Plant Disease • 2019 • 103:1685-1692 • https://doi.org/10.1094/PDIS-09-18-1486-RE

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Soilborne Inoculum Density and Environmental Parameters Influence the Development of Pythium Stunt Caused by *Pythium tracheiphilum* in Head Lettuce Crops

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Abstract

In Quebec muck soils, Pythium stunt (*Pythium tracheiphilum* Matta) is responsible for important yield losses in head lettuce crops each year, which can reach up to 50% in certain cases. Despite the significance of the disease, factors influencing its development remain poorly documented, and no disease risk indicators are available, which makes the development of management strategies difficult. Hence, growers systematically use chemical fungicides throughout the growing season to reduce crop losses. However, it is known that soilborne disease incidence or severity may be influenced by soil inoculum density and environmental parameters. Therefore, the objectives of this study were to investigate the influence of inoculum density on lettuce growth under controlled conditions and evaluate the influence of soil inoculum density, air temperature, relative humidity, and rainfall on disease incidence under field conditions. In particular, this study aims to develop accurate predictors for Pythium stunt incidence. Results showed that, under controlled environment, thresholds of inoculum density of 97 and 46 propagules per gram of dry soil were needed to reduce lettuce dry weight by one-half for cultivars Estival and Prestige, respectively. These results were confirmed under field conditions, where a soil inoculum density >132 propagules per gram of dry soil combined with air temperatures <18°C for the first 2 weeks and rain accumulation >64 mm for the first 3 weeks after transplanting accurately predicted disease incidence 79% of the time. These relationships improve understanding of seasonal Pythium stunt development and will provide useful tools to develop sustainable management strategies.

Keywords: binary recursive partitioning, oomycetes, TaqMan.

In Canada, >90% of head lettuce (*Lactuca sativa* var. *capitata*) production is grown in the province of Quebec, mainly in the muck soils (histosols) of Napierville County (Massicotte et al. 2016). Pythium stunt caused by *Pythium tracheiphilum* Matta was reported in lettuce fields for the first time in Quebec in 1983 and resulted in up to 24% yield losses (Gracia et al. 1991). In 2018, *P. tracheiphilum* was identified to be the predominant species associated with Pythium-induced diseases in head lettuce crops, and related yield losses have increased to reach >50% (Van der Heyden et al. 2019). *P. tracheiphilum* is known to infect vascular vessels of its host, the xylem in particular, where it spends most of its lifecycle (Jacquet 1979). Young plants (7 to 14 days old) are generally more susceptible to infection, and above-ground symptoms involve stunting and wilting of lettuce plants (Jacquet 1979). The reduced root system is characterized by a great loss of secondary roots, root hairs, and fine feeder rootlets. Even if plants survive the infection, they usually do not reach a marketable size (Gracia 1989; Gracia et al. 1991; Jacquet 1979). *P. tracheiphilum* produces oospores that could remain latent in soil from several months to years. Sporangia are another structure capable of infection; however, they are known to stay viable for a shorter period compared with oospores (Martin and Loper 1999). Because Quebec's growing season is interrupted by a 6-month cold period, it is likely that pathogen structures recovered from the soil in the spring would be long-term survival structures, mostly oospores. Accordingly, the concentration of oospores found in soil in the spring could be a good indicator of subsequent Pythium stunt incidence in head lettuce crops. Moreover, Van der Heyden et al. (2019) reported the development of a sensitive real-time quantitative polymerase chain reaction (qPCR) assay and suggested that a relationship between *P. tracheiphilum* soil inoculum concentration and Pythium stunt disease may exist.

When evaluating the influence of temperature and moisture on the growth of *P. tracheiphilum* growing on culture media, Jacquet (1979) showed that temperatures between 10 and 18°C favor its growth, multiplication, and sexual reproduction. Similarly, temperatures oscillating between 18 and 24°C as well as a 36-h period under water were optimal for the sporangia germination. Under field conditions, severity of diseases caused by *P. tracheiphilum* in lettuce fields seemed to increase with high rainfall, with overwatering, and in zones of poor drainage (Gracia et al. 1991; Jacquet 1979).

Despite the significance of *P. tracheiphilum*-induced disease in Quebec and elsewhere, factors influencing disease development, such as soil inoculum and environmental conditions, remain poorly documented, making disease management difficult. Hence, to prevent yield losses caused by *P. tracheiphilum*, head lettuce growers of Napierville County systematically use chemical fungicides (cyazofamid or metalaxyl) throughout the growing season, resulting in an increase of production and environmental costs. Moreover, the repeated use of fungicides over time is also known to reduce soil biodiversity and promote pathogen resistance (Sullivan 2004). For example, cases of resistance to metalaxyl were reported for *Pythium ultimum* isolated from potato crops in Idaho, Oregon, Washington (Porter et al. 2009), and Minnesota (Taylor et al. 2002) and *Pythium aphanidermatum* recovered from turfgrass in eastern Pennsylvania (Sanders 1984).

The first step to rationalize disease management strategies is to identify the conditions for which the use of chemicals fungicides is necessary. Therefore, the aim of this study was to characterize biotic and abiotic factors influencing the development of Pythium stunt in head lettuce production. More specifically, the objectives of the

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Funding: Funding was provided by Phytodata Inc. and the Ministère de l'Agriculture, des Pêcheries et de l'Alimentation du Québec through the Innov'Action program and the Cultivons l'avenir 2 program.

*The ***e*-Xtra** logo stands for "electronic extra" and indicates that two supplementary tables are published online.

The author(s) declare no conflict of interest.

