qPCR as a decision-making tool for Plasmodiophora brassicae forecast in the field

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Abstract

Clubroot caused by *Plasmodiophora brassicae* is a very destructive soilborne disease of brassica plants worldwide. The pathogen survives in soil with resting spores, that are produced in large numbers inside infected roots and can persist in the soil for up to 17 years. These resting spores can build up in the soil when brassicas are repeatedly grown, increasing the inoculum potential, leading to increased disease incidence and severity in subsequent crops. Infection by this pathogen can result in considerable yield and quality losses in susceptible crops, accounting for a 20–90% yield loss of broccoli, cauliflower, and cabbage crops in Colombia. A national survey to determine the extent of clubroot in Colombia was done in 2017, and it was found that 53.7% of the visited fields had clubroot disease on the crops, but the pathogen DNA was detected by PCR methods on 91.3% of the surveyed fields in all the departments where crucifers are grown. At the time of the survey, no symptoms of clubroot disease were observed in Nariño department, despite finding the pathogen DNA at concentrations varying from $2x10^3$ to $2x10^4$ resting spores per gram of soil. However, during the year 2020, Nariño farmers reported the presence of a disease, causing wilting and plant death of their cruciferous crops. Here we report that *P. brassicae* is the causal agent of the newly appeared disease in brassica crops in Nariño. The aims of this study were to determine the inoculum threshold needed to cause disease and the inoculum concentration that can be detected in both artificially and naturally infected soils to use them as tools for *P. brassicae* management and control. We propose the use of qPCR as a decision-making tool for cruciferous growers.

Introduction

Plasmodiophora brassicae Woronin is a soil-borne obligate biotroph pathogen, which causes clubroot on plants of the Brassicaceae family, posing a serious economic threat in oilseed rape and vegetable brassicas growing areas worldwide. Crop losses due to clubroot are estimated at 10–15% worldwide (Dixon, 2009, 2014). The disease has become a serious threat to canola industry in Canada, as heavily infected fields cause yield losses of 30–100% (Strelkov & Hwang, 2014), while in cruciferous crop production zones from China, the fast spread of the disease has caused 20–30% annual yield losses (Ning et al., 2018). In Colombia, infection by *P. brassicae* can result in considerable yield and quality losses in susceptible crops, accounting for 20–90% yield loss of broccoli, cauliflower, and cabbage crops (Riascos et al., 2011; Tamayo & Jaramillo, 2004). Because Cruciferous crops in the country are mostly grown by smallholder farmers in areas with a national average of three hectares, clubroot disease is a significant threat that impacts food security (Botero-Ramírez et al., 2022).

The typical symptoms of clubroot include enlarged and deformed roots (root galls) due to cellular hyperplasia and hypertrophy impeding water and nutrient uptake, causing wilting, chlorosis, stunting and plant death when symptoms are very severe (Riascos et al., 2011). The pathogen survives in soil through resting spores that are produced in large numbers inside infected roots. When host roots decay, these resting spores are released into the soil and can persist in it for up to 17 years (Ernst et al., 2019; Wallenhammar, 1996). These resting spores can build up in the soil when brassicas are repeatedly grown, increasing the inoculum concentration, which leads to higher disease incidence and severity in

subsequent crops (Faggian & Strelkov, 2009; Murakami et al., 2002). This is a major challenge for clubroot management since the pathogen is very difficult to control once it has been established in a field (Strelkov & Hwang, 2014). Clubroot management involves strategies such as crop rotation with non-host crops, application of lime to increase soil pH which is unfavorable for the pathogen, sanitation of field equipment, strategic application of fungicides and the use of resistant cultivars (Donald & Porter, 2014). However, these strategies are not sufficient because *P. brassicae* can spread easily, which is facilitated by the transportation of contaminated material (farm machinery, soil, plants, grazing animals, people) or by natural dispersal through water or wind.

Because of the complexity of clubroot management in fields infested with the pathogen, it is essential to implement control measures based on prevention and exclusion of *P. brassicae*. However, this is not always possible, and once the pathogen is in the field, early detection, and quantification of *P. brassicae* is crucial as a decision-making tool for farmers. Pathogen quantification in the field may be used for clubroot forecasting, for designing agronomic management strategies to control disease and develop an economically and environmentally sustainable approach (Wallenhammar et al., 2012; Yang et al., 2022).

Different methods with varying precision and sensitivity have been developed to identify and quantify resting spores of *P. brassicae* in both soil, water, and plant tissue (Xing et al., 2021). However, because the pathogen is an obligate biotroph, and cannot be cultivated *in-vitro*, most of the detection methods are complex, time-consuming, and require extensive glasshouse space for bioassays, while others require personnel with a high level of expertise or may result in underestimates as is the case of fluorescent microscopy or serological methods (Faggian & Strelkov, 2009). Therefore, research has focused on designing sensitive, reliable, specific, and rapid tests that can detect and quantify the amount of *P. brassicae* inoculum in the soil so that it can be integrated into a clubroot control strategy. To this end, several PCR-based tools have been used to detect and quantify resting spores in environmental samples (Czubatka-Bieńkowska et al., 2020; Gossen et al., 2019; Li et al., 2013; Wen et al., 2020).

