Risk Potential of Clubroot Disease on Winter Oilseed Rape

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Abstract

Strehlow, B., de Mol, F., and Struck, C. 2015. Risk potential of clubroot disease on winter oilseed rape. *Plant Dis.* 99:667-675.

Clubroot disease caused by *Plasmodiophora brassicae* is an important disease of cruciferous plants. Although the pathogen is widespread and has been reported to cause high yield losses, the impact on winter oilseed rape (OSR) has not been experimentally verified. To quantify the risk potential of *P. brassicae*, we conducted two closely linked experiments. A semicontrolled experiment used artificial soil infestation at inoculum densities between 10^6 and 10^8 spores liter⁻¹ of soil to detect the impact on seed yield and yield components of a susceptible and resistant OSR cultivar. A greenhouse experiment was implemented using the soil of the semicontrolled experiment after cropping the two cultivars to quantify the influence of

cultivar resistance on soil inoculum. According to cumulative link mixed models, disease rating was positively correlated with the amount of inoculum. Linear regression analyses revealed a negative correlation between seed yield and inoculum density. Yield losses of 60% already appeared at the lowest inoculum density. Plant losses and reduced seed per pod were accountable for yield losses. Although the resistant cultivar showed clubroot symptoms, seed yield was not affected by the pathogen. The greenhouse experiment revealed that clubroot severity in subsequent OSR was reduced after cropping the resistant cultivar. This study showed significant yield damage of *P. brassicae* already at low infestation levels.

The soilborne pathogen *Plasmodiophora brassicae* causes clubroot, an important disease in cruciferous crops worldwide. Clubroot of winter and summer oilseed rape (OSR; *Brassica napus*) has become an increasing threat (Diederichsen et al. 2014; Dixon 2009; Korbass et al. 2009; Rastas et al. 2012; Strelkov et al. 2007; Wallenhammar et al. 2012) due to an increasing area of OSR cropping and a relatively short crop rotation. Strong seasonal and environmental effects such as soil moisture (Narisawa et al. 2005; Rastas et al. 2012; Thuma et al. 1982), pH value, or temperature (Einhorn and Bochow 1990; Gossen et al. 2013; Sharma et al. 2011a,b) have been reported to affect disease incidence (DI) and disease severity (DS) of *P. brassicae*. These complex interactions hamper the prediction of the risk potential of *P. brassicae*. Yield losses of spring OSR or canola due to *P. brassicae* have been reported to range between 10 and 100%. These reports are estimates based on observations under field conditions or on greenhouse experiments carried out with dilutions of naturally infested soil (Hwang et al. 2011a; Pageau et al. 2006; Tewari et al. 2005; Wallenhammar et al. 2000). Although it is widely accepted that yield losses of winter OSR could be substantial, to our knowledge, the impact of *P. brassicae* on the most widespread winter OSR in Europe has not been studied in detail thus far. Clubroot development is affected by inoculum density (Hwang et al. 2011a; Murakami et al. 2001, 2002; Voorrips 1996), environmental conditions (Gossen et al. 2013; Sharma et al. 2011a), and the interaction of the host and the pathogen (Buczacki et al. 1975; Crute et al. 1980; Somé et al. 1996; Williams 1966). As a result, experiments carried out with different hosts, genotypes, spore concentrations, field isolates, or environmental conditions cannot be compared with each other (Dixon 2014). Data about the risk potential of *P. brassicae* on canola or in canola-growing areas cannot be transferred to winter OSR or winter OSR-growing areas due to completely different environmental conditions and cropping practices. Field experiments to quantify yield effects are particularly hampered by the fact that *P. brassicae*, as a soilborne pathogen, requires an artificial soil infestation system with defined inoculum densities. Because DS correlates with inoculum density (Hwang et al. 2011a; Murakami et al.