Accepted for publication 12 February 2019.

study were to (i) determine the relationship between soilborne inoculum density (ID) and the development of Pythium stunt under controlled conditions, (ii) examine the relationship between soil ID and disease incidence (DI) under field conditions, and (iii) evaluate the influence of environmental parameters on DI under field conditions.

Materials and Methods

Controlled condition experiments. *Pathogen isolation.* Symptomatic lettuce plants were collected from commercial fields in Napierville County (45°08′37″ N and 73°34′22″ W) during the 2015 growing season. Whole plants were first washed under running tap water; pieces of root were then cut and soaked for 1 min in distilled water, 1 min in 1% sodium hypochlorite, and 1 min in distilled water. Infected root tissues were selected, cut into 1- to 2-mm-length pieces, and placed on the surface of potato dextrose agar (PDA; Becton, Dickinson and Company) plates. Pure cultures were obtained by transferring 6-mm plugs containing hyphal tips on PDA, and identification of *P. tracheiphilum* was made by Sanger sequencing using the universal barcode Internal Transcribed Spacer as described in Robideau et al. (2011). Pure cultures were stored in sterilized soils and placed at 4°C until use.

Preparation of P. tracheiphilum *inoculum and inoculation procedures.* Four different isolates of *P. tracheiphilum* (GenBank accession numbers MH0704869, MH023328, MH023361, and MH023331) were used in the following experiments. To induce the production of propagules (i.e., sporangiaand oospores), 6-mm plugs taken from 3-day-old PDA plates were placed on 90-mm petri dishes containing V8 agar medium (200 ml of V8 juice, 3 g of $CaCO_3$, 15 g of agar, and tap water to complete to 1 liter) and set at room temperature. After 14 days, inoculum was prepared by soaking the culture with 7 ml of distilled water and scraping the agar surface using a scalpel blade to recover mycelium and propagules. The suspension was first vortexed and then filtered through two double layers of cheesecloth to remove mycelium. To recover as many propagules as possible, the cheesecloth was washed with 100 ml of distilled water. A stock solution was prepared, and propagule concentration was enumerated using a hemacytometer. Aliquots were taken from the stock suspension and added to 100 ml of water to prepare the following IDs: 0, 10, 25, 50, 100, 500, 1,000, or 5,000 propagules per gram of dry soil. Each suspension was added to the pasteurized soil (Mix 3; muck soil, manure, and loam; Les sols Isabelle Inc.) and thoroughly mixed before transplanting. The soil pasteurization was performed twice at 90°C for 30 min to reduce the possible effect of biological antagonism within the soil. Two-week-old lettuce plants were transplanted in individual 4-inch square plastic pots containing the artificially inoculated soil and grown in a growth chamber for 2 weeks with temperature set to 21 ± 3°C (day) and 15 ± 3°C (night) and relative humidity set to 70 ± 5% (Supplementary Table S1). The photoperiod was adjusted to 14 h with a light intensity of 1,280 $\mu mol^{-1}\ m^2\ s^{-1}$. Lettuce plants were individually watered with 100 ml the first day followed by 50 to 75 ml every 2 days. After 2 weeks, whole plants, including the roots, were harvested and carefully washed under running tap water. Plants were dried at 60°C, and dry weight was measured.

Two different cultivars of head lettuce were tested: cultivar Prestige (especially sensitive to *P. tracheiphilum*) and cultivar Estival, which is widely used by head lettuce growers from Napierville County. For each cultivar, experiments were conducted twice in a randomized complete block design with six blocks and eight IDs per block.

Statistical analysis. First, the assumption of a normal distribution of the data was tested using the Kolmogorov–Smirnov goodness-of-fit tests (SAS PROC Univariate; SAS V9.3). Second, an analysis of variance (ANOVA) was conducted using a two-way ANOVA model with three factors (PROC GLM; SAS V9.3). ID was specified as the treatment factor, blocking was specified as the block factor, and the experimental replicates (trials 1 and 2) were specified as the time factor. A least significant difference (LSD) test was performed when there was a significant treatment effect to identify the differences between IDs.

To define a disease threshold based on soilborne inoculum, the relationship between ID and plant dry weight (y) was described by the following sigmoidal dose-response function:

$$y = \min + \frac{\max - \min}{1 + 10^{(\log EC_{50} - x)}} \quad (1)$$

where x is the inoculum concentration (propagules per gram of dry soil on an ln scale), min is the minimum response, max is the maximum response, and EC_{50} is the concentration providing a response halfway between both asymptotes. Fitting of the sigmoidal dose-response curve was performed using Sigma plot (Systat Software Inc.). ID threshold was calculated according to the EC_{50} value obtained.

Field experiments. *Data collection.* In 2016 and 2017, a total of 134 experimental plots were established in 20 fields from May to July distributed among six lettuce growers in Napierville County (spatial coordinates related to each field are presented in Supplementary Table S2). Each experimental plot consisted of a quadrat of 5 × 5 m containing 192 lettuce plants. To make sure that only long-term survival structures (i.e., propagules) were sampled, soil sampling was conducted before cropping. Hence, 250-g soil samples were taken from each plot and treated on sampling. Otherwise, each sample was placed in plastic bags and frozen at −20°C until use. Within each experimental plot, soil samples were made from 15 random subsamples taken with an auger from the first 15 cm of soil.

DI was evaluated twice: the first evaluation was conducted when lettuce plants were at the 14-leaf stage, and the second evaluation was when the head had reached a diameter of 10 cm. At each evaluation, the number of symptomatic plants (wilted or stunted plants presenting yellow-brown discoloration of the root xylem within each plot) was counted. Dying plants affected by other diseases, such as bottom rot (*Rhizoctonia solani*), white rot (*Sclerotinia sclerotiorum*), or Fusarium wilt (*Fusarium oxysoprum*), were not counted. Symptomatic plants were removed from the plots during each evaluation to avoid recounting. The sum of each evaluation was used to calculate final DI.