A national survey to determine the extent of clubroot in Colombia in 2017 reported that 53.7% of the visited fields had clubroot disease on the crops, but the pathogen DNA was detected by PCR methods on 91.3% of the surveyed fields in all the departments where crucifers were grown. However, at the time of the survey, no symptoms of clubroot disease were observed in Nariño department, despite finding the pathogen DNA at concentrations varying from 2×10^3 to 2×10^4 resting spores per gram of soil (Botero-Ramírez et al., 2022). At that time, these spore concentrations were possible at the lower end of spore density required for disease development under field conditions (Botero-Ramírez et al., 2022; Hwang, Ahmed, Zhou, et al., 2011; Hwang et al., 2019). However, symptoms of wilting and subsequent plant death in brassica crops were reported by farmers in Nariño department in 2020. Here we report that *P. brassicae* is the causal agent of the newly appeared disease in brassica crops in Nariño and is posing a threat to cruciferous vegetables growing farmers due to its high incidence and severity, suggesting that the unawareness of the disease promoted the inoculum buildup in the fields from 2017 to 2022. We used qPCR as a tool to assess the level of risk for planting cruciferous crops, based on resting spores' quantification both in artificially and naturally infested soil. The aims of this study were to determine i)

the inoculum threshold needed to cause disease under controlled conditions, ii) the effect of inoculum density on plant development, iii) the increase of resting spores in soil under controlled conditions, and iv) quantification of naturally infected soils in three regions of the country to use them as decision-making tools for *P. brassicae* forecasting.

Materials and Methods

Plasmodiophora brassicae isolation and inoculum preparation

Mature galls with *Plasmodiophora brassicae* were obtained from naturally infected broccoli (*Brassica oleracea* var. *italica*) plants from fields located in the municipality of Gualmatán, Nariño Department. For inoculum preparation, the galls were extracted from the infected plants, washed using tap water, and disinfected with 70% alcohol for 5 minutes followed by 5% sodium hypochlorite for 20 minutes. Subsequently, the galls were rinsed with sterile distilled water three times. Following the procedure described by Fei et al. (2016) the galls were homogenized in a blender using 10% sucrose. The homogenate was then filtered with cheesecloth to remove root fragments, and then was centrifuged for 15 minutes at a speed of 4,000 rpm. The pellet was resuspended in sterile, deionized water, followed by another centrifugation of the suspension. The freshly obtained pellet was suspended in a solution of 20% glycerol and carefully stored at a temperature of -80°C. The concentration of the spore suspension was determined by counting on a hemocytometer under a microscope.

Plant material for pathogen maintenance

The cultivar of broccoli (*B. oleracea*) *Legacy* susceptible to *P. brassicae* was used in this study. Seeds were sown in peat in 50-well seeding trays under commercial nursery conditions. Thirty days old seedlings were transplanted in a mix of soil: rice husk (3:1) in 2kg bags. One week after transplanting, plants were drench-inoculated with *P. brassicae* resting spores at a concentration of 10^8 spores g-1 soil, for maintaining the pathogen alive. Plants were maintained in a greenhouse with 12 hours day/night, temperatures that ranged from 10°C at night to 25°C during the day, and a relative humidity of 50% on average.

Pathogenicity assay to determine inoculum threshold for P. brassicae disease development and spore accumulation in soil

To determine inoculum threshold for *P. brassicae* development and pathogen quantification, a greenhouse assay was conducted using an experimental unit of 10 bags disposed in a plastic tray, each containing 800 g of soil and rice husk (3:1) mix. The spore suspension was incorporated homogenously into the soil to an inoculum density of 10^1, 10^2, 10^3, 10^4 and 10^5 resting spores per gram of soil. The non-inoculated controls were treated with tap water. Then, 15 days-old broccoli seedlings were transplanted into the bags eight days after soil inoculation. The experiment was conducted as a complete randomized block design (CRBD) with ten plants (repetitions) per treatment. The whole experiment was done three

times. Plants were grown for eight weeks under greenhouse conditions as described above. Plants were irrigated to maintain soil moisture and fertilized weekly.

Plants were evaluated for clubroot incidence and severity eight weeks after inoculation. Clubroot severity was assessed based on a visual scale of grades ranging from 0 to 4, where 0 = no visible disease symptoms, 1 = a few small clubs on lateral secondary roots, 2 = larger galls on lateral roots, 3 = enlarging gall on the main and lateral roots and 4 = large singular gall on root neck without secondary roots (Cubeta et al., 2008; Wesołowska, 2014). The disease severity index (DSI) was calculated using the five-grade scale according to the formula:

$$\mathbf{DSI= [(\Sigma} Si * Ni\mathbf{) / (4} * N\mathbf{)] * 100}$$

Where *Si* represents the severity grade of the symptoms, *Ni* is the number of plants in each severity grade, 4 represents the number of grades minus 1, and N is the number of plants in the experimental unit. The disease indexes were calculated according to plant susceptibility at the eight-week plant stage.