2001, 2002; Voorrips 1996), we conducted experiments with defined inoculum densities in order to better understand the interaction of *P. brassicae* and winter OSR. These experiments were implemented under semicontrolled conditions to simulate field conditions. In addition to the damage potential, the enrichment of persistent resting spores in the soil is another important issue concerning the risk potential of the pathogen. Different methods exist to quantify the soil inoculum of *P. brassicae* but every method has some limitations and drawbacks. Soil inoculum can be measured directly by counting of spores with a microscope or by detection of genomic DNA of *P. brassicae* by quantitative polymerase chain reaction (Hwang et al. 2012; Wallenhammar et al. 2012) and indirectly by clubroot development on subsequent susceptible hosts (Hwang et al. 2012, 2013). Whereas the direct quantification methods detect infectious and non-infectious spores, the bioassay exclusively detects infectious spores. Nevertheless, as a semiquantitative method, it cannot specify the absolute amount of spores in the soil samples. The amount of spores in a soil sample can only be specified relative to other samples. There is little knowledge about the effect on the soil inoculum after cropping susceptible and resistant OSR. Conflicting experimental data on the influence of cropping resistant cultivars on the soil inoculum have been obtained. There are studies supporting the idea that cropping of resistant cultivars increases resting spore population in the soil (Hwang et al. 2011b, 2013), contradicting studies that resulted in a reduction of the spore load (Kroll et al. 1984; Hwang et al. 2012; Wallenhammar et al. 2000) or experiments that neither increased nor reduced the soil inoculum (Hwang et al. 2013). Therefore, data are needed on the effect of cropping resistant OSR cultivars on the soil inoculum level.

The objectives of this study were to (i) detect disease-crop loss relationships for a resistant and a susceptible winter OSR cultivar, (ii) identify yield components that were affected by *P. brassicae*, and (iii) evaluate the effect of cropping resistant and susceptible OSR cultivars on clubroot severity of subsequent susceptible OSR cultivars.

Materials and Methods

Semicontrolled experiments—2012. In miniplot experiments under semicontrolled conditions, the impact of different levels of *P. brassicae* inoculum densities on DS and yield and yield components of a resistant and a susceptible OSR cultivar was investigated during the growing season 2012—13.

Inoculum was prepared from infested OSR plants collected from a field in North Germany, a traditional OSR cropping area. The field isolate was multiplied in *B. napus* 'Oase' and infected roots were

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Accepted for publication 6 December 2014.

stored at -20°C . Resting spores were isolated from homogenized clubbed roots and spore concentration was estimated with a hemocytometer and diluted to the appropriate density. This inoculum was added to a soil mix (Einheitserde, Rhododendronerde, Quarzsand at a ratio of 0.70:0.25:0.05; pH 5.5 to 6) to produce inoculum densities of 0, 10^6 , 10^7 , and 10^8 spores liter $^{-1}$ of soil mix and filled into miniplots. These miniplots, consisting of plastic containers (diameter = 46 cm), were filled with 70 liters of sterilized soil and then filled up with 10 liters of the inoculated soil. The treatments were factorial combinations of the four inoculum densities, the susceptible OSR 'Visby', and the resistant OSR 'Mendelson'. In order to simulate field conditions, the experiment was set up outdoors, arranged in a randomized block design with 10 replicates (containers). Fifteen seedlings (cotyledon stage) were transplanted on 23 August 2012 into each container. The containers were maintained in a tray filled with water for the first week after planting to keep a high level of soil moisture. Fertilizers, fungicides, growth regulators, and insecticides were applied according to common agricultural practice. Average daily air temperature was recorded (Fig. 1).

After 6 weeks, 10 plants of each container were uprooted and disease symptoms and DI assessed. The remaining plants (five plants/container) were left until maturity to evaluate seed yield and yield components. DI was expressed as percentage of infected plants. Disease rating was recorded on a scale from 0 to 3, with 0 = no disease; 1 = tiny to small clubs, mainly on lateral roots; 2 = medium sized to bigger clubs, also including the main root; and 3 = main root heavily clubbed, few or no intact lateral roots, according to Diederichsen and Sacristan (1996). Disease ratings were weighted and combined as DS, with n_0 to n_3 = number of plants in the indicated class and N = total number of plants tested:

$$DS = \frac{0 \times n_0 + 1 \times n_1 + 2 \times n_2 + 3 \times n_3}{3 \times N}$$

Seed yield and yield components were only surveyed in 2012. Plant height and plant growth of the remaining plants were scored eight times during the growing season. A standardized growth stage (GS) scale, called the BBCH decimal system, was used to describe the growth stages of the OSR cultivars (Lancashire et al. 1991). Plants were maintained until maturity and harvested at GS 83 to 85 (30 to 50% of pods ripe, seed black and hard) to evaluate single-plant yield and the yield components, plant losses per container (percent), pods per plant, seed per pod, and thousand seed weight (TSW) per plant (grams). Yield of the inoculated treatments were related to

the noninoculated control. After the harvest of the mature plants, the roots were left in the container to decay.