Weather conditions were collected from the closest weather station (Watchdog 2700; Spectrum Technologies, Inc.) belonging to the local weather network. These stations collected hourly data related to air temperature (degrees Celsius), relative humidity (percentage), and rainfall (millimeters). These three meteorological parameters were, therefore, chosen for the analysis.

DNA extraction and qPCR analysis. From the 250 g sampled, 100 g of soil was air dried for 48 h and thoroughly homogenized using a mortar and pestle. Total DNA was extracted from 0.20 g of dry soil using the FastDNA SPIN kit for soil (MP Biomedicals) following the manufacturer's instructions. The concentration and quality of the obtained DNA were measured by spectrophotometry using a Nanodrop lite instrument (Thermo Scientific).

The real-time qPCR protocol described in Van der Heyden et al. (2019) was used for the detection and quantification of *P. tracheiphilum* in soil. A CFX connect real-time qPCR instrument (Biorad) was used for the qPCR assay. Each reaction (25 µl) contained 300 nM each primer (909F, PTMGB-R, EIPC100F, and EIPC100R), 100 nM each probe (PTMGBP and EIPC100P), 0.06 ng of bovine serum albumin (BSA), 6 mm of $MgCl_2$, 1× PerfeCTa qPCR ThoughMix (Quanta Bioscience), and 3 µl of gDNA. Cycling conditions were set to 95°C for 5 min and 40 cycles at 95°C for 15 s and 62°C for 20 s. To measure qPCR inhibition, a negative control with only the internal control was included in each qPCR run (Fall et al. 2015a; Van der Heyden et al. 2019).

Correlation analysis. Correlation between DI and soilborne ID, air temperature, relative humidity, and rain accumulation was assessed using a Spearman's rank correlation procedure (PROC CORR; SAS 9.4). Because each experimental plot had a specific transplanting date, the averages of daily air temperature during the first week (ATW1), the first 2 weeks (ATW12), and the first 3 weeks (ATW123) after transplanting in the field were calculated for each experimental plot. The same correlations were made for the air relative humidity (RHW1, RHW12, and RHW123, respectively) and

precipitation (RW1, RW12, and RW123, respectively). ID was ln transformed [ln(ID + 1)] before the correlation analysis procedure.

Determination of conditions favoring DI. Based on field data collected in 2016 and 2017, experimental plots were classified into two groups: those with <5% DI were designated as healthy (zero), and those with >5% DI were designated as diseased (one). Based on the most correlated parameters, a binary recursive partitioning (BRP) analysis (JMP 13 Pro; SAS Institute) was used to classify the predictors. Briefly, in a BRP analysis, the algorithm predicts a response variable by successively partitioning (splitting) the explanatory variables into two subgroups (a node is split into a right branch and a left branch) (Merkle and Shaffer 2011), where the optimal cut-point value for a split is the one for which the logworth statistic [$-\log_{10}(P$ value)] is maximized (SAS Institute Inc. 2017). Therefore, this type of analysis allows us to set threshold values (cut points in each split) that will distinguish the "healthy" group from the "diseased" group. In the creation of a decision tree involving a categorical response, the variable chosen at each split is the one that maximizes the likelihood ratio χ^2 (candidate G^2) (SAS Institute Inc. 2017). The number of splits was predetermined to be at least three, because each of the three parameters (ID, ATW12, and RW123) needed to be assessed in the partition model and needed to be involved only one time to get a simpler decision tree. The minimum size split was set to 10, which means that the node was not partitioned if there were <10 observations (counts) in each branch.

In BRP analysis, no significance tests are involved; therefore, the predictive accuracy is used to evaluate the goodness of fit of the tree (Merkle and Shaffer 2011). Accordingly, a contingency table was calculated to assess the reliability of using the thresholds found with the BRP analysis for the prediction of DI. Seven combinations of predictive parameters were included in the contingency table. First, each predictive parameter was evaluated alone; second, all pairs of combinations were evaluated, and third, a combination of the three parameters was evaluated. Each experimental plot with DI of ≥5% was classified as observed (O+), and those with DI of <5% were classified as not observed (O–). Disease was predicted (P+) when the predictive parameters (DI, ATW12, and RW123) were greater than their respective threshold, whereas the disease was not predicted (P–) when one or more predictive parameters were greater than their respective threshold. Hence, four different cases were possible. First, a true positive (TP) was obtained when the disease was predicted (P+) and observed (O+); second, a true negative (TN) was obtained when the disease was not predicted (P–) and was not observed (O–). Third, a false positive (FP) was obtained when the disease was predicted (P+) but was not observed (O–), and fourth, a false negative (FN) was obtained when the disease was not predicted (P–) but was observed (O+). Sensitivity of the test was designated by the TP proportion: [TP/(TP + FN)]. Specificity of the test was designated by the TN proportion: [TN/(TN + FP)]. The proportion of correct assessment [TP + TN/(TP + TN + FP + FN)] was calculated to provide the overall accuracy of the test (Shaikh 2011).

