of *P. graminis* was verified based on the presence of various developmental stages in infected barley roots under microscope. In addition, the population of resting spores in host tissue was determined by establishing standard curve between manually counted number of spores and C_t values of 18S rDNA amplification using quantitative real-time PCR. Furthermore, it was validated that standard curve generated was also applicable to estimate the abundance of *P. graminis* in soil environments. In conclusion, the present study would help to generate a system to investigate the etiological causes as well as management of plant diseases caused by *P. graminis* and BaYMV in tissue and soil.

Keywords Barley · *Polymyxa graminis* · Real-time PCR · Resting spore · Zoospore

Introduction

Polymyxa graminis is a soil-borne intercellular parasite of plant roots and belongs to the order Plasmodiophorales [1]. *P. graminis* itself causes no detrimental effects on its host plant. However, several plant viruses from the genera *Benyvirus*, *Bymovirus*, *Furovirus*, and *Pecluvirus*, including *barley yellow mosaic virus* (BaYMV), *barley mild mosaic virus* (BaMMV), *wheat spindle streak mosaic virus* (WSSMV), *wheat yellow mosaic virus* (WYMV) and *soil-borne wheat mosaic virus* (SBWMV) were reported to utilize *P. graminis* as a vector for the host infection [2, 3]. These viruses are pathogenic and cause yield reduction of cereals in both temperate and tropical region [4, 5]. *P. graminis* is found in almost all types of soils where grasses are grown. *P. graminis* infects and spreads from plant to plant by forming motile zoospores and survived in the soil for many years as thick-walled resting spores or cystosori [2, 6–9].

Polymyxa graminis exhibits the following distinctive life cycle (Fig. 1). In soil, biflagellate primary zoospores (PZo) are induced from resting spores (RS). Motile primary zoospores are penetrated into root hairs, encysted, enlarged within the host cell and undergo several cycles of synchronous mitotic nuclear divisions (cruciform) which develop into multinucleate structure called as primary plasmodium (PP) [7, 10]. As the root hair elongates, the plasmodia also increase in size/number and turn into primary zoosporangium (PZ) [7, 10–12]. The segments of primary zoosporangium form small exit tubes (ET) which release secondary zoospores (SZo). Secondary zoospores

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Fig. 1 The short diagrammatic representation of the life cycle of *P. graminis* in barley root tissues

are either released into the soil or infect into the root hair of other hosts. Alternatively, secondary zoospores also possibly enter the cortex of the hosts and instigate a new sporangial phase, resulting in the production of sporogenic plasmodia and resting spores (RS). In the cortex, zoospores are developed into secondary multinucleate plasmodium (mSP). This structure is propagated into sporulating secondary plasmodia (sSP) which further transformed into the resting spores having individual cell walls and nucleus [13]. The clusters of resting spores are either present in root tissue or released into the soil [10–13].

In general, in vitro culturing is essential to investigate the pathogenesis-related properties of microbes and develop the efficient methods to control the diseases. Previous studies attempted to maintain *P. graminis* on various synthetic media or in sand. However, due to its obligate nature and relatively long life cycle, it is known to be difficult to culture in vitro or in artificial nutrient media in a pure fashion [6, 7, 14]. In addition, many approaches to isolation were also found contaminated

with soil microorganisms like *Olpidium radicale* and other fungi [14–17].

On the other hand, the accurate measurement of population of pathogens around hosts is a useful parameter to predict the disease incidence. In case of *P. graminis*, the quantification of resting spores will be more accurate than zoospores which are unstable and variable depending on the soil conditions. Up to date, bait plant bioassays and determination of the most probable numbers (MPN) of infective propagule of resting spores have been used to estimate abundance of *P. graminis* in a variety of ecological and epidemiological studies [7, 18–21]. However, these methods are indirect, inaccurate, and time-consuming, which require more than 8 weeks to complete a single soil sample. In recent years, alternatively, advanced approaches using molecular biology to quantify *P. graminis* by serological methods [1, 19], conventional and real-time PCR have been simplified and used [22–24]. Mutasa-Gottgens et al. [19] expressed *P. graminis* c-DNA in in vitro condition, purified protein and used these purified

proteins to raise antibodies against *Polymyxa*. On other side, polyclonal antibodies were developed by Delfosse et al. [1] when resting spore suspension was used as antigen. Both methods were able to detect very low level of inocula in root material but found to be less sensitive when compared with PCR based methods [3, 23]. The real time PCR assay developed by Vaianopoulos et al. [4] and Ward et al. [23] and others [3, 22] was found 50–100 times more sensitive than ELISA techniques [1, 19, 24, 25]. However, most of studies were established based on pure spores isolated from infected host tissues [3, 4, 25, 26], which need to be validated for the application to soil environment where diverse fungal species present. Therefore, the development of a novel, quick, and simple technique to quantify the population of *P. graminis* directly in soil would be useful for epidemiological studies and assessment of disease risk.