Semicontrolled experiments—2013. The containers from the first experimental year were used again in order to detect the effect of cropping resistant and susceptible OSR cultivars on clubroot severity in subsequent crops. For this purpose, 10 seedlings (cotyledon stage) were transplanted on 7 September 2013 into each container. After 6 weeks, the plants were uprooted and disease symptoms assessed as previously described. Except for the initial inoculum density and the weather conditions, the experimental conditions were like those the first experimental year. Soil moisture and temperature affect initial infection and symptom development (see above). Soil moisture was kept constant between the treatments and between the years until disease assessment. Average daily air temperature after planting until symptom assessment is shown in Figure 1.

Greenhouse experiments. After the first experimental year, soil samples were taken from the pot experiments to test the influence of cultivar resistance and inoculum density on soil inoculum by a bioassay. The level of the *P. brassicae* soil infestation was measured by assessing disease ratings and DS of the subsequent susceptible OSR cultivar. Soil samples were taken after decay of the clubbed roots and then pooled for each factorial combination (inoculum density OSR cultivar). The bioassay was conducted with 35 seedlings of the susceptible OSR Visby used in each soil sample. Control plants were transplanted into pots filled with soil mix without resting spores. The containers were placed in a greenhouse at day and night temperatures of 20 ± 2 and $16 \pm 2^{\circ}\text{C}$, respectively, with a 16-h photoperiod. For the first week, the plants were kept under conditions of high soil humidity. Symptoms were assessed as mentioned above.

Statistical analyses. For ordinal measurements (disease rating on a 0-to-3 scale), cumulative link mixed models (CLMM) were fitted to the data and log-likelihood estimation provided the estimates for the model's parameters. A separate model was fitted for each experimental year (2012 and 2013) and each OSR cultivar (resistant and susceptible). The data were analyzed separately because the experimental set up was different in 2012 and 2013 and different cultivar resistance leads to characteristically different associations between inoculum density and symptom development. In 2012, the models describe the relationships between disease rating and the initial inoculum density [$\log(x + 1)$] whereas, in 2013, the explanatory variables were initial inoculum density ($\log[x + 1]$), disease rating of the previous year, and their interaction. All models were complemented with the variable "replication" as random factor to account for the variation of the experimental design. As goodness-of-fit test for the CLMM, residual deviance was defined by extent and significance.

To describe the influence of OSR resistance on the soil inoculum, a cumulative link model (CLM) was fitted to the data. The model describes the relationships between disease rating and the previous OSR cultivar (resistant or susceptible) at different levels of inoculum densities. As goodness-of-fit test for the CLM, residual deviance was defined by extent and significance.

Linear and generalized linear models (LM and GLM) were fitted to the yield data and maximum-likelihood estimation provided the estimates for the model's parameters. The models describe the relationships between seed yield and yield components (plant losses per container, pods per plant, seed per pod, and TSW per plant) as response variables and the treatment (inoculum density) as explanatory variable. The data were checked for normality using Shapiro-Wilk test prior to the analysis. Inoculum density was $\log(x + 1)$ -transformed. LM was fitted to the response variables seed yield, pods per plant, and TSW per plant. GLM with a binomial resp. poisson probability distribution was fitted to the response variables plant losses per container and seed per pods, which are appropriate theoretical distributions to consider for proportions and counts. Multiple comparisons of treatments by means were determined by Tukey's honestly significant difference (HSD) posthoc test on data (normal distributed data) or by Tukey's HSD posthoc test on ranks of data (ordinal data and not normally distributed data).