Table 1. Effect of *Pythium tracheiphilum* inoculum density (propagules per gram of dry soil) on lettuce dry weight (grams)

Source of variation and soil inoculum concentrations[w]	Cultivar Prestige[x]		Cultivar Estival: Trials 1 and 2
	Trial 1	Trial 2	
ANOVA			
Model	<0.0001	0.0007	<0.0001
Treatment	<0.0001	<0.0001	<0.0001
Block	0.998	0.8263	0.0002
Time	—	—	0.9676
Treatment × time	—	—	0.839
LSD			
0[y]	0.37[z] a	0.44 a	0.39 a
10	0.35 a	0.42 a	0.37 a
25	0.30 b	0.36 abc	0.35 a
50	0.24 c	0.39 ab	0.33 a
100	0.28 bc	0.27 cde	0.3 b
500	0.25 c	0.24 de	0.27 bc
1,000	0.24 c	0.32 cdb	0.27 bc
5,000	0.17 d	0.2 e	0.25 c

[w] ANOVA, analysis of variance; LSD, least significant difference.
[x] Because trials 1 and 2 had significant time effects when pooled, each trial was analyzed separately.
[y] Inoculum densities are expressed as the number of propagules per gram of dry soil.
[z] Lettuce dry weight is expressed in grams (mean of six replicates). Means with the same letter are not significantly different from each other ($P > 0.05$).

Results

Controlled conditions experiments. For the growth chamber experiments conducted with Prestige, the ANOVA shows a significant effect of ID ($P < 0.0001$) but no block effect ($P = 0.8843$) on dry

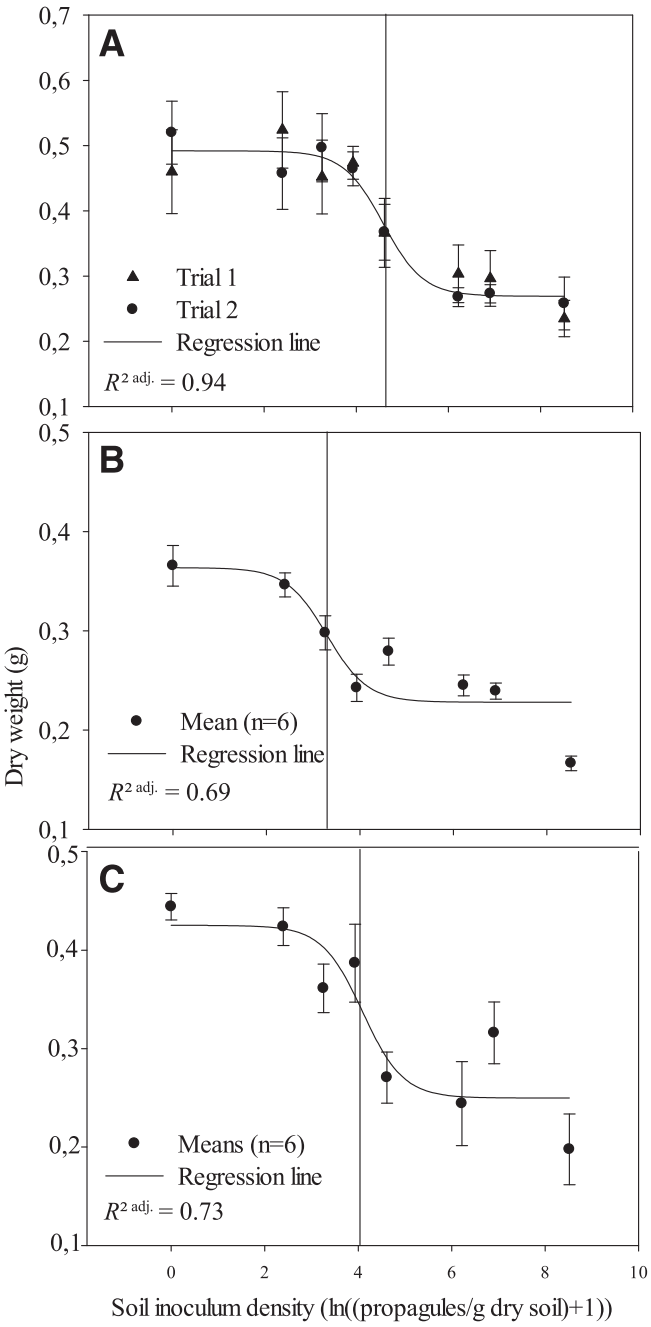

Fig. 1. Relationship between *Pythium tracheiphilum* inoculum density and lettuce dry weight for **A,** the combination of both trials for the cultivar Estival; **B,** for the first and **C,** second trial for the cultivar Prestige. Points are the means of six replicates. The vertical lines represent the concentration providing a response halfway between both asymptotes.

weight. However, because time effect was significant ($P > 0.001$) (Table 1), the LSD test was performed on each independent replicated trial. In the first trial, the dry weight of lettuce grown in 10 propagules per gram of dry soil was not significantly different from the noninoculated controls (Table 1), whereas weights of plants grown at higher concentration were significantly lower than the controls ($P < 0.0001$). In the second trial, the dry weight of lettuce grown with ID of 10, 25, or 50 propagules per gram of dry soil was not significantly different from the noninoculated controls, but weight decreased significantly at higher concentrations (Table 1).

For growth chamber experiments conducted with Estival, because the time effect was not significant ($P = 0.9676$), the two trials were

Table 2. Coefficient, standard error, and adjusted coefficient of determination (R^2_{adj}) for sigmoidal dose-response function describing the relation between *Pythium tracheiphilum* soil inoculum density and lettuce dry weight

Cultivar, trial, and parameters	Coefficient	Standard error	*t*	*P* value	Propagules per gram dry soil[z]	R^2_{adj}
Cultivar Prestige						
1						0.692
Min	0.363	0.030	12.261	<0.0001	—	
Max	0.228	0.018	12.614	<0.0001	—	
$logEC_{50}$	3.292	0.482	6.827	0.001	26	
2						0.726
Min	0.425	0.031	13.733	<0.0001	—	
Max	0.230	0.026	9.484	0.0002	—	
$logEC_{50}$	4.092	0.459	8.921	0.0003	61	
Cultivar Estival						
1 and 2						0.936
Min	0.492	0.011	44.717	<0.0001	—	
Max	0.269	0.011	24.553	<0.0001	—	
$logEC_{50}$	4.587	0.150	30.569	<0.0001	97	

[z] Equivalent to the concentration providing a response halfway between both asymptotes (EC_{50}) value in number of propagules per gram of dry soil.