In this study, to investigate the various properties and accurate quantitation of *P. graminis*, we established the conditions required for maintenance of the pure culture of *P. graminis* within barley roots in vitro. In addition, we developed a method to measure the population of *P. graminis* that can be rapidly performed using real-time PCR.

Materials and Methods

Preparation of Zoospore Inoculum

To infect *P. graminis* into barely roots in nearly pure fashion, we standardized the protocol for the pre-treatments and fractionation processes of zoospore inoculum (Fig. 2). Barley plants showing BaYMV disease symptoms were obtained from the field at Iksan (35°56′24.5″N 126°55′29.7″E), Korea in May, 2014 and 2015. Root samples were collected and examined for the presence of

Fig. 2 The standard procedure of the preparation of nearly pure zoospore inoculum of *P. graminis*. The barley plants infested by BaYMV and showing yellow stripe symptoms in the field were chosen for the collection of *P. graminis* infected root, these infected roots were washed and processed and used for isolation of zoospore of *P. graminis* (see also “Materials and Methods” section)

resting spores of *P. graminis* using Leica EC3 stereo microscope (Leica Microsystems, Switzerland). The sporosori-containing root parts were cut and surface sterilized by vortexing in 0.1 % mercuric chloride for 2 min, followed by washing with sterile distilled water for five times and grinded to fine powder in liquid nitrogen. Afterwards, 1 g of fine root powder was mixed with 14 ml of distilled water in 50 ml polystyrene tube, covered with aluminum foil, and then incubated overnight at 4 °C to induce zoospores. Root powder suspension was centrifuged either at 4000, 6000, or 8000 rpm for 10 min and then supernatant (~8 ml) was retrieved carefully to a fresh tube. Subsequently, the supernatant was layered on the top of the two layers of sucrose solutions composed of 20 % (top) and 60 % (bottom), respectively. Then, it was centrifuged at 6000 rpm for 5 min. Final supernatant was examined for the quantity and purity of zoospores. The average numbers of zoospores were counted using haemocytometer (Neubauer-improved, Lauda-Königshofen, Germany) from three independent experiments containing triplicates for each.

Inoculation of Zoospore into Barely

Barley seeds were disinfected by soaking in 0.1 % mercuric chloride for 1 min, followed by rinsing three times with sterile distilled water [27]. The seeds were pre-germinated on the moistened filter paper at 20 °C for 3 days in dark conditions. Germinated seedlings were then transferred into hydroponic culture system containing perlite, MS salts (Murashige and Skoog media) 4.4 g/l, MES 0.5 g/l at 22 °C [28]. In the hydroponic system, plant roots were easily manipulated without disturbance. After 10 days, the roots were inoculated with zoospore inoculum ($\sim 1 \times 10^5$ zoospores/ml) by directly dipping for 1 h at room temperature. Roots of inoculated plants were surface sterilized again with 0.1 % mercuric chloride for 10 s, followed by washing with sterile distilled water three times. Post-inoculated sterilized plants were transferred back to MS media containing 0.3 % agar and further incubated. The seedlings without zoospore inoculation under the same conditions were also kept as an uninfected negative control.

Microscopic Observation of Zoospore Infected Roots

After inoculation of zoospore, barley roots were removed from MS-containing magenta boxes at various time courses and vigorously washed in sterile water. Plant roots were cut to be 1 cm in length and examined under a Carl Zeiss Axio Imager A2™ light microscope with bright field illumination. The infection level of *P. graminis* in root system was assessed by following Legrève et al. [29] with

modifications. Twenty plants were examined for the presence of different stages of *P. graminis* in the root tissues. The subsequent infection (i.e. secondary infection) of *P. graminis* from primarily infected barley to fresh barley roots were also assessed in this study. Infection rate was determined by three independent experiments having triplicates for each.