All statistical analysis and model fittings were performed using the software R version 3.0.1 (R Core Team 2013) and basic packages

Fig. 1. Average daily air temperature from August to November in 2012 and 2013. Arrows indicate planting date in the two experimental years.

were complemented by the packages “ordinal” (Christensen 2013) and “agricolae” (de Mendiburu 2013).

Results

Effect of *P. brassicae* on plant growth, DS, seed yield, and yield components. During the entire cropping season, plant height of the susceptible OSR was reduced relative to the control when inoculated with resting spores of *P. brassicae* (Fig. 2A). Plant height decreased significantly with increasing spore density (F test, $P < 0.0001$, $R^2 = 0.81$). Susceptible OSR plants inoculated with the highest inoculum density of 10^8 spores liter⁻¹ of soil did not survive the winter season due to severe *P. brassicae* infection (Fig. 2A). In contrast, *P. brassicae* did not affect plant height of the resistant OSR (Fig. 2C). The pathogen had no significant influence on growth stages for either the susceptible or resistant OSR (Fig. 2B and D).

In 2012, mean DS of the susceptible OSR increased from 0.58 to 0.86 with increasing spore concentrations between 10^6 and 10^8 spores liter⁻¹ of soil (Table 1; Fig. 3A), whereas DI was very high ($\geq 99\%$) at any inoculum level (Table 1). No differences in mean DS between the treatments were observed for the resistant cultivar (Table 1); mean DS was 0.11 to 0.14 (Table 1; Fig. 4A), and 16 to 24% of these plants were infected by *P. brassicae*.

The Kruskal-Wallis test showed a significant effect of inoculum density on DS for susceptible and resistant OSR in 2012. Significant differences among the inoculated treatments were detected for the susceptible OSR whereas, in the case of the resistant OSR, the inoculated treatments were significantly different from the noninoculated control (Table 1).

The illustration of the CLMM for the susceptible OSR shows the cumulative probabilities of an observation to fall in each of the

response categories as a function of different inoculum densities (Fig. 3B); high spore concentrations significantly led to increased probabilities of observations in high disease ratings (χ^2 test, $P < 0.001$). The analysis of deviance table shows the partitioning of the total deviance for the data (Table 2). Most of the deviance was explained by the treatments while residual deviance was not significant (goodness-of-fit test, $P = 0.127$; Table 2). Even for the resistant OSR, CLMM revealed a significant association between inoculum density and disease rating; the probabilities of observations in high disease ratings slightly increased with increasing spore concentrations (χ^2 test, $P < 0.001$; Fig. 4B). Only a small portion of the deviance was explained by treatments; however, the residual deviance was not significant (goodness-of-fit test, $P = 0.735$; Table 2).

Effect of cropping resistant and susceptible OSR cultivars on DS of the subsequent OSR in the second experimental year. In 2013, after 1 year of OSR cropping, DS and DI of the subsequent susceptible OSR were lower compared with DS and DI in 2012. Mean DS was 0.34 to 0.49, and 55 to 69% of the plants were infected with *P. brassicae* (Table 3; Fig. 5A). The data displayed a high level of dispersion (Fig. 5A). Only one resistant OSR plant was infected at the highest level of inoculum. The Kruskal-Wallis test showed a significant effect of inoculum density on DS for the susceptible cultivar in 2013. However, the inoculated treatments were only significantly different from the noninoculated control (Table 3). CLMM with DI of 2012 as explanatory variable and the replicate as random effect provided the best fit to the data. The initial inoculum density did not affect model performance significantly (χ^2 test, $P > 0.05$) and, therefore, was deleted from the model. Plants belonging to high rating categories in 2013 were associated with high DI of the previous year (χ^2 test, $P < 0.02$; Fig. 5B; Table 2), regardless of the disease

Fig. 2. Effect of inoculum density on plant height and plant development of the susceptible (A and B) and resistant (C and D) oilseed rape cultivar during the growing season from November 2012 to June 2013 (inoculum density: ● = 0, * = 10^6 , ▲ = 10^7 , and ■ = 10^8 spores/liter of soil). Bars indicate standard deviations.

ratings in 2012. Residual deviance was very high and almost significant (goodness-of-fit test, $P = 0.053$; Table 2).