After disease rating, the plant height (cm), fresh and dry shoot and root weight (g) at the different inoculum concentrations were measured.

Sample collection, DNA extraction and quantitative PCR Analysis

Soil samples for each spore concentration from the pathogenicity assay were collected at two time points: the day of transplanting (T1) and two months after transplant (T2). The total soil DNA was extracted from a subsample of 250 mg from each pot using the DNAeasy PowerSoil PRO® kit from Qiagen following the manufacturer's instructions. The DNA quality was determined in a 1% agarose gel and the concentration was measured with a spectrophotometer Nanodrop 2000 (Thermoscientific) at 260/280 nm.

For PCR analyses, all DNA samples were adjusted to a concentration of 10 ng/µl. For detection and initial confirmation of *P. brassicae*, conventional PCR was done using the specific primers TC1F (5´-GTGGTCGAACTTCATTAAATTTGGGCTCTT-3´) and TC1R (5´-TTCACCTACGGAACGTATATGTGCATGTGA-3´) (Cao et al., 2007). The amplification was done in a BioRad Thermocycler, in a 20 µl final volume containing 1U of Taq DNA polymerase, 2 mM MgCl2, 1X PCR buffer, 0.4 µM each primer, 0.2 mM each dNTP, and 10 ng of total *P. brassicae* DNA extracted from soil. As positive control, DNA extracted from *P. brassicae* resting spores was used, and water was included as negative control in each PCR assay. The PCR conditions used were as described by Cao et al., (2007). Briefly, an initial heat denaturation step at 94°C for 2 min; followed by 45 cycles of 94°C for 30 s, 65°C for 1 min, and 72°C for 1 min; and a final extension at 72°C for 10 min. PCR amplification was verified by 1% agarose gel electrophoresis.

To calculate resting spore concentration in the soil, a quantitative real time PCR (qPCR) was performed with primers DC1F (5´-CCTAGCGCTGCAT CCCATAT-3´) and DC1R (5´-CGGCTAGGATGGTTCGAAAA-3´) designed by Rennie et al., (2011). The soil DNA samples were used undiluted or diluted tenfold if necessary. The reaction mixture of a 10 µL final volume contained 1 µl template DNA, 0.25 µl of each

primer at 10 µM, and 5 µl Luna® Universal qPCR Master Mix, containing SYBR Green as the detection dye. Each DNA sample was measured in three technical replicates. Reaction conditions consisted of an initial heat denaturation step at 95°C for 2 min, followed by 35 cycles of 95°C for 15 s and 60,5°C for 60 s. A melting point analysis was conducted at the end of each reaction, and the presence of a single amplification product confirmed.

Standard curves for the quantification of *P. brassicae* were generated with DNA isolated from known quantities of purified resting spores. Total DNA was extracted from 10^9 resting spores/ml of *P. brassicae* using the kit Quick DNA Fungal/Bacterial Miniprep kit (ZYMO Research) and serial dilutions were done with sdH_2O. DNA samples from each dilution were used in triplicate to determine the standard deviation (SD) of cycle threshold values (Ct value). The average Ct value, linear regression coefficient (r^2), line equation and PCR efficiency (E) were calculated. Standard curves were generated by plotting Ct values against the logarithm of the starting spore concentration. The amplification efficiency (E) of the qPCR assay was calculated from the slope of the standard curve using the following equation: $E = 10^{(-1/slope)}$.

Detection of P. brassicae from naturally infested fields from three departments: Boyacá, Cundinamarca, and Nariño

Soil samples from naturally infested fields were collected from three departments Boyacá (B), Cundinamarca (C), and Nariño (N) in 2023 (Table 1). Soil samples were dried at 40°C for 8 hours, sieved to remove excess of plant residues and kept at -20°C until processed. DNA was extracted, conventional PCR and qPCR were done for pathogen detection and quantification in each sample as described above.

Table 1
Description of soil samples collected from *P. brassicae* naturally infected fields in three departments, Boyacá (B), Cundinamarca (C), and Nariño (N).