Table 3. Soil inoculum density of *Pythium tracheiphilum* and disease incidence for each field evaluated in the study

Year, farm code, and field code	*N*[w]	Inoculum density[x]				Disease incidence[y]			
		Average	σ[z]	Max	Min	Average	σ	Max	Min
2016									
A									
G1	4	42	41	97	4	4.6	5.2	12.0	0.0
B									
B1	6	111	64	210	44	0.2	0.3	0.5	0.0
B5	3	40	49	97	10	0.0	0.0	0.0	0.0
VN14	2	0	0	0	0	1.3	0.4	1.6	1.0
VN301	3	0	0	0	0	0.0	0.0	0.0	0.0
VN308	3	8	8	16	0	0.0	0.0	0.0	0.0
VN31	1	19	—	—	—	0.0	—	—	—
VN32	2	26	24	44	9	1.3	1.8	2.6	0.0
C									
BAB	6	329	221	725	77	1.3	0.8	2.6	0.5
MOQ3	3	80	79	164	7	1.2	2.1	3.6	0.0
MOQ5	3	113	103	223	20	4.0	2.9	7.3	2.1
MOQ6	3	521	430	890	48	5.4	3.3	7.8	1.6
D									
24	3	179	46	223	131	2.7	1.7	4.6	1.1
27	8	128	77	229	22	3.2	4.0	12.0	0.0
31	3	26	5	31	20	1.2	1.3	2.6	0.0
35	23	341	182	810	112	5.9	5.7	18.5	0.0
5	3	436	230	684	229	1.9	1.1	3.1	1.0
2017									
B									
1.3	7	483	175	740	188	3.9	2.4	8.9	2.1
2.4	14	310	240	720	32	1.9	2.1	6.3	0.0
VN240	4	0	0	0	0	0.3	0.5	1.0	0.0
E									
Lab2	3	471	454	950	46	0.0	0.0	0.0	0.0
C									
A3C3	5	4,998	7,678	18,650	200	0.3	0.5	1.0	0.0
D									
24	11	980	423	1,810	463	8.5	3.7	15.6	5.2
51	11	1,154	564	2,110	318	2.9	2.4	8.3	0.5

[w] Number of experimental plots in each field.
[x] Expressed in propagules per gram of dry soil.
[y] Percentage of diseased plants within the experimental plot.
[z] Standard deviation of the mean.

pooled together for the analysis. There was a significant effect of ID ($P < 0.0001$) and block ($P = 0.0002$) on the measured dry weight (Table 1). Dry weight of lettuce grown at ID of 10, 25, or 50 propagules per gram of dry soil was not significantly different compared with the noninoculated controls (Table 1). However, plants grown at higher concentrations had significantly lower dry weights. The highest concentrations of 100, 500, and 1,000 propagules per gram of dry soil were not significantly different from each other (Table 1).

For both cultivars, the relationship between ID and dry weight (y) was described by a sigmoidal dose-response function (Fig. 1). Coefficient, standard error, t, and P value for equation parameters as well as adjusted R^2 are shown in Table 2. For the first trial with Prestige, the coefficient of determination was slightly lower ($R^2_{adj} = 0.692$) compared with the second trial ($R^2_{adj} = 0.726$). Calculated EC_{50} values corresponded to 26 propagules per gram of dry soil and 61 propagules per gram of dry soil for trial 1 and trial 2, respectively; and for Estival, the estimated EC_{50} was 97 propagules per gram of dry soil ($R^2_{adj} = 0.936$) (Table 2).

Field trials. In 2016, DI ranged from 0 to 19%, with an average of 2% over the 79 experimental plots evaluated. ID ranged from 0 to 890 propagules per gram of dry soil, with a median of 139 propagules per gram of dry soil. In 2017, DI ranged from 0 to 16%, with an average of 2.5% over the 55 experimental plots evaluated, and ID ranged from 0 to 18,650 propagules per gram of dry soil, with a median of 545 propagules per gram of dry soil (Table 3).

In both years, DI was positively correlated with soilborne ID ($r = 0.46$ and $P < 0.0001$ in 2016; $r = 0.32$ and $P = 0.016$ in 2017; $r = 0.44$ and $P < 0.0001$ for the combined data of 2016 and 2017). In 2016 and 2017, air temperature recorded during the first 2 weeks (ATW12) after transplanting was negatively correlated with DI ($r = -0.58$ and $P < 0.0001$; $r = -0.47$ and $P = 0.0003$, respectively) (Table 4).

Accumulation of rain was positively correlated with DI in both years, but accumulation during the first 2 weeks (RW12) was better correlated in 2016 than the other periods ($r = 0.32$ and $P = 0.0038$), whereas accumulation during the first 3 weeks (RW123) was better correlated with DI in 2017 ($r = 0.59$ and $P < 0.0001$) (Table 4). In 2016, relative humidity for the first 3 weeks (RHW123) was the only period correlated with DI ($r = -0.37$ and $P = 0.0009$). In 2017, the first week (RHW1) and the first 2 weeks (RHW12) were negatively correlated with DI ($r = -0.48$ and $P = 0.0002$; $r = -0.30$ and $P = 0.0249$, respectively) (Table 4).