Effect of cultivar resistance on soil inoculum. Using a bioassay under greenhouse conditions, we tested how the initial soil inoculum of 10^6 , 10^7 , and 10^8 spores liter⁻¹ of soil had changed after cropping susceptible and resistant OSR. The greenhouse experiments revealed that mean DS of the test plants was 0.83 after cropping the susceptible cultivar and 0.39 after cropping the resistant cultivar. Mean DS did not increase with increasing inoculum density (Table 4). According to CLM, the cultivar (χ^2 test, $P < 0.001$) and the initial inoculum density (χ^2 test, $P < 0.001$) were significant predictors of disease ratings. Furthermore, cropping susceptible OSR after high initial inoculum density increased the likelihood of higher disease ratings of the test plants (Fig. 6). This relationship was observed for both cultivars. In the case of the susceptible OSR, infected plants were already

observed at low initial inoculum density in contrast to the resistant cultivar at equal spore densities (Fig. 6).

Yield effects and disease-crop loss relationships. The yield components, plant losses per container, and seed per pods were affected by *P. brassicae* in the case of the susceptible OSR. Pods per plant and TSW per plant were not different from the noninoculated control or among the inoculated treatments (Table 1). The effect of *P. brassicae* on the survival rate of the susceptible OSR was very high: 60, 92, and 100% of the plants died due to severe *P. brassicae* infection (Table 1). Regression analysis revealed that spore density was a significant predictor for the survival rate of susceptible OSR plants (t test, $P < 0.001$; $R^2 = 0.813$). Regression analysis indicated a weak and negative relationship between inoculum density and seed per pod (t test, $P = 0.03$, $R^2 = 0.177$). However, seed per pod were only affected by *P. brassicae* at high spore concentrations of at least

Table 1. Effect of inoculum density (ID) on disease incidence (DI), disease severity (DS), yield components, and seed yield of the resistant and the susceptible oilseed rape (OSR) cultivars induced by *Plasmodiophora brassicae* in 2012 (semicontrolled experiment)^y

ID (spores liter ⁻¹ of soil)	OSR cultivar	DI (%)	DS	Plant losses (%)	Pods/plant	Seed/pod	TSW (g)	Yield (%) ^z
0	Susceptible	0 a (0.00)	0 a (0.00)	2 a (0.00)	200 a (44.51)	22 a (3.32)	4.3 a (0.34)	100 a (28.39)
10^6		99 b (3.16)	0.58 b (0.12)	60 b (0.30)	203 a (105.32)	22 a (2.73)	3.6 a (0.42)	35 b (58.35)
10^7		99 b (2.85)	0.68 b (0.08)	92 c (0.10)	186 a (220.59)	11 b (6.91)	3.9 a (1.09)	7 c (189.73)
10^8		100 b (0.00)	0.86 c (0.06)	100 d (0.00)	-	-	-	0 c
0	Resistant	0 a (0.00)	0 a (0.00)	6 a (0.10)	188 a (37.75)	26 a (3.79)	3.8 a (0.28)	100 a (22.92)
10^6		24 b (12.49)	0.14 b (0.07)	14 a (0.21)	106 a (123.57)	25 a (3.54)	3.7 a (0.23)	98 a (33.26)
10^7		16 b (6.13)	0.11 b (0.05)	26 a (0.23)	229 a (109.44)	24 a (2.07)	3.8 a (0.26)	97 a (43.06)
10^8		17 b (8.34)	0.12 b (0.07)	24 a (0.16)	204 a (46.93)	24 a (1.82)	3.7 a (0.28)	83 a (25.30)

^y Values within a column followed by different letters differ significantly at $P < 0.05$ according to Tukey's honestly significant difference (HSD) posthoc test on data (normal distributed) or on Tukey's HSD posthoc test on ranks of data (otherwise). Values in parentheses are standard deviations.