Sample	Origin	Pathogen history
BS1	Tibasosa (Boyacá)	Soil with no history of *P. brassicae*
BS2	Duitama (Boyacá)	Soil from a field where clubroot was reported the previous year
BS3	Duitama (Boyacá)	Soil from a field with symptomatic broccoli plants (75 days after sowing)
BS4	Duitama (Boyacá)	Soil from a field where clubroot was reported
CS1	El Rosal (Cundinamarca)	Soil with no history of *P. brassicae*
CS2	Mosquera (Cundinamarca)	Soil from a field where clubroot was reported the previous year. Agrosavia, Tibaitatá plot 14
CS3	Mosquera (Cundinamarca)	Soil from a field with symptomatic broccoli plants. Agrosavia, Tibaitatá plot 31.
NS1	Gualmatán (Nariño)	Soil from a field with symptomatic broccoli plants
NS2	Pasto (Nariño)	Soil with no history of *P. brassicae*
NS3	Gualmatán (Nariño)	Soil with no history of *P. brassicae*
NS4	Gualmatán (Nariño)	Soil from a field with history of losses by *P. brassicae*
NS5	Gualmatán (Nariño)	Soil from a field with history of losses by *P. brassicae*

Using qPCR as a decision-making tool for brassica growers –proof of concept

A field with high clubroot inoculum pressure and report of crucifers yield losses of 100% in previous years in Gualmatán Nariño, and the data obtained from pathogenicity assay and the impact on plant development were used to evaluate if we could predict whether the grower would have yield or not when planting crucifers based on the quantification of *P. brassicae* spores per gram of soil. Resistant cultivars for both broccoli (cv. Monclano) and cauliflower (cv. Clapton) were used as controls.

Half of selected field was treated to reduce the pathogen inoculum with a scheme of disease management that consisted in the application of lime at a concentration of three tons/hectare and the commercial fungicide Tizca® (Fluazinam) in dose of 2 ml/L (Kowata-Dresch & May-De Mio, 2012; Struck et al., 2022). The other half was kept untreated. Pathogen concentration was determined with qPCR as mentioned above before and after soil treatment. Then, 30 days-old seedlings of broccoli cv. Legacy and

cauliflower cv. Crenique both highly susceptible to *P. brassicae* were transplanted in the treated and untreated plots at a density of 24.000 plants per hectare. Fertilization was done during transplanting and one month after transplant. Disease and insect management was done by local farmers. Three months after transplanting yield expressed in tons per hectare was determined in both treated and untreated plots.

Statistical analysis

Clubroot severity, DSI and plant development traits were checked for normal distribution by the Shapiro-Wilk test, and then checked for equality of variance by Levene's test. Based on normality and equality of variance tests, Kruskal-Wallis or ANOVA with Nemenyi's or Tukey's post hoc test was employed for multiple comparisons (McKight & Najab, 2010; Nemenyi, 1963). All analyses were conducted using RStudio Software version 4.1.3 with the PMCMRplus, agricolae, and stats packages (De Mendiburu, 2023; Pohlert, 2022; R Core Team, 2022).

Results

Determining inoculum threshold for disease development under controlled conditions

To determine the inoculum threshold for disease development, soil samples were inoculated at different known concentrations of *P. brassicae* resting spores from 10^1 to 10^5 spores per gram of soil. The Kruskal-Wallis' test showed that the spore concentration was highly significant for disease severity ($p < 0.001$) and these were positively correlated ($r = 0.89$ $p = 0.001$). Clubroot symptoms were observed at the lowest concentration 10^1 spores/g soil in 60% of the plants and at 10^2 spores/g soil, the disease was observed in 89% of the plants. The incidence increased to 100% of the plants when the inoculum density was equal to or greater than 10^3 spores/g soil and no significant differences were observed on incidence at inoculum densities from 10^2 to 10^5 spores/g soil (Fig. 1A).

Similarly, disease severity index (DSI) for clubroot increased proportional to inoculum density and there were significant differences among all spore concentrations (Fig. 1B). The DSI for 10^1 spores per gram of soil was 20.8%, which presented a few small clubs on secondary roots. The DSI remained below 45% for 10^2 spores per gram of soil, while the severity was higher than 60% at 10^3 and 10^4 spores/g soil and reached more than 80% at the highest inoculum concentration tested 10^5 spores/g soil (Fig. 1B). From an inoculum density of 10^4, plants with a large singular gall on root neck were observed.

Effect of inoculum density on plant development

Plant growth was affected by clubroot, and significant differences were found among inoculum density for plant height, fresh and dry weight of aerial part and fresh weight of roots traits (Fig. 2). Plant height decreased significantly as inoculum concentration increased. No differences in plant height were

observed at 10^1 and 10^2 spores/g soil compared to non-inoculated control, but it was significantly reduced at 10^3 spores/g soil, with 18% of plant heigh reduction. Plant height reduction increased at higher inoculum densities, 22% and 33% at 10^4 and 10^5 spores/g soil, respectively (Fig. 2B). Similarly, the fresh weight of the aerial parts did not have a significant difference between the non-inoculated control, and plants inoculated at 10^1 and 10^2 spores/g soil, but it was significantly reduced at 10^3 spores/g soil. This reduction increased to 54% and 64% at 10^4 and 10^5 spores/g soil, respectively (Fig. 2C). As expected, the same trend was observed for dry weight (Figs. 2D).