The correlation between DI and ATW12 was the strongest ($r = -0.51$ and $P < 0.0001$) followed by the correlation between DI and ID ($r = 0.44$ and $P < 0.0001$) and the correlation between DI and RW123 ($r = 0.36$ and $P < 0.0001$) (Table 4).

Based on the parameters with the highest correlation with DI (ATW12, ID, and RW123), a BRP analysis was used to build the decision tree (Fig. 2). The "diseased" subgroup is represented in the left branches of the tree, whereas the "healthy" subgroup is in the right branches. For the first split, the selected candidate was ATW12 ($G^2 = 34.6$), and the cut-point value was estimated to be 18.2°C (Table 5). The G^2 value related to the "diseased" subgroup for the first split is zero, confirming that no disease cases were found at

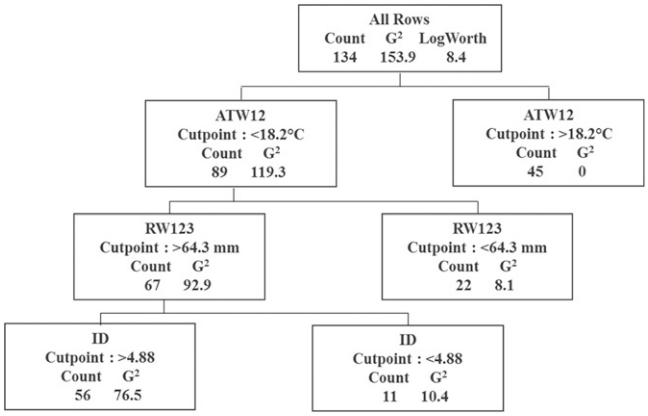

Fig. 2. Decision tree for the prediction of a disease incidence ≥5%. The decision tree was constructed using a binary recursive partitioning analysis (JMP 13 Pro; SAS Institute). The "disease" subgroup is represented in the left branches of the tree, and the "healthy " subgroup is in the right branches. ATW12 is the average daily air temperature (degrees Celsius) for the first 2 weeks after planting. RW123 is the rain accumulation (millimeters) for the first 3 weeks after planting. ID is the soil inoculum density within the experimental plot [ln(propagules per gram of dry soil) + 1]. Count refers to the number of observations within each subgroup.

Table 5. Candidate G^2 and cut-point values for each candidate at each split

	ID[v]		ATW12[w]		RW123[x]	
Split	**G^2[y]**	**Cut point**	**G^2**	**Cut point**	**G^2**	**Cut point**
First	19.4	3.86	34.6[z]	18.2	24.1	64.3
Second	10.8	3.86	3.5	13.9	18.3[z]	64.3
Third	6.0[z]	4.88	3	10.9	2.1	89.8

[v] Inoculum density (ID) cut point is expressed in propagules per gram of dry soil on an ln + 1 scale.
[w] ATW12 is the average daily air temperature for the first 2 week after planting. Cut point is expressed in degrees Celsius.
[x] RW123 is rain accumulation for the first 3 weeks after planting. Cut point is in millimeters of rain.
[y] Candidate G^2.
[z] Candidate that maximizes the G^2 at each split.

Table 4. Correlation between disease incidence of lettuce and weather parameters in field studies conducted in Quebec

	2016		2017		2016 and 2017	
Parameters[u]	**r[v] (P value)**	**n**	**r (P value)**	**n**	**r (P value)**	**n**
ID[w]	0.46 (<0.0001)	79	0.32 (0.0156)	55	0.44 (<0.0001)	134
ATW1[x]	−0.32 (0.0044)	79	−0.24 (0.0734)	55	−0.31 (0.0002)	134
ATW12	−0.58 (<0.0001)	79	−0.47 (0.0003)	55	−0.51 (<0.0001)	134
ATW123	−0.39 (0.0003)	79	−0.47 (0.0003)	55	−0.42 (<0.0001)	134
RW1[y]	0.30 (0.0074)	79	−0.17 (0.2105)	55	0.18 (0.0429)	134
RW12	0.32 (0.0038)	79	0.16 (0.2452)	55	0.28 (0.001)	134
RW123	0.20 (0.0791)	79	0.59 (<0.0001)	55	0.36 (<0.0001)	134
RHW1[z]	0.23 (0.041)	79	−0.48 (0.0002)	55	−0.07 (0.3898)	134
RHW12	0.02 (0.8775)	79	−0.30 (0.0249)	55	−0.07 (0.4211)	134
RHW123	−0.37 (0.0009)	79	−0.26 (0.0553)	55	−0.34 (<0.0001)	134

[u] W1 refers to the first week after lettuce planting, W12 refers to the first 2 weeks after lettuce planting, and W123 refers to the first 3 weeks after lettuce planting.
[v] Spearman's rank correlation coefficient r.
[w] Inoculum density (ID) of *Pythium tracheiphilum* in soil. ID is expressed in ln(propagules per gram of dry soil) + 1.
[x] AT refers to the average daily air temperature (degrees Celsius).
[y] R refers to the rain accumulation (millimeters).
[z] RH refers to the average daily air relative humidity (percentage).

>18°C (Fig. 2). For the second split, the selected candidate was RW123 (G^2 = 18.3), and the cut point was estimated to be 64.3 mm (Table 5). Finally, for the third split, the selected candidate was ID (G^2 = 6.0), and the cut point was set to 4.88 (propagules per gram of dry soil on an ln + 1 scale), which correspond to 132 propagules per gram of dry soil. Because all three parameters were selected in the first three splits, no additional split was necessary.