Extraction and Counting of Resting Spores of *P. graminis*

The powder (100 mg) of roots or soil (1 g) potentially containing *P. graminis* were soaked in 5 ml of 1 % sodium meta hexa phosphate buffer containing Tween 20 (0.25 %) and phenyl acetic acid (final concentration 50 µg/µl) [30–32]. Then, the suspension was homogenized using Tissuelyser LT (Qiagen, Hilden, Germany) and subjected to centrifugation at 16,000 rpm for 5 min. Supernatant was filtered through eight layers of Miracloth (Calbiochem, San Diego, CA), followed by centrifugation at 3000 rpm for 5 min. Then, the filtrates were mixed (1:1 = v/v) with 200 µg/ml of Fluorescent Brightener-28 (Sigma, Saint Louis, MO) [31] to visually distinguish resting spores. The number of resting spores emitting 405 nm of fluorescence was counted on haemocytometer (Neubauer-improved, Lauda-Königshofen, Germany) under fluorescent microscope.

PCR-Mediated Detection of *P. graminis* in Infected Barley Roots and Soil

Total DNA was extracted from barley roots and soil using Exgene™ Plant SV mini kit (GeneAll, Seoul, Korea) and UltraClean Soil DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA), respectively. Both methods were processed by following the manufacturers' instructions. A pair of primers-Pgfw2 (5'-GGA AGG ATC ATT AGC GTT GAA T-3') and Pxrev7 (5'-GAG GCA TGC TTC CGA GGG CTC T-3')-was used to amplify a 270 bp fragment of the nuclear ribosomal DNA of *P. graminis* [33]. The standard PCR protocols used for the detection of *P. graminis* were adapted from Ward and Adams [33]. The PCR reaction was performed in a 50 µl reaction volume containing 100 ng DNA, 5 µl ExTaq reaction buffer, 4 µl dNTPs (0.25 mM), 0.5 µl Pxrev7 primer (10 pM), 0.5 µl

Pgfw2 primer (10 pM), and 1U ExTaq polymerase (TaKaRa ExTaq® DNA Polymerase, China). Amplification was performed using a GeneAmp 9700 PCR thermocycler (Applied Biosystems, CA, USA) using program as 95 °C for 5 min, 35 cycles of 95 °C for 30 s, 53 °C for 30 s and 72 °C for 30 s and final synthesis for 7 min at 72 °C. Barley roots free from *P. graminis* were used as a negative control. The amplified products were visualized by ethidium bromide staining on 1.0 % of agarose gel.

In the case of the quantitative real-time PCR, total DNA was extracted from resting spore suspension using Exgene™ Plant SV mini kit (GeneAll, Seoul, Korea). PCR reaction was performed in Rotor-Gene™ 6200 real-time thermal cycler (Corbett Research, Mortlake, NSW, Australia). The PCR mixture (20 µl) was prepared with 10 µl of SYBR Green Master mixture (Applied biosystems, San Diego, CA), 0.5 µl of reverse and forward primer (10 pM), and 100 ng of template DNA. The amplification reaction was conducted based on thermo cycles (three step melt) as follow: with 40 cycles, each composed of (1) 95 °C for 10 s, (2) 55 °C for 30 s and (3) extension at 72 °C for 30 s. The reaction mixture without DNA template was used as negative control.

Statistical Analysis

Data were analyzed either by independent sample *t* tests or analysis of variance (ANOVA) with a Tukey's multiple comparison tests using the statistic package of software GraphPad Prism 5 In-Stat version 5.02.

Results

Isolation of *P. graminis* from Barley Roots

To isolate *P. graminis* in a pure fashion, we primarily extracted the zoospores from the *P. graminis* infested barley roots showing the *barley yellow mosaic virus* disease symptoms and enriched them using various centrifugation methods (Fig. 2). Then, the purity and the number of zoospores at each step were examined in quantitative fashion (Table 1). When root powder suspensions were subjected to centrifugation with 4000, 6000, or 8000 rpm. However, even after centrifugation, the supernatants still

Table 1 The efficiency of zoospore fractionation of *P. graminis* under different centrifugation conditions

Centrifugation	4000 rpm	6000 rpm	8000 rpm
Zoospore(s)/ml	$1.5 \pm 0.21 \times 10^5$ ^a	$0.5 \pm 0.26 \times 10^5$ ^b	$0.3 \pm 0.21 \times 10^5$ ^c