The fresh and dry weight of roots increased with the inoculation of *P. brassicae* in soil, but it was not statistically significant. The fresh root weight for the non-inoculated control was 2.2 g, while in the inoculated roots it increased to 4 or 5 g. The same result was observed for dry root weight where the non-inoculated control presented 0.5 g and increased to a maximum of 0.8 g for diseased roots (Fig. 2E-F). DSI were negatively and significative correlated with plant height (r=-0.93; $p < 0.0001$), fresh weight of the aerial part (r=-0.89; $p < 0.0001$) and dry weight of the aerial part (r=-0.91; $p < 0.0001$). No correlation was found between DSI and fresh and dry root weight.

Quantification of P. brassicae spore accumulation in soil

The standard curve generated from purified resting spores from 10^2 to 10^9 spores, allowed the spore quantification in soil samples with an amplification efficiency that range from 94 to 102%. Samples with a concentration below 10^2 spores/g soil were not accurately detected; being 10^2 spores/g soil the detection limit achieved in this study.

To determine pathogen accumulation over time, soil samples at transplanting (T1) and eight weeks after transplanting (T2) were collected at different inoculum concentrations. Differences between T1 and T2 were found at all initial inoculum densities except 10^1 spores/g soil where qPCR quantification was not possible. At initial inoculum of 10^2 spores/g soil, T1 was detected with 9.48×10^2 and the T2 was detected with a spore concentration of 1.99×10^3 spores/g soil, suggesting probably a two-fold increase in spores. At an initial inoculum of 10^3 spores/g soil, the T2 increased 5.0 times, going from 2×10^3 to 10^4 spores/g soil. For 10^4 spores/g soil the increase was 6.7 times, from 2.54×10^3spores/g soil to 1.70×10^4 at T2. And for an initial inoculum density of 10^5 spores/g soil the pathogen spores increase 9.4 times, from 1.18×10^4 at T1 to 1.11×10^5 at T2 (Table 2).

Table 2
Spore accumulation in soil over time

Initial spore concentration spores/g soil	qPCR quantification T1 spores/g soil	qPCR quantification T2 spores/g soil
10^1	Not detected	Not detected
10^2	$9.48\times10^2 \pm 5,7$	$1.99\times10^3 \pm 72$
10^3	$2.00\times 10^3 \pm 178,9$	$1.00\times10^4 \pm 335$
10^4	$2.54\times 10^3 \pm 187$	$1.70\times10^4 \pm 1175,4$
10^5	$1.18\times 10^4 \pm 204$	$1.11\times 10^5 \pm 11645,9$

Detection and quantification of P. brassicae in naturally infected soils

Early detection and quantification of *P. brassicae* spore concentration under field conditions is essential for clubroot forecasting and for designing agronomic strategies to manage this disease. Using conventional PCR, soil samples with a concentration of *P. brassicae* of 10^3 spores/g soil were detectable with TC2 primers set and from 10^4 – 10^5 spores/g soil for TC1 primers set (Figure S1).

Once the detection limit with qPCR was determined in soil samples under controlled conditions at 300 spores/g soil, 12 samples collected from different locations were assessed for *P. brassicae* detection and quantification. Soil samples were collected in three departments: Boyacá (B), Cundinamarca (C), and Nariño (N). *P. brassicae* was detected on BS2, CS3, NS1, NS4 and NS5 samples with conventional PCR, while it was not detected on BS1, BS3, BS4, C1, C2, N2 and N3 samples (Table 3). When using qPCR, *P. brassicae* was detected on all positive samples by PCR and on sample BS3 at a concentration of 3.86×10^2 spores/g soil, indicating a higher sensitivity of qPCR, as expected. The other negative samples remained negative when evaluated with qPCR (Table 3).

The soil samples NS1, NS4 and NS5 from Nariño showed the highest concentration of *P. brassicae* with 2.62×10^7, 2.70×10^7 and 1.40×10^5 spores/ g of soil, respectively (Table 3). In these plots, other brassica crops have been planted in the previous years and the disease has been reported continuously, leading to an increase of pathogen inoculum in the soil.

Samples BS1, CS1, NS2 and NS3 had no history of the disease, thus, they could be considered negative samples. However, samples BS4 and CS2 came from plots where the disease has been reported in years prior to sampling, so careful management and monitoring is needed to avoid the spread of the pathogen. This result indicates that the pathogen concentration in these two samples could be below the detection level of the qPCR technique.

