



Reaction of clubroot-resistant genotypes of *Brassica rapa*, *Brassica napus* and *Brassica oleracea* to Polish *Plasmodiophora brassicae* pathotypes in laboratory tests

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Abstract The *Brassica* genotypes selected for the experiments were previously found to be resistant to various *Plasmodiophora brassicae* pathotypes (Pb). Their interaction with pathotypes Pb2, Pb3 and Pb9 isolated in Poland was studied, using macroscopic observation for the presence of root galls, microscopic observations of *P. brassicae* plasmodia in the root hairs and quantitative PCR for determination of the pathogen's quantity in plant roots and growing media. Of the *Brassica* genotypes studied, only *B. rapa* var. *capitata* line AABBcc was fully resistant to all the Polish pathotypes of *P. brassicae*. Some of the other “clubroot-resistant” genotypes tested were resistant to selected pathotypes, e.g. *Brassica napus* var. *rapifera* ‘Wilhelmsburger’ to Pb 2 and Pb3, *Brassica oleracea* var. *capitata* ‘Kilaton F₁’ to Pb2 and *Brassica rapa* subsp. *pekinensis* ‘Bilko F₁’ to Pb3, but were susceptible to others. *B. oleracea* var. *capitata* ‘Bindsachsener’, *B. oleracea* var. *acephala* subvar. *lacinata* ‘Verheul’ and *B. napus* var. *napus* ‘Mendel F₁’ were moderately to highly susceptible to all

Polish *P. brassicae* pathotypes. These results show that the classification of virulence of *P. brassicae* pathotypes selected in various areas differs significantly toward the same *Brassica* genotypes and puts in question the practical value of pathotype classification determined with differential sets for farmers and plant breeders. Our results showed that *B. rapa* var. *capitata* AABBcc line, *B. napus* var. *rapifera* ‘Wilhelmsburger’, *B. oleracea* var. *capitata* ‘Kilaton F₁’, *B. rapa* subsp. *pekinensis* ‘Bilko F₁’ could be considered in *Brassica* breeding programmes as a source of resistance to Polish *P. brassicae* isolates.

Keywords *Brassica* genotypes · Clubroot resistance · *Plasmodiophora brassicae* · Pathotypes

Introduction

Clubroot is a very dangerous disease affecting most of *Brassica* species and many other cruciferous plants. Its causative agent is the obligate parasite *Plasmodiophora brassicae* Wor. (Dixon 2009). The pathogen has a complex life cycle comprising three stages: 1) root hair infection by resting spores present in soil, 2) cortical infection, and 3) formation of resting spores and their release to the soil from decaying root galls (Kageyama and Asano 2009; McDonald et al. 2014). The primary phase of the life cycle of *P. brassicae* (root hair infection) occurs in both susceptible and resistant cultivars of host species, and even in non-host plants (Deora et al. 2013). However, the infection rate and further development of the pathogen in root hairs differ among *Brassica*

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species, with development occurring quicker in susceptible lines and slower in the resistant ones. Characteristic disease symptoms appear on roots in the form of irregular galls, initially small, light yellow and fragile and then dark and decaying. Diseased tissues lose their ability to uptake water and nutrients from the soil, which results in wilting and stunting of the above ground part of plants (Strelkov et al. 2006). Decaying diseased roots release resting spores into the soil, where they can remain viable for at least 20 years (Dixon 2009).