Values are means of three experiments containing triplicates for each. Statistical significance of data was differentiated as lower case of characters by one way ANOVA at *P* value (<0.05), followed by Tukey–Kramer Multiple Comparison Test

contained lots of cell debris. To separate zoospores from other contaminants further, we subsequently applied additional centrifugation at 6000 rpm in presence of 20–60 % of sucrose solution. Sucrose density removed most of cell debris. In the result, when supernatant from 4000 rpm-centrifugation followed by sucrose density centrifugation exhibited the highest number of zoospores (Table 1). When the isolated zoospores were infected to barley roots, neither fungal nor bacterial contamination was observed in the media (data not shown). Based on these, a procedure of the preparation of zoospore inoculum was standardized (Fig. 2).

Maintenance of Zoospores into Barley Roots

The infection efficiency of prepared inoculum (i.e. *P. graminis* zoospores suspension) was monitored by examining the presence of various morphological features of *P. graminis* in the root cells of infected barley seedlings (Fig. 3a). In the root sections, various developmental features such as multiplied forms of primary zoospores (PZom), primary plasmodia (PP) associated with host wall (HW), primary zoosporangia (PZ) and mature primary zoosporangium (mPZ), exit tube (ET), secondary plasmodium (mSP), sporulating plasmodium (sSP) and resting spores (RS) were observed (Fig. 3b–i). These structures were seen in the primary root cortex as well as in the root hairs. The efficiencies of infections were quantitatively determined based on the percentage of infected epidermal cells out of total epidermal cells in cm² of root. According to the infection, the resting spores of *P. graminis* were observed in approximately 60–80 % of root epidermal cells. On the other hand, to verify whether such endophytic features were due to the infection of *P. graminis*, the zoospores were re-isolated and subjected to PCR reaction using *P. graminis* rRNA specific primer set. As a result, 270 bp of amplicon was obtained from infected barley roots whereas no product was amplified in uninoculated control plants (Fig. 3j). The nucleotide sequencing of the amplicon verified the PCR product was the fragments of *P. graminis* rRNA. Additionally, we also attempted to detect presence of bacteria by using universal primers for bacterial 16S rRNA sequences, but no amplicon was produced (data not shown). This further indicated that the zoospore inoculum prepared in current study was efficiently infected to the barley roots without bacterial contamination.

To investigate whether the inoculation of zoospore is sustainable throughout multiple rounds of infection, we re-isolated zoospores from primarily infected roots and inoculated to the fresh barley seedlings. In the case of the secondary infection, the roots were found to contain encysted zoospores (EZ) and plasmodium (PP) of *P. graminis* after 21 days of inoculation (Fig. 3k–m). Combined

all, artificial infection of zoospores provided a reliable system to maintain *P. graminis* in barley seedlings.

Quantification of *P. graminis* Resting Spores Using Real-Time PCR

In current study, zoospore suspension was shown to be efficient to infect barley roots in vitro condition. On the other hand, in field condition, zoospores are very small in size and their numbers are not stable, which hampers to estimate the population of *P. graminis* in accurate fashion. Alternatively, it will be informative to examine the population of resting spores rather than zoospores to predict the *P. graminis* mediated BaYMV disease incidence in soil. To measure the population of *P. graminis*, we isolated the resting spores from infected. In lysis buffer, phenyl acetic acid was added to prevent zoospore induction. Tween 20 and sodium hexa-metaphosphate were also treated to facilitate the dissociation of resting spore cluster. Under these conditions, nearly pure resting spores were retrieved without zoospores and plant debris (data not shown). Spores were easily distinguished from soil particles by adding fluorescent dye (see “Materials and Methods” section) and appeared as round—irregular shaped granules, 5–9 µm in diameter. The number of resting spores in lysis suspension was assessed using haemocytometer. The numbers of resting spores were ranged from 1.2×10^5 to 2.6×10^6 (Fig. 4) in 100 mg of root powder of the individual barley plants.