To assess the reliability of these predictors to predict DI ≥ 5%, a contingency table was built (Table 6). The results showed that each individual predictor provides good sensitivity (between 0.89 and 1.00) but poor specificity (between 0.42 and 0.45), whereas the overall accuracy ranged between 0.56 and 0.60. Air temperature provides a better prediction (0.60) than rain accumulation (overall accuracy of 0.57) or ID (overall accuracy of 0.56) when evaluated alone. The combination of the predictors by pair increases the overall accuracy (between 0.69 and 0.75) and the specificity (between 0.63 and 0.67) in all cases, whereas the sensitivity remains constant (between 0.89 and 0.97). Finally, the combination of the three predictors increases both overall accuracy (0.79) and specificity (0.76). As visually represented with a scatterplot (Fig. 3), almost all of the disease cases occurred at <18°C with an inoculum concentration of >132 propagules per gram of dry soil and >64 mm of rain.

Discussion

Each year, Pythium stunt caused by *P. tracheiphilum* leads to considerable yield losses for Quebec head lettuce growers. This disease is gaining in importance, because 90% of Canadian lettuce production is located in the province of Quebec (Massicotte et al. 2016). However, despite the significance of the disease, the effect of soilborne ID and weather conditions on Pythium stunt remains poorly documented. Because no risk estimation model is currently available, growers systematically use seed treatments (metalaxyl) and preplantation fungicides applications (cyazofamid). Therefore, the objectives

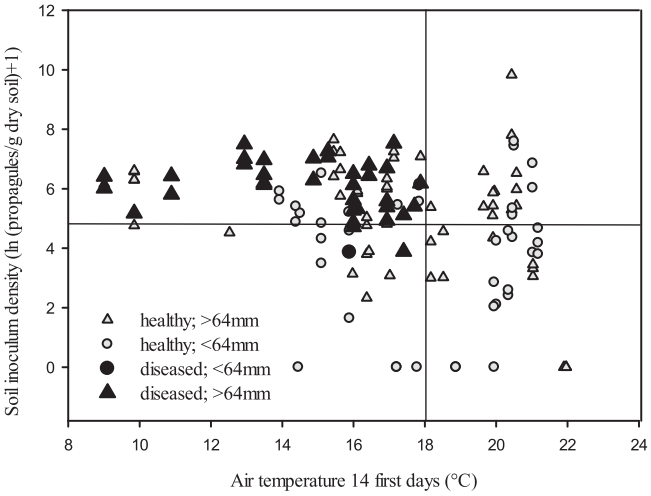

Fig. 3. Soil inoculum density [ln(propagules per gram of dry soil) + 1] plotted against air temperature for the first 14 days (degrees Celsius). Black indicates the disease cases (disease incidence [DI] > 5%); gray indicates the healthy cases (DI > 5%). Circles are cases in which rain accumulation was <64 mm during the first 3 weeks after planting; triangles are cases in which rain accumulation was >64 mm during the first 3 weeks after planting. The vertical line represents the threshold for air temperature during the first 2 weeks, and the horizontal line represents the threshold for inoculum density.

Table 6. Contingency table for assessment of using air temperature (AT), rain accumulation (R), and soil inoculum density (ID) as predictive parameters for Pythium stunt incidence

Predictive parameters[w] and observed	Predicted		Total	Overall accuracy[x]	Sensitivity[y]	Specificity[z]
	P+	P−				
ATW12						
O+	35	0	35	0.60	1.00	0.45
O−	54	45	99			
Total	89	45	134			
RW123						
O+	34	1	35	0.57	0.97	0.42
O−	57	42	99			
Total	91	43	134			
ID						
O+	31	4	35	0.56	0.89	0.44
O−	55	44	99			
Total	86	48	134			
ATW12 + RW123						
O+	34	1	35	0.75	0.97	0.67
O−	33	66	99			
Total	67	67	134			
ATW12 + ID						
O+	31	4	35	0.70	0.89	0.64
O−	36	63	99			
Total	67	67	134			
RW123 + ID						
O+	31	4	35	0.69	0.89	0.63
O−	37	62	99			
Total	68	66	134			
ATW12 + ID + RW123						
O+	31	4	35	0.79	0.89	0.76
O−	24	75	99			
Total	55	79	134			

[w] W12 refers to the first 2 weeks after lettuce planting, and W123 refers to the first 3 weeks after lettuce planting. ATW12 < 18.2°C, RW123 > 64.3 mm, and ID > 4.88 (corresponding to 132 propagules per gram of dry soil) are considered predictive thresholds for having a disease incidence ≥5%.

[x] Overall accuracy was calculated as the number of correct assessments (observed [O+] and predicted [P+]; not observed [O−] and not predicted [P−]) divided by the total number of assessments.

[y] Sensitivity was calculated as the proportion of true positives (O+ and P+).

[z] Specificity was calculated as the proportion of true negatives (O− and P−).

of this study were to investigate the influence of ID on lettuce growth under controlled conditions and evaluate the influence of soilborne ID and weather conditions on DI under field conditions.

Under controlled conditions, ID of *P. tracheiphilum* was always well correlated with lettuce dry weight. As expected, the threshold found for Prestige, especially susceptible to Pythium stunt, was lower than the threshold obtained for Estival. Results from this study suggest that average concentrations of 97 propagules (Estival) and 46 propagules (Prestige) per gram of dry soil were necessary to cause infection leading to a reduction of 50% of lettuce total dry weight. Similarly, when assessing the effect of *P. tracheiphilum* soilborne ID (sporangia per gram of soil) on 2-week-old lettuce, Gracia et al. (1991) found that leaf and root weights of lettuce (cultivar Ithaca) grown in artificially infested soil with 200 sporangia per gram of soil were significantly lower compared with noninoculated controls. However, in their study, concentrations <200 sporangia per gram of soil were not tested. Similar results were found for other Pythium species, such as *Pythium polymastum* on cabbage and *Pythium aphanidermatum* on cotton, for which 43 or 24 oospores per gram of soil were needed to cause 50% DI (Mitchell 1978). Sauvage et al. (2007) also compared severity of root rot caused by the oomycete *Aphanomyces euteiches* in pea grown in artificially infested soil with different IDs and found significant difference at a concentration equivalent to 50 oospores per gram of dry soil compared with noninoculated control.