The disease causes severe losses on cruciferous vegetable and rapeseed plantations in Poland and worldwide (Robak 1991; Wallenhammar 1996; Dixon 2014). Depending on soil conditions, infestation rate, pathogenicity of *P. brassicae* local strains and susceptibility of a crop's variety, yield losses can range from 30% to even 100% (Wallenhammar 1996). In control of the disease, the most important are preventive treatments including usage of pathogen-free fields, early pathogen detection, non-host crop rotation, use of the bait, decoy or catch crops, maintaining the appropriate soil pH and soil drenches with fungicides (Murakami et al. 2000; Peng et al. 2014; Hwang et al. 2015). However, these methods would not reduce clubroot severity in heavily infested fields and they are not cost effective. Besides, soil drenches with fungicides have a very negative impact on the environment. Thus, the use of resistant varieties is considered as the most efficient method for control of the disease (Rahman et al. 2014). However, the choice of resistant cultivars is limited and the varietal resistance can be easily broken due to high pathogenic variability of *P. brassicae* (Diederichsen et al. 2014). Therefore, resistance to clubroot is one of the mayor tasks in *Brassica* breeding programmes.

P. brassicae consists of numerous physiological pathotypes (races), differing in their ability to infect specific host genotypes (Robak 1991). Several sets of plant hosts have been proposed to determine these pathotypes (Williams 1966; Buczacki et al. 1975; Strelkov and Hwang 2014). The most commonly used is European Clubroot Differential Set (ECD), developed by Buczacki et al. (1975), which consist of three subsets: *B. rapa* (five hosts), *B. napus* (five hosts) and *B. oleracea* subset (five hosts) and allows to select different pathotypes of *P. brassicae*. The differential set proposed by Williams (1966) uses four differential genotypes: *B. napus* (two host) and *B. oleracea* (two

host), which can differentiate sixteen pathotypes. The pathogenicity of *P. brassicae* pathotypes is highly variable within and between field populations. According to Robak (1991), in Poland the most common are pathotypes 2, 3 and 9.

In clubroot resistance breeding programs it is of a great importance to evaluate the available sources of resistance. There are several resistant *Brassica* species and cultivars harbouring resistance genes, but their resistance may vary depending on a pathotype of the pathogen. The aim of this study was: 1) to evaluate the degree of resistance of several *Brassica* genotypes carrying various resistance genes to Polish isolates of *P. brassicae* by the use of micro- and macroscopic observation of the disease symptoms and molecular tests of the presence of spores in roots of the tested plants; and 2) to determine whether cultivation of selected plants can lead to the reduction of the pathogen's quantity in the soil.

Materials and methods

Plant material

The experiments were done on five genotypes of *B. oleracea*, *B. napus* and *B. rapa*, selected from European Clubroot Differential Set, and on three commercial cultivars. These genotypes have been found to be highly resistant to several *P. brassicae* pathotypes (Kuginuki et al. 1999; Diederichsen et al. 2006; Strelkov et al. 2006; Cebula et al. 2010; Cao et al. 2014) and are recommended as a source of resistance traits in breeding (Hasan et al. 2012). The Chinese cabbage (*B. rapa* ssp. *pekinensis*) cv. Granaat, extremely susceptible to all known pathotypes of clubroot (Kopecký et al. 2012), was used as a negative control for resistance to clubroot. The list of the genotypes used in the study is presented in Table 1. The seeds of genotypes belonging to European Clubroot Differential Set (ECD) were obtained from the Horticulture Research International, Genetic Resources Unit (Wellesbourne, Warwick, UK). The seeds of Chinese cabbage (*B. rapa* subsp. *pekinensis*) cv. Bilko F₁ were purchased from Bejo Seeds Inc. (Konotopa, Poland) and seeds of headed cabbage (*B. oleracea* var. *capitata*) cv. Kilaton F₁ and rapeseed (*B. napus* var. *napus*) cv. Mendel F₁ were obtained from the collection of Research Institute of Horticulture (Skierniewice, Poland).

Source of the *P. brassicae* isolates

Infected root samples, with visible galls, were collected from the commercial plantations of *Brassica oleracea* var. *capitata* cv. Kamienna Głowa located in various parts of Poland. The samples were washed, cleaned, air dried and catalogued. The pathotypes of the isolates were determined as *P. brassicae* (Pb) 2, 3 and 9 based on the reaction of the four genotypes included in differential set of Williams (1966) to the pathogen (Table 2).