^z Seed yield relative to the noninoculated control

Fig. 3. Disease severity (DS) of the susceptible oilseed rape cultivar induced by different spore concentrations of *Plasmodiophora brassicae* in 2012 under semicontrolled conditions. **A**, Boxes and whiskers indicate interquartile ranges and subsequent 1.5-fold interquartile ranges. **B**, Relationship of *P. brassicae* inoculum density and cumulative probability of disease ratings of the susceptible oilseed rape cultivar according to cumulative link mixed models in 2012.

10^7 spores liter⁻¹ of soil (Table 1). There were no significant differences in yield components between the treatments in the case of the resistant OSR. Plant losses increased with increasing spore density but this trend was not significant (Table 1).

The effect of *P. brassicae* on seed yield of the susceptible OSR was generally very high: there was a 65, 93, and 100% reduction of mean yield relative to the noninoculated control at spore concentrations of 10^6 , 10^7 , and 10^8 spores liter⁻¹ soil, respectively (Table 1). The regression analysis revealed that spore concentration (*F* test, $P < 0.001$, $R^2 = 0.81$) and DS (*F* test, $P < 0.001$, $R^2 = 0.77$) were

significant predictors for seed yield of the susceptible OSR (Fig. 7). Multiple comparison according to Tukey's HSD posthoc test showed that seed yield was significantly different among the inoculated treatments (Table 1). Seed yield of the resistant OSR was not affected by *P. brassicae* (Table 1).

Discussion

Up to now, the evaluation of the damage potential of *P. brassicae* on yield and yield components of winter OSR is lacking. This study revealed that clubroot severity is positively correlated with inoculum

Fig. 4. Disease severity (DS) of the resistant oilseed rape cultivar induced by different spore concentrations of *Plasmodiophora brassicae* in 2012 under semicontrolled conditions. Boxes and whiskers indicate interquartile ranges and subsequent 1.5-fold interquartile ranges (A). Relationship of *P. brassicae* inoculum density and cumulative probability of disease ratings of the resistant oilseed rape cultivar according to cumulative link mixed models in 2012 (B).

Table 2. Partitioning of the total deviance into treatment and residual deviance according to cumulative link mixed models for the semicontrolled experiments in 2012 and 2013 and cumulative link model for the bioassay in the greenhouse

Experiment	OSR cultivar ^v	Source	df ^w	deviance	<i>P</i> value
Semicontrolled, 2012	Susceptible	Total	120	614.54	<0.001
		Treatment, ID ^x	5	485.28	<0.001
		Residual	115	129.27	0.1265
	Resistant	Total	117	124.53	<0.001
		Treatment, ID ^x	5	22.31	<0.001
		Residual	112	102.22	0.7352
Semicontrolled, 2013	Susceptible	Total	117	275.872	<0.001
		Treatment, DI ^y	5	138.701	<0.02
		Residual	112	137.171	0.053
Bioassay, greenhouse		Total	12	242.45	<0.001
		Treatment, Cult. + ID ^z	5	241.58	<0.001
		Residual	7	0.8657	0.9967

^v Oilseed rape.

^w Degrees of freedom.

^x Explanatory variable = initial inoculum density (ID).

^y Explanatory variable = disease incidence (DI) of 2012.

^z Explanatory variables = cultivar and initial inoculum density.

density; DS increased from 0.6 to 0.9 with increasing inoculum density between 10^6 and 10^8 spores liter⁻¹ in the case of the susceptible OSR. Previous greenhouse experiments support these findings (Hwang et al. 2011a; Murakami et al. 2002; Voorrips 1996). For example, in canola, DS increased from 0.4 to 1.0 with increasing inoculum density of 10^5 to 10^8 resting spores cm⁻³ of soil (Hwang et al. 2011a). DS of *B. rapa* increased from approximately 0.4 to 1.0 at spore concentrations between 10^4 and 10^5 spores g⁻¹ of soil, and DS of *B. oleracea* increased from 0.1 to 0.9 with increasing spore concentrations between 10^4 and 10^6 spores g⁻¹ of soil (Murakami et al. 2002). The DS of all these cruciferous crops are lower at all inoculum levels compared with the susceptible OSR cultivar used in this study. Murakami et al. (2002) concluded that the role of