Table 3
Assessment of *P. brassicae* presence in soil samples in three departments

Sample	Detection by conventional PCR	Concentration *P. brassicae* (spores/g soil) by qPCR
BS1	Not detected	Not detected
BS2	Positive	$1.14\times10^{3} \pm 50$
BS3	Not detected	$3.86\times10^{2} \pm 25$
BS4	Not detected	Not detected
CS1	Not detected	Not detected
CS2	Not detected	Not detected
CS3	Positive	$2.29\times10^{3} \pm 91$
NS1	Positive	$2.62\times10^{7} \pm 5500$
NS2	Not detected	Not detected
NS3	Not detected	Not detected
NS4	Positive	$2.70\times10^{7} \pm 6100$
NS5	Positive	$1.40\times10^{5} \pm 1005$
Departments: Boyacá (BS1-BS4), Cundinamarca (CS1-CS3) and Nariño (NS1-NS5)		

Using qPCR as a decision-making tool for brassica growers

Because *P. brassicae* can be a devastating pathogen in highly infested fields leading up to 100% yield loss, it is important to have a tool to help farmers to make decisions about sowing cruciferous crops based on the amount of pathogen inoculum in the field. The quantification of *P. brassicae* on soil artificially infested with spores ranging from 10^{1} to 10^{5} indicated that a concentration above 10^{4} spores/g soil significantly impacts the incidence, severity, and development of the plant.

The *P. brassicae* inoculum of a highly infested field was quantified by qPCR. Before treatment, $1.09\text{x}10^{5}$ spores per gram of soil were quantified in this field. After treatment with lime and fungicide, the spore concentration decreased to $9.41\text{x}10^{4}$ spores per gram of soil, indicating a reduction of 15.190 spores per gram of soil. Both broccoli and cauliflower susceptible cultivars were sown in the untreated and treated plots. Plants from the untreated field were seriously affected by the pathogen and no production was achieved for either broccoli or cauliflower, while the treated soil yielded 23 tons per hectare for broccoli and 20 tons per hectare for cauliflower (Fig. 3). Resistant cultivars were used as controls. These results suggest yield losses can reach up to a 100% when inoculum density is above 10^{5} spores per gram of soil.

Discussion

Development of clubroot disease in a susceptible broccoli cultivar was directly associated with inoculum density. Under controlled conditions, with the pathogen incorporated into the soil, clubroot symptoms were observed in 60% of the plants at the lowest density tested (10^1 spores/g soil) and the incidence increased to 100% of the plants at 10^3 spores/g soil. The present results on broccoli confirm the early work of Hwang, Ahmed, et al. (2011), who observed a direct correlation between spore density and disease severity on canola. However, these results differ to the reported by Botero-Ramírez et al. (2022), who mentioned that soils with $2x10^3$ to $2x10^4$ spores per gram of soil did not cause visible symptoms on susceptible plants. This might be due to several reasons. We hypothesize that measurement of DNA in the soil under field conditions can be of non-viable resting spores, thus, overestimating the amount of effective inoculum, or the inoculum is distributed in patches, or because the plants at these concentrations show mild symptoms that are difficult to detect without comparing to a non-inoculated control.

Plant height and fresh and dry weight of aerial parts did not change at 10^1 or 10^2 spores/g soil compared to the control plants but started to decrease at 10^3 spores/g soil. At a higher concentration (10^5 spores/g soil) the severity reached 80% and the plant height and foliar fresh weight was reduced 22% y 54%, respectively. This is to be expected considering that the pathogen obstructs the absorption of water and nutrients to the plant, so its development is reduced (Kageyama & Asano, 2009). Similarly, Hwang, Ahmed, Strelkov, et al. (2011) reported that plant height and seed yield decreased with increasing inoculum density in *Brassica napus*.

We used real-time qPCR assay to detect and quantify *P. brassicae* from soil samples both artificially and naturally infected by the pathogen. The qPCR was specific and enabled the quantification of *P. brassicae* spores in soil and allowed the detection of 300 spores/g soil. Using this technique, we were able to quantify resting spores' buildup in artificially infected soils. A significant correlation was found between the initial resting spores' concentration and the final spores' concentration after two months with a susceptible host. This finding highlights the importance of taking measurements to reduce pathogen accumulation in soil overtime, such as crop rotations or soil treatments. Additionally, qPCR was used to assess the concentration of resting spores of *P. brassicae* in naturally infested fields in three departments, where crucifers are grown. The samples from Nariño showed a high density of *P. brassicae*, with a minimum of 1.4×10^5 spores/g soil and a maximum of 2.62×10^7 spores/g soil. This study presents concentrations of the pathogen higher than reported by Botero-Ramírez et al., (2022) who reported a maximum concentration of the pathogen of 2.1×10^4 spores/g soil during a field survey in 2017. Suggesting that consecutive planting of cruciferous crops without the implementation of crop rotation or soil treatments could lead to an accumulation of this pathogen in the field.