Inoculum preparation

Spore suspension was prepared as described by Voorrips (1992). The clubbed roots were homogenized with distilled water (1:3, w:v) at high speed in a mechanical blender. The suspension was filtered four times through 5 layers of cheesecloth. The spore concentration in the filtrate was quantified in haemocytometer and was adjusted to approximately 10^8 spores per mL through dilution with sterile distilled water. Obtained spore suspension was stored at 4 °C until use. To propagate these pathotypes for further studies, seeds of the susceptible *B. rapa* cv. Granaat were sown in growing medium inoculated with resting spores (10^7 spores per mL of the medium) of each *P. brassicae* pathotype and grown in a greenhouse under 14-h light/10-h dark photoperiod at 22 °C. After 5 weeks of growth in the inoculated medium, the plants were dug out, cleaned and then the galls were separated and stored at –20 °C until use.

Glasshouse bioassay of *P. brassicae* resistance – Macroscopic observation

The growing medium used in the tests was prepared by mixing the peat substrate (Klasmann) at pH 5.2 with the

Table 2 Genotypes used to identify *Plasmodiophora brassicae* pathotypes according to Williams (1966)

ECD No.	Differential host
ECD 10	<i>Brassica napus</i> var. <i>rapifera</i> cv. Wilhelmsburger
ECD 11	<i>Brassica oleracea</i> var. <i>capitata</i> cv. Badger Shipper
ECD 13	<i>Brassica oleracea</i> var. <i>capitata</i> cv. Jersey Queen
–	<i>Brassica napus</i> var. <i>napobrassica</i> cv. Laurentian

low peat (pH 5.9) in 1:1 proportion (v:v), and then sterilizing at 100 °C for 20 min. After 2 weeks, the resting spore suspension of *P. brassica* pathotypes Pb2, Pb3 and Pb9 was added to growing medium in quantity to obtain final spore concentration of about 10^7 spores per mL and mixed thoroughly. The experiments were carried out in plastic trays filled with 2 L of inoculated growing media. The trays filled with non-inoculated medium served as a control. 40 seeds from each genotype were seeded into the trays in three replicates. Plants were grown in a glasshouse under natural light, photoperiod 14-h/10-h and temperature 22 °C. The growing medium moisture was maintained by regular watering. 10, 20 and 35 days after sowing the sample consisted of 10 randomly selected plants was uptaken, the roots were washed under the running water and blotted on paper towel. The occurrence of root galls was assessed individually on each plant on a 0–3 scale according to Buczacki et al. (1975) and Kuginuki et al. (1999), where: 0 – no symptoms visible on roots, 1 – single galls on lateral roots, 2 – numerous galls on lateral and/or main roots, 3 – distortion of the entire root system, uniform galls on the main root. The trials were conducted as a complete randomized block design with three replications per a treatment. For

Table 1 Genotypes used for micro- and macroscopic observation and for molecular tests

Genotype	ECD number
<i>Brassica rapa</i> var. <i>capitata</i> line AABBcc (turnip)	ECD 03
<i>Brassica rapa</i> ssp. <i>pekinensis</i> cv. Granaat (Chinese cabbage)	ECD 05
<i>Brassica napus</i> var. <i>rapifera</i> cv. Wilhelmsburger (swede)	ECD 10
<i>Brassica oleracea</i> var. <i>capitata</i> cv. Bindsachsener (headed cabbage)	ECD 12
<i>Brassica oleracea</i> var. <i>acephala</i> subvar. <i>lacinata</i> cv. Verheul (kale)	ECD 15
<i>Brassica oleracea</i> var. <i>capitata</i> cv. Kilaton F ₁ (headed cabbage)	–
<i>Brassica rapa</i> subsp. <i>pekinensis</i> cv. Bilko F ₁ (Chinese cabbage)	–
<i>Brassica napus</i> var. <i>napus</i> cv. Mendel F ₁ (rapeseed)	–

statistical analysis, disease index (ID) for each genotype was calculated, using the formula of Strelkov et al. (2006):

$$ID = \frac{\sum(n \times 0 + n \times 1 + n \times 2 + n \times 3)}{N \times 3} \times 100\%$$

where: n is the number of plants in each class; N is the total number of plants; 0, 1, 2 and 3 are the symptoms severity indices.