The results obtained in this study suggest that an increase in inoculum concentration to >500 propagules per gram of dry soil does not further reduce the lettuce dry weight. The effect of such a high inoculum concentration was discussed for *Pythium* spp. in Fukui et al. (1994). In their study, an increase of ID above 200 propagules per gram of soil did not further increase wheat embry infection.

Among the weather parameters evaluated in this study, air temperature was most correlated with DI. Used alone, air temperature <18°C was found to predict DI with an overall accuracy of 0.60, suggesting that cooler temperatures are more favorable for the disease development. This is consistent with results obtain by Jacquet (1979), which found that growth, multiplication, and sexual reproduction of *P. tracheiphilum* are optimal between 10 and 18°C on growing media. Several other pathogenic *Pythium* species are also known to cause more severe disease at cooler temperatures. This is the case of *Pythium violae*, the causal agent of carrot cavity spot, which was reported to be more virulent at 15°C (Vivoda et al. 1991). In muck soils, air temperatures were also showed to be negatively correlated with carrot cavity spot, and this negative relationship was stronger with increasing number of days with temperature >30°C (Saude et al. 2014). Similarly, root infections of alfalfa caused by *Pythium irregulare* were more severe between 16 and 21°C compared with higher temperatures (Hancock 1991).

As expected, rain accumulation, especially the accumulation during the first 3 weeks after planting, was positively correlated with DI, suggesting that a wet environment is essential for disease development. Numerous studies reported the influence of soil moisture on the development of diseases caused by *Pythium* spp. (Biesbrock and Hendrix 1970; Bratoloveanu and Wallace 1985; Hancock and Grimes 1990; Saude et al. 2014; Stanghellini and Burr 1973). However, total rainfall during the first 3 weeks was not as accurate as expected to predict DI, with an overall accuracy of only 0.57. For other crops, such as alfalfa, soil moisture was more important for zoospore-forming *Pythium* spp. but was less significant for *P. ultimum* and *P. irregulare*, which are not producing zoospores (Hancock and Grimes 1990). In controlled conditions, the rate of sporangia germination for *P. tracheiphilum* is known to be influenced by the time spent in water (Jacquet 1979), and motile zoospores were formed in free water when root exudates are present (Martin and Loper 1999). Hence, it was expected that rainfall accumulation would have played a greater role in disease development.

In this study, soilborne ID was found to be positively correlated with DI, and the BRP analysis suggested a threshold ID of 132 propagules per gram of dry soil. These results are consistent with results obtained by Van der Heyden et al. (2019), who suggested a positive relationship between ID and DI with an apparent threshold rounding of 100 oospores per gram of dry soil. For pea, Gangneux et al. (2014) found a similar threshold for soil ID under natural conditions for the oomycete *Aphanomyces euteiches* causing root rot. They found a linear relationship between inoculum potential (IP) of the pathogen and the concentration of oospores in the soil, for which a concentration of about 185 oospores per gram of dry soil corresponded to an IP of three (>90% of the root system is brown without discoloration of epicotyl or hypocotyl). Similarly, Almquist et al. (2016) suggested that, for *Aphanomyces cochlioides*, an inoculum concentration of about 10 oospores per gram of soil would result in a disease severity index of <60, which is considered to be a low-risk threshold.

In this study, the decision tree combining the three predictors (ID, ATW12, and RW123) provides overall accuracy of 79%. However, soilborne ID can be measured before crop implantation unlike with the weather parameters, which are difficult to predict and always susceptible to change. Nevertheless, in a context where soilborne ID would not be available, an accurate prediction of the DI could be made based on a combination of air temperature and rain accumulation. Alone, ID provided a reliable tool in a decision support system, because it allows us to distinguish between fields with higher risk and those with low risk of disease development before crop establishment. Model accuracy could be increased by acquiring, among other things, knowledge of the role of *Pythium* community composition and interactions between present species.

The BRP modeling approach used in this study provided an intuitive decision tree describing the relationship between the predictors and DI. This approach was also effective for other pathosystems in determining best predictors and thresholds for disease, such as is the case for, among others, *Bremia lactucae* in lettuce (Fall et al. 2015b), *S. sclerotiorum* in soybean (Fall et al. 2018), *R. solani* AG3- and *Streptomyces scabies*-induced diseases in potato (Tegg et al. 2015), and *Cercospora zeae-maydis* in maize (Paul and Munkvold 2004).

In this study, three hypotheses were validated. A threshold of ID under controlled conditions was defined for the widely used lettuce Estival and the susceptible lettuce Prestige. Under field conditions, factors influencing the incidence of Pythium stunt were defined and can accurately be used to predict DI. Different thresholds for soilborne ID, air temperature, and rainfall were developed and proved to be good predictors of Pythium stunt disease in head lettuce grown in muck soils.

Acknowledgments

The authors thank the Compagnie de recherche Phytodata Inc. for the utilization of equipment and space. Acknowledgments are also given to lettuce grower members of PRISME Consortium (Productions en Régie Intégrée du Sud de Montréal enr.) who gave access to their farms and summer students who participated in soil sampling and DNA extraction. The authors are also grateful to Dr. Pierre Dutilleul for statistical advice.

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