Subsequently, we isolated total DNA from the same root powder and measured the C_t values of amplification of *P. graminis* 18S rRNA coding gene by real time PCR. The calculated C_t values of the samples tested were ranged from 18 to 29 cycles (Fig. 4). Then, finally, we established the standard curve between manually counted spore numbers and C_t values in real time PCR (Fig. 4). As expected, the spore numbers were proportional to C_t values in reciprocal way. The resulted plot was well fitted into the first order of regression of which correlation coefficient (R²) was 0.966 (Fig. 4). On the other hand, the standard curve was developed based on the density of resting spores isolated from barley root tissue. To validate this method in field conditions, we examined the number of resting spores present in soil samples from four different locations by manual counting. Then, we compared it to the predicted population extrapolated from standard curve generated in this study (Table 2). According to manual counting, resting spores were present around $1.8 \pm 0.3 \times 10^6$ to $2.3 \pm 0.5 \times 10^6$ in 1 g of soil samples. The predicted numbers of resting spores were ranged from $2.0 \pm 0.1 \times 10^6$ to $2.4 \pm 0.3 \times 10^6$, which were similar to the values obtained by manual counting. In statistical aspects, the numbers of resting spores measured by manual

Fig. 3 Different stages of *P. graminis* in barley roots infected by zoospore inoculum. **a** Infected barley plant after 4 weeks of inoculation. **b** The multiplication of primary zoospore (PZom) in barley root tissues after 6 days of inoculation with *P. graminis* zoospore suspension. Line indicating the crucifer division of encysted zoospore. **c** Primary plasmodium (PP) in close association with host nucleus (HN). **d, e** Developing primary zoosporangium (PZ) after 4 days of incubation. At this time primary zoosporangium wall (PZW) is clearly visible and differentiated from host wall (HW). Primary zoosporangium turned into multinucleate mature primary zoosporangium (mPZ). **f** Exit tube (ET) formation towards HW from PZW. **g** Exit tube abutted with HW. **h** Secondary mature plasmodium (mSP) and sporulating plasmodium (sSP) with distinct plasmodium wall (PW). **i** Resting spore cluster formation observed after 5–6 weeks of incubation. Lines indicates resting spore (RS), resting spore wall (RW) distinct from host wall (HW). **j** PCR-mediated detection of *P. graminis* from infected barley root. M, molecular markers; P0 un-infected barley control P1 *P. graminis* infected roots. **k** Formation of encysted zoospore (EZ) in infected barley root hair from secondary infection, lines indicates encysted zoospore in root hair (RH). **l** Primary plasmodium (PP). **m** Mature secondary plasmodium (mSP) in infected barley roots

Fig. 4 The standard curve between the numbers of resting spores and C_t values of real-time PCR mediated amplification of *P. graminis* DNA fragments. The resting spores were isolated from barley roots infected by nearly pure zoospore inoculum of *P. graminis*, followed by counting using haemocytometer. Total DNA was also isolated from the resting spores and subject to *P. graminis* DNA fragment specific real-time PCR. The plots were regressed by first order and its equation was designated on the graph. R^2 correlation coefficient

counting and prediction by real time PCR showed the no significant difference at 95 % confidence level in student t tests (Table 2). Combined all, the standard curve driven

by the correlation between resting spore numbers and C_t values of real time PCR for 18S rRNA fragments provided the reliable prediction to determine the abundance of *P. graminis* in host tissues or soil.

Discussion

Traditional etiology and quantification of pathogenic microbials largely rely on the pure culturing, followed by counting of colonies or spores. However, such techniques are not feasible for absolute obligate organisms like *P. graminis* which has been known to be recalcitrant for in vitro culturing on artificial media. Current study reported the practical approaches to solve such problems on *P. graminis* by developing (1) a method to isolate and maintain *P. graminis* in nearly pure fashion in barley roots, and (2) a method to rapidly quantitate *P. graminis* population in soil/roots using real-time PCR.

To prepare nearly pure *P. graminis* culture in barley roots, an efficient surface sterilization of *Polymyxa* infected roots must be critical. In our study, disinfection of roots with mercuric chloride at low concentration (0.1 %) annihilated the most of unwanted microbes present on the surface of roots and other endophytic bacterial population. In contrast, it caused no impact on the viability of *P. graminis* spores. It also should be noted that sucrose density gradient during fractionation steps of zoospores efficiently removed cell debris of barley tissues (Fig. 2). The application of dense sucrose arrested the plant debris and other contaminants in the pellets at low centrifugation speed and placed zoospores in upper layer (Table 1). Successful preparation of nearly pure zoospore preparation allowed maintenance and propagation of *P. graminis* within the barley roots in contamination free. When using total DNA isolated from infected barley roots as a template in PCR reaction with primer sets targeting for fungal 18S rRNA and bacterial 16S rRNA, no amplified product was obtained except for the one of *P. graminis*, further verifying the absence of other microbial contaminants (Fig. 3j).