All roots were then packed in plastic bags and stored at -70° for further qPCR analysis. The bioassays were conducted twice.

qPCR analyses of *P. brassicae* in plant roots and growing medium

The research was carried out on the roots obtained from plants grown for 10, 20 and 35 days, as described above. In the case of plants growing in non-inoculated growing medium (control), the samples were collected only once, after 35 days. The samples of growing media were collected twice, before sowing and after the experiment was completed. Before DNA extraction, root samples were thoroughly washed with distilled water and blotted on paper towel. Both the roots and growing media were pulverized in liquid nitrogen with mortar and pestle. DNA extraction from plant material was made using DNeasy Plant Mini Kit (Qiagen) and from soil samples using NucleoSpin Soil Kit (Macherey Nagel), according to manufacturer's instructions.

Real-time PCRs were performed using PbF and PbR primers and fluorescently labelled PbP probe specific for *P. brassicae* (Wallenhammar et al. 2012) (Table 3). All qPCR amplifications were conducted using a LightCycler 480 Probe Master (Roche) in 25 μ L volume containing 5 μ L template DNA, 0.45 μ L primer F, 0.15 μ L primer R, 0.5 μ L probe, 12.5 μ L Master Mix and 6.4 μ L H_2O . DNA extracts were diluted five-fold before analysis and were analysed in triplicate. All reactions were run at $95^{\circ}C$ for 10 min followed by 45 cycles at $95^{\circ}C$ for 15 s and finally $59^{\circ}C$ for 1 min. The standard curve for calculating the number of *P. brassicae* genome copies in analysed samples was constructed using plasmid carrying the *P. brassicae* target sequence as a template in qPCR, prepared according to Wallenhammar et al. (2012). A 10-fold dilution series of the plasmid standard were analysed in triplicate using the real-time PCR assay and Ct values were then plotted

against the amount of plasmid DNA to create a standard curve. The pathogen is present in plants and soil in various development forms. To simplify the further calculations, the results of quantitative determination of its DNA copy numbers were presented as equivalents of resting spores, as described by Wallenhammar et al. (2012).

Microscopic observation of *P. brassicae* plasmodia in root hair

The plants were grown in Phytotoxkit test plates (Tigret sp. z o. o.) filled with growing medium inoculated with *P. brassicae* resting spores at density of 10^7 per mL. Ten seeds of each plant genotype studied were sown per one plate in three replications and germinated in growing chamber (Sanyo) with relative humidity of 60%, at $22^{\circ}C$ in the light for 14 h and at $20^{\circ}C$ in the dark for 10 h. Microscopic observations of plants roots were conducted using Olympus BX 41 microscope from 4th to 20th day after sowing. The roots were carefully extracted from the growing medium, washed several times in sterilized water and cut into 1 cm fragments. Three fragments of each root were examined, beginning from the fragments situated nearest of the seedling's stem. The number of plasmodia containing mature, spherical zoosporangia was estimated in 1 cm fragments of roots. Average number and maximum number of plasmodia was estimated in 18 pieces of root hairs for each genotype. The experiment was conducted twice for each *P. brassicae* pathotype and each plant genotype.

Data analysis

The data were analysed by standard statistical procedure. Significance of differences between means was established by one-way analysis of variance and Newman-Keuls test at $P < 0.05$.