Symptoms were visible on plants when the spore concentration under field conditions was 10^3 but cause severe damage at 10^5 spores/g soil. Thus, these results allowed us to predict that cruciferous crops can

be grown on fields with *P. brassicae* concentrations lower than 10^4 spores per gram of soil with damage from the pathogen but can still get production. While fields with 10^5 or higher, should not be used for cruciferous crops, unless the soil is treated to lower the inoculum concentration or there is a period of crop rotation with no cruciferous species. As a proof of concept, a field with a high inoculum concentration where the grower had reported 100% yield loss the previous year was used. We measured the pathogen concentration to be $1.09x10^5$ spores per gram of soil. Half of the plot was treated to reduce pathogen concentration with lime and fungicide, and we were able to reduce it to $9.41x10^4$ spores per gram of soil. We hypothesized that the treated plot would produce yield while the untreated plot will not. A 100% yield loss was reported for both broccoli and cauliflower in the untreated plot, while the treated soil gave a yield of 23 tons per hectare for broccoli and 20 tons per hectare for cauliflower. Despite the high concentration of pathogen on the treated field, the actual viable spores could be less due to inhibition or death due to lime and fungicide treatments. In future work, measuring spore viability could give an insight into the mechanism by which soil treatments affected pathogen spores. Our results indicate that qPCR can be used as a tool for *P. brassicae* forecast in the field and as a decision-making tool for cruciferous growers.

Determining the pathogen concentration in the field allows the deployment of control measurements required to minimize farmers' losses in an appropriate and timely manner, by assessing the level of risk in a region or within a field, developing forecasting models and helping to stop the spread of the pathogen. This study found that the use of susceptible cultivar contributes to accumulation and development of new resting spores, generating a considerable increase in the concentration of the pathogen. Also, we demonstrated that soil treatment with lime and fungicide can reduce viable spores, although the mechanism was not determined. Finally, the benefit of using qPCR as a decision-making tool for cruciferous growers was shown.

Declarations

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Author contribution

Francy Liliana García Arias: Conducted experimental research, Data collection and analysis, Writing – original draft, figures, revised, and edited. Edwin Rodríguez: Conducted experimental research, data collection and analysis, writing – review and editing. Lorena Dávila: Conducted experimental research, data collection and analysis, Writing – review and editing. Donald Riascos: Conducted experimental research, writing – review and editing. Eliana Revelo: Conducted experimental research, and critical reading of the manuscript. Alejandro Villabona Gelvez : Conducted experimental research, and critical reading of the manuscript. Carlos Andrés Moreno-Velandia : Conducted experimental research, critical reading of the manuscript and editing. Paola Zuluaga: Conceptualization of the research, Methodology, Supervision analyses– advice on experimental design, conducted experimental research, and analyzed data. Project administration, wrote and reviewed the manuscript as corresponding author. All authors have read and agreed to the published version of the manuscript.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Data Availability

All data generated or analysed during this study are included in this published article [and its supplementary information files].

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Figures

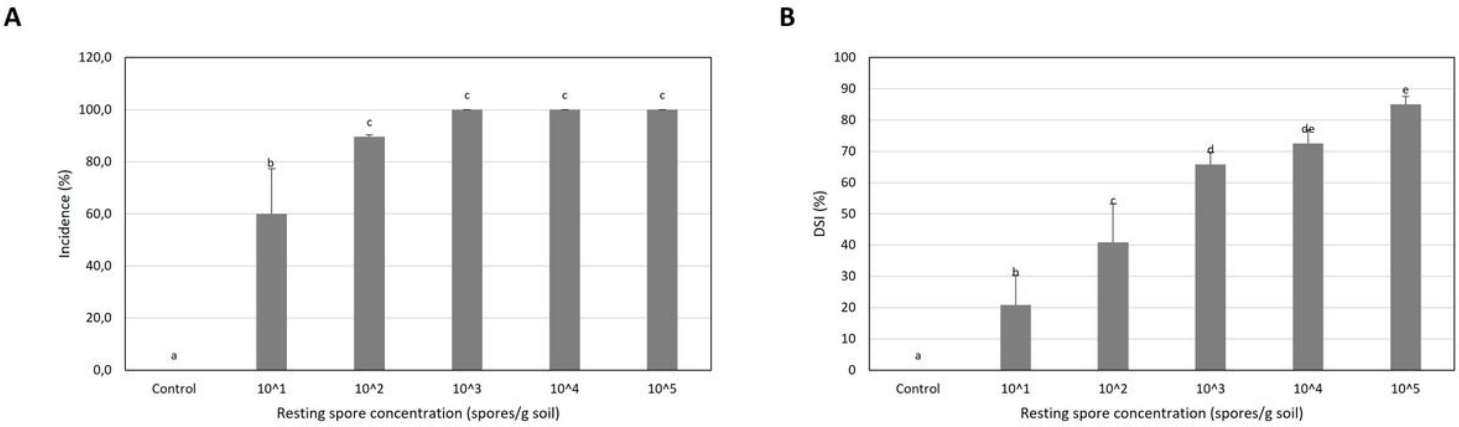

Figure 1

Evaluation of broccoli plants inoculated at different spore concentrations of *P. brassicae*. Disease incidence (A) and Disease Severity Index – DSI (B). Different letters indicate statistically significant differences according to Kruskal-Wallis.