For the epidemiological study of BaYMV, it is essential to develop a reliable method to estimate the abundance of

Table 2 Validation of real-time PCR method to determine the abundance of *P. graminis* in soil samples

Experiment	C_t values	Number of resting spores/g of soil (by standard curve)	Number of resting spores/g of soil (manual counting)	P value (<0.05)
1	19.81 ± 0.07	$2.06 \pm 0.01 \times 10^6$	$1.92 \pm 0.04 \times 10^6$	0.51
2	19.86 ± 0.45	$2.05 \pm 0.10 \times 10^6$	$1.88 \pm 0.32 \times 10^6$	0.48
3	18.11 ± 0.06	$2.46 \pm 0.14 \times 10^6$	$2.34 \pm 0.42 \times 10^6$	0.50
4	18.31 ± 0.14	$2.41 \pm 0.32 \times 10^6$	$2.26 \pm 0.52 \times 10^6$	0.41

Values are means of three experiments containing triplicates for each. Statistical significance of data was differentiated by unpaired t tests

P. graminis in soil. The accurate estimation of the total number of sporosori in soil would be informative to predict the incidence of BaYMV causing disease. However, since resting spores usually grouped in a three dimensional compact forms such a cystosori or resting spore clusters, it is difficult and tedious to determine the abundance of spores in the field. In our study, efficient determination of resting spore population was developed using several combinatorial treatments of chemicals in extraction buffer. Tween 20, a non-ionic surfactant, facilitated the dissociation of resting spore clusters [30]. PAA inhibited zoospore germination from resting spores [32]. Mixture of these chemicals assisted the release of spores from root tissues, reduced the chance of zoospore induction and made resting spores disable to form cluster and stick to root debris. In this way, maximum number of resting spore could be extracted and their counting was easily achieved using haemocytometer (data not shown).

Previously, quantification of *P. graminis* using serological methods [1, 19], conventional and real-time PCR have been reported [3, 4, 9, 22–26]. Monoclonal and polyclonal antibodies developed by Delfosse et al. [1] and Mutasa-Gottgens et al. [19] respectively were found to detect one zoospore per gram of root material. However, the accuracy was variable depending on the amount of sample. On the other hand, the real-time PCR assays reported the high sensitivity and specificity to detect a variety ribotypes of *P. graminis*. However, most of methods were based on the pure spores isolated from infected host tissues. Considering high level of fungal and bacterial diversity present in field conditions, such methods still need to be verified in soil samples. In our study, after we establishing the correlation between spore numbers and C_t values of 18S rDNA using purely isolated resting spores in barley root tissue (Fig. 4), we further validated the standard curve was also well fitted into prediction of spore abundance in soil conditions (Table 2). This quantification method could give accurate measurement about presence of the population of vector in the soil so it would be useful for epidemiological studies and assessment of disease risk prior to planting.

Conclusion

In current study, we developed short, efficient and sensitive methods to maintain and quantify *P. graminis* in living barley root cells. Contamination free *P. graminis* maintained within barley roots can render the controllable systems for the artificial infection of obligate autotrophic fungi to understand the various characteristics of *P. graminis* and the tripartite interaction among vector parasites, host plants, and pathogenic viruses like BaYMV (*Barley yellow*

mosaic virus) and WSSMV (*Wheat spindle streak mosaic virus*) etc. On other hand, previously, many versatile and advent approaches available to detect spores in infested host root tissues by *P. graminis*. However, whether those techniques were applicable to assess the abundance in soil environment were yet to be validated. Our study verified that the standard curve developed based on the resting spores in host root tissue was also applicable to real field conditions. This will provide as a potential application in early diagnosis, screening of field soil and identification of virus reservoirs in agricultural environments.

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Author's contribution S. Tyagi, and R. Sultana, contributed equally to conceived the study, carried out experiments, and wrote the manuscript. H. J. Ju, W. H. Lee, B. Lee, and K. J. Lee, aided the interpretation of results and participated in editing. K. Kim supervised the experiments and edited the manuscript.

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

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