Table 3 Primers and probe sequences used in real-time PCR quantitation of *P. brassicae* (according to Wallenhammar et al. 2012)

Primer/probe	Sequence 5' – 3'
PbF (forward)	AAA CAA CGA GTC AGC TTG AAT GC
PbR (reverse)	TTC GCG CAC AAG CAC TTG
PbP (probe)	CGC GCC ATG CGA CAC TGT TAA ATT G

Results

Glasshouse resistance bioassay – Macroscopic observations

The first galls on roots appeared 20 days after sowing on the susceptible Chinese cabbage ‘Granaat’ growing in media inoculated with *P. brassicae* pathotypes 2 and 9 and on resistant swede ‘Wilhelmsburger’ and kale ‘Verheul’ growing in media inoculated with Pb9 and on Chinese cabbage ‘Bilko F₁’ growing in media inoculated with Pb3 and Pb9 (Table 4, Fig. 1). After 35 days from sowing, disease symptoms were visible in almost all genotypes, although their severity varied greatly depending on a pathotype of the pathogen. Swede ‘Wilhelmsburger’ and headed cabbage ‘Kilaton F₁’ were fully resistant to Pb2, but only partially resistant to Pb3 and highly susceptible to Pb9. Chinese cabbage ‘Bilko F₁’ showed high resistance to Pb2 but intermediate to Pb3 and low to Pb9. Only turnip line AABBcc showed nearly complete resistance to all the pathotypes of *P. brassicae* tested. Disease symptoms were not observed in all control trays during the entire assay.

qPCR analyses of *P. brassicae* in plant roots and growing medium

The pathogen was present in the roots of all the tested genotypes grown in media inoculated with pathotypes 2, 3 and 9 on all sampling times (Table 5). In the case of Pb2, the lowest quantity of spores was observed in turnip line AABBcc. After 35 days the highest quantity of spores was observed in the roots of kale ‘Verheul’ and headed cabbage ‘Bindsachsener’ and in the susceptible Chinese cabbage ‘Granaat’. In three genotypes: turnip line AABBcc, swede ‘Wilhelmsburger’ and headed cabbage ‘Kilaton F₁’ the quantity of spores in roots after 35 days from sowing (third sampling time) have decreased as compared to the quantity of the spores found during the first sampling time, whereas in the remaining genotypes the spore quantity in roots increased during this period. In the case of Pb3, the lowest quantity of spores was observed in turnip line AABBcc, swede ‘Wilhelmsburger’ and rapeseed ‘Mendel F₁’ (Table 5). After 35 days the highest quantity of spores was observed in the roots of the four cultivars: resistant headed cabbages ‘Bindsachsener’ and ‘Kilaton F₁’ and Chinese cabbage ‘Bilko F₁’ and in the susceptible Chinese cabbage ‘Granaat’. Between 10th and 35th

day the level of spores has decreased in swede ‘Wilhelmsburger’, but increased in the remaining genotypes, including susceptible Chinese cabbage ‘Granaat’. In plants inoculated with Pb9, the lowest quantity of spores was observed in turnip line AABBcc and kale ‘Verheul’ after 35 days (third sampling time). The highest quantity of spores was detected in the roots of clubroot resistant rapeseed ‘Mendel F₁’, swede ‘Wilhelmsburger’ and in susceptible Chinese cabbage ‘Granaat’ (Table 5). The level of spores decreased between 10th and 35th day after sowing in the roots of turnip line AABBcc, kale ‘Verheul’ and headed cabbage ‘Kilaton F₁’, but increased in the roots of remaining genotypes. More complicated was the time course of root infection in the case of Pb9. Analysis of the roots collected after 20 days from sowing showed a decrease of spore numbers in reference to quantity of spores in the roots collected at the first sampling date, but after additional 15 days of growth the quantity of spores increased, in some genotypes even exceeding the quantity detected in the roots after 10 days of growing (Table 5).