inoculum density on symptom development changes with soil type, soil pH, plant species, cultivar resistance, and inoculum used. Therefore, estimating the damage potential of *P. brassicae* is difficult on the basis of the inoculum density in the soil alone. Little is known about the resting spore population in the soil of commercial fields. In Japan, the soil infestation level of the fields was usually below 10^6 spores g⁻¹ of soil (Tsushima et al. 2010). On Swedish fields, soil infestation levels above 1.3×10^6 spores g⁻¹ of soil were detected (Wallenhammar et al. 2012) and the concentration of *P. brassicae* spores on Canadian fields had been estimated to exceed 10^8 spores g⁻¹ of soil (Hwang et al. 2011b). These high soil infestation levels demonstrate the severe potential impact of *P. brassicae* on cruciferous crops on commercial fields.

In 2012, about 14% of the plants of resistant Mendelson developed galls. The field isolate selected in this study is not virulent against Mendelson. In other studies 11 to 14% of the plants of resistant canola

Table 3. Effect of inoculum density (ID) on disease incidence (DI) and disease severity (DS) of the resistant and the susceptible oilseed rape (OSR) cultivars induced by *Plasmodiophora brassicae* in 2013 (semicontrolled experiment)^z

ID (spores liter ⁻¹ soil)	OSR cultivar	DI (%)	DS
0	Susceptible	0 a (0.00)	0.00 a (0.00)
10^6		69 b (36.58)	0.34 b (0.27)
10^7		67 b (34.72)	0.49 b (0.33)
10^8		55 b (27.28)	0.34 b (0.19)
0	Resistant	0 a (0.00)	0.00 a (0.00)
10^6		0 a (0.00)	0.00 a (0.00)
10^7		0 a (0.00)	0.00 a (0.00)
10^8		1 a (3.95)	0.01 a (0.04)

^z Values within a column followed by different letters differ significantly at $P < 0.05$ according to Tukey's honestly significant difference posthoc test on ranks of data. Values in parentheses are standard deviations.

Table 4. Effect of inoculum density (ID) and cropping of susceptible and resistant oilseed rape (OSR) on disease severity (DS) and disease incidence (DI) of the subsequent susceptible oilseed rape according to the bioassay.

ID (spores liter ⁻¹ soil)	OSR cultivar	DI (%)	DS
0	Susceptible	0	0.00
10^6		100	0.83
10^7		100	0.82
10^8		100	0.84
0	Resistant	0	0.00
10^6		63	0.42
10^7		79	0.38
10^8		88	0.47

Fig. 5. Disease severity (DS) of the susceptible oilseed rape cultivar induced by different spore concentrations of *Plasmodiophora brassicae* in 2013 under semicontrolled conditions. **A**, Boxes and whiskers indicate interquartile ranges and subsequent 1.5-fold interquartile ranges. **B**, Relationship of *P. brassicae* inoculum density and cumulative probability of disease ratings of the susceptible oilseed rape cultivar according to cumulative link mixed models in 2013.

Fig. 6. Effect of inoculum density of *Plasmodiophora brassicae* and previous crop (resistant and susceptible oilseed rape cultivar) on disease ratings of the subsequent susceptible cultivar according to cumulative link model. Disease rating: light gray = 0 (no clubs), medium gray = 1 (small clubs), dark gray = 2 (medium sized clubs), and black = 3 (heavily clubbed) in the greenhouse experiment.

Fig. 7. Effect of *Plasmodiophora brassicae* inoculum density and disease severity on seed yield of the susceptible oilseed rape cultivar in 2012.

cultivars developed some clubroot symptoms (Hwang et al. 2011b, 2013). The authors speculate that this may be caused through susceptible off-types, segregation, or incomplete gene penetrance. Because the resistance of Mendelson is race specific (Diederichsen et al. 2009), it might be possible that, due to an increase of inoculum density, a pathotype which is virulent against Mendelson and which occurred at a very low frequency within our field isolate reached a threshold level and led to an increased development of clubroot symptoms in the resistant OSR. In 2013, the resistant OSR showed no clubroot symptoms. The climatic conditions, with colder conditions during the first 6 weeks in the second experimental year (Fig. 1), could have prevented disease outbreak in the resistant OSR. Particularly under unfavorable environmental conditions, plants of the resistant OSR Mendelson were not or only slightly infected even in fields with pathotypes virulent against Mendelson (Diederichsen et al. 2014).