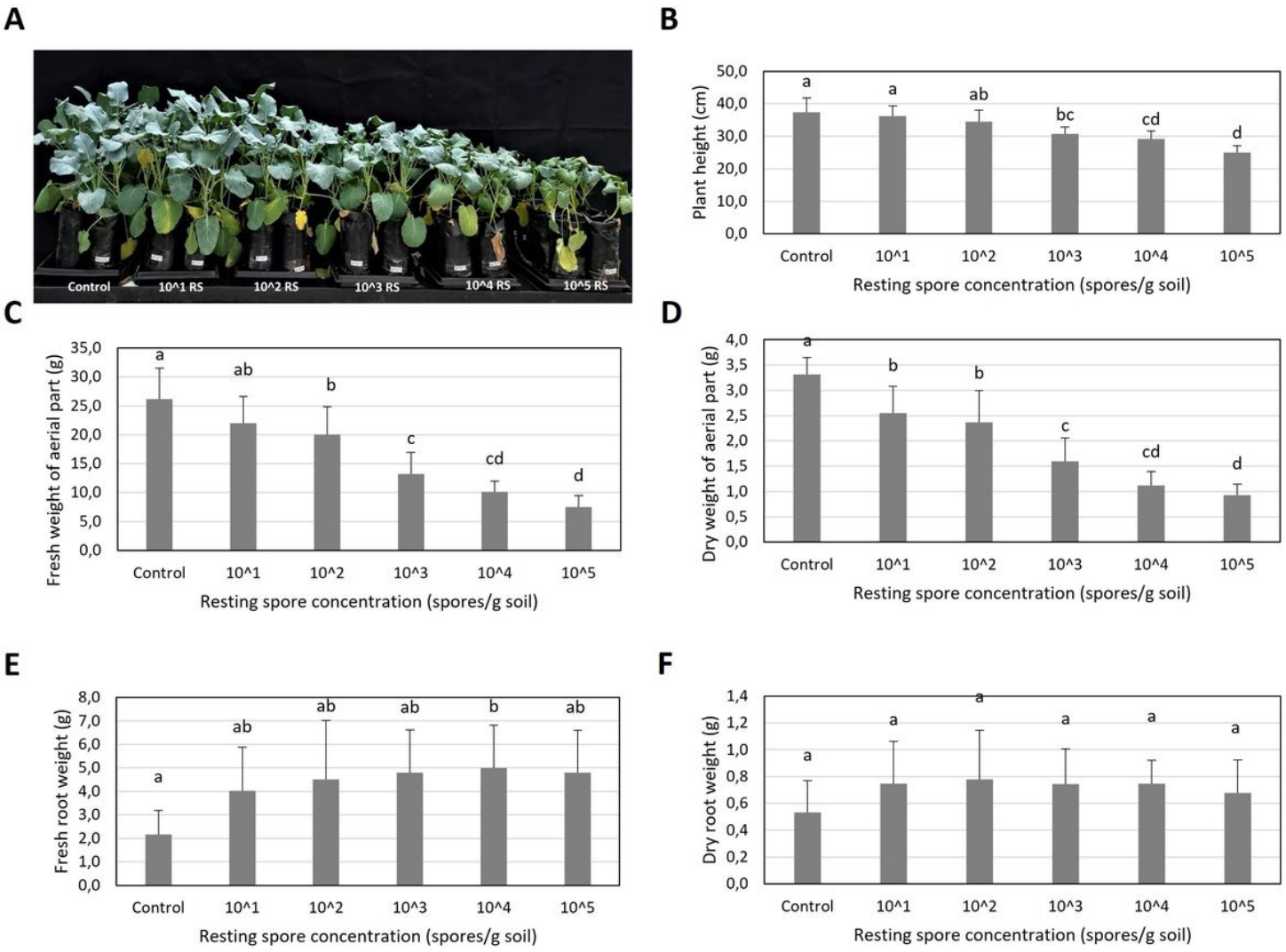

Figure 2

Plant development traits of broccoli inoculated at different inoculum concentrations of *P. brassicae*. A) phenotype of broccoli plants of control (non-inoculated plants) and inoculated treatments at different spore concentrations; B) Plant height, C-D) Fresh and dry weight of aerial part; E-F) Fresh and dry weight of roots. Data was pooled across two bioassays. Significant differences were calculated by Tukey test $p<0.05$; same letters indicate no significant difference.

Figure 3

Field assay showing the effect of soil treatment on *P. brassicae*. Field assay showing both broccoli cv. Legacy and cauliflower cv. Crenique crops grown on an untreated plot (lower panel), a treated plot where fungicide and lime were used prior to sowing (middle panel). Resistant cultivars for both crops were used as controls, broccoli cv. Monclano and cauliflower cv. Clapton (upper panel).

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