In the second experimental year, increased clubroot severity in the subsequent OSR was expected due to the multiplication of *P. brassicae* spores in the soil. However, DS was lower compared with 2012 and the data displayed a high level of dispersion in the case of the susceptible OSR. This confirms previous studies showing that a successful infection and clubroot severity are influenced by environmental factors. In 2013, the average air temperature was 4°C colder compared with 2012 and the daily air temperature was at no time above 15°C (Fig. 1). Greenhouse experiments revealed that root hair infection and symptom development depend strongly on temperature (Gossen et al. 2012, 2013; Sharma et al. 2011a,b; Thuma et al. 1982).

Regression analyses revealed a significant influence of inoculum density and DS on seed yield of the susceptible OSR. Murakami et al. (2002) concluded that disease seldom occurred at an inoculum density of 10^3 spores g^{-1} of soil in any crop or cultivar. In this study, mean DS and seed yield losses were high; yield losses of about 60% already occurred at the generally accepted threshold level for symptom development of 10^3 spores g^{-1} of soil (Faggian et al. 2009). Hwang et al. (2011a) detected seed yield losses of about 80% at an inoculum density of 10^5 spores cm^{-1} soil. In this study, all plants died due to severe clubroot infection at a similar spore concentration. This could be attributed to a high pathogenicity of the inoculum used in this study. Hwang et al. (2011a) studied the effect of inoculum density of a Canadian field isolate on canola; for similar DS, they had to use at least 100× higher inoculum densities to get clubroot severity similar to our study. Field isolates of the New World seemed to be less virulent against *B. napus* compared with field isolates of Europe (Donald et al. 2006). Thus, already low levels of soil inoculum could become an important limiting factor for crop profitability, especially in regions where *P. brassicae* possess a high pathogenicity against *B. napus*. Strehlow et al. (2014) showed that *Plasmodiophora* field isolates in Germany are genetically highly diverse. This indicates that *P. brassicae* displays great pathogenic variation. Therefore, information about the pathogenicity of the predominant field isolate is necessary when characterizing the damage potential of *P. brassicae*.

The greenhouse experiment using the previous-year soil revealed that mean DS was twice as high in the soil that was cultivated with the susceptible OSR compared with the soil that was cultivated with the resistant OSR (0.83 versus 0.39). After cropping the resistant OSR, mean DS of the subsequent susceptible OSR was even lower compared with DS before repeated cropping of OSR at the lowest initial inoculum density. Mean DS did not differ significantly between the initial inoculum densities. All these findings suggest that (i) cropping susceptible cultivars will greatly increase the soil inoculum, as expected; (ii) the propagation of resting spores in the soil was more efficient at low initial inoculum density in the case of the susceptible OSR; and (iii) the cropping of resistant OSR reduced the soil inoculum. Hwang et al. (2013) counted the number of resting spores in clubs of susceptible canola after inoculation with different spore concentration. This study revealed that the spore load increased by over 60-fold at low and by less than 3-fold at high inoculum levels and supports the finding that the propagation of spores was density dependent in the case of the susceptible cultivar.

In conclusion, inoculum density affected DS, seed yield, and yield components of susceptible winter OSR and demonstrated a significant

yield damage potential of *P. brassicae*. Nonetheless, the impact on seed yield has to be confirmed by conducting experiments of sufficient size carried out on naturally clubroot-infested fields. Already low levels of soil inoculum became an important limiting factor for crop profitability. Weather conditions, particularly low temperature, seemed to be responsible for hampering disease outbreak in the second experimental year. All factors such as environmental conditions or pathogenicity of the field isolate that may influence clubroot disease, as well as the nonuniform distribution of the pathogen, prevent the use of inoculum density to predict clubroot severity and its impact on the yield.

Acknowledgments

This research was partially supported by the Evangelisches Studienwerk Vilgert. We thank J. Jäger and I. Gliege for their excellent technical assistance.

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