At the beginning of the experiment, in all the tested trays there was about 10⁷ resting spores in 1 g of growing medium (Table 6). After 5 weeks of growth of susceptible Chinese cabbage ‘Granaat’ the highest number of resting spores was observed in growing media infested with all the pathotypes tested. Cultivation of the other tested genotypes contributed to the increase of the number of spores only in the growing media inoculated with pathotypes 2 and 9, which was especially visible in case of turnip line AABBcc and headed cabbage ‘Bindsachsener’ grown in the medium infected by Pb2 as well as in case of swede ‘Wilhelmsburger’ and rapeseed ‘Mendel F₁’ grown in medium infected by Pb9. It was noted that the quantity of spores of Pb3 decreased after 35 days of growth of all the genotypes in the infested media. The largest decrease was observed in Pb3 inoculated medium where rapeseed ‘Mendel F₁’ was cultivated.

Microscopic observations of *P. brassicae* plasmodia in root hairs of the genotypes tested

The first plasmodia in hair roots appeared within 4–10 days from sowing the seeds (Fig. 2). The average number of plasmodia in the observed fragments of the roots depended on the plant genotype and on the *P. brassicae* pathotype. For the Pb2, the highest average

Table 4 Reaction of *Brassica* genotypes to *P.brassicae* pathotypes in macroscopic observation

Genotype	Disease Index (%)					
	Pb2		Pb3		Pb9	
	Days after sowing					
	20	35	20	35	20	35
<i>B. rapa</i> var. <i>capitata</i> line AABBcc	0 a	3 a	0 a	1 a	0 a	4 a
<i>B. napus</i> var. <i>rapifera</i> cv. Wilhelmsburger	0 a	0 a	0 a	20 b	10 b	100 c
<i>B. oleracea</i> var. <i>capitata</i> cv. Bindsachsener	0 a	80 b	0 a	66 e	0 a	66 bc
<i>B. oleracea</i> var. <i>acephala</i> subvar. <i>lacinata</i> cv. Verheul	0 a	71 b	0 a	46 d	2 a	40 b
<i>B. oleracea</i> var. <i>capitata</i> cv. Kilaton F ₁	0 a	0 a	0 a	68 f	0 a	72 bc
<i>B. rapa</i> subsp. <i>pekinensis</i> cv. Bilko F ₁	0 a	7 a	2 b	66 e	2 a	80 c
<i>B. napus</i> var. <i>napus</i> cv. Mendel F ₁	0 a	21 a	0 a	32 c	0 a	100 c
<i>B. rapa</i> ssp. <i>pekinensis</i> cv. Granaat	6 b	100 c	0 a	100 g	47 c	100 c

Means in columns followed by the same letter do not differ at $P < 0.05$ based on Newman-Keuls test

and maximum number of plasmodia was observed in root hairs of turnip line AABBcc and headed cabbage ‘Kilaton F₁’. The lowest average number of plasmodia was observed in root hairs of kale ‘Verheul’ and the lowest maximum number of plasmodia was recorded in swede ‘Wilhelmsburger’. In the case of Pb3 the highest average and maximum number of plasmodia was observed in root hairs of kale ‘Verheul’. Also, high maximum number of plasmodia in root hairs was observed in Chinese cabbage ‘Bilko F₁’. The lowest number of plasmodia was observed in case of swede ‘Wilhelmsburger’. For Pb9, the highest average and maximum number of plasmodia was observed in root hairs of rapeseed ‘Mendel F₁’. Similarly, high average and maximum numbers of plasmodia per 1 cm of root were observed in turnip line AABBcc

(Table 7). The lowest number of plasmodia was observed in case of kale ‘Verheul’.

Discussion and conclusions

Resistance to clubroot is one of the most important objectives in *Brassica* breeding programmes. The main problems in this task is large variations of pathogenicity of *Plasmodiophora brassicae*, which can quickly break the resistance of cultivars intended for planting on large areas (LeBoldus et al. 2012; Tanaka and Ito 2013; Diederichsen et al. 2014). Therefore, in clubroot resistance breeding programmes it is of a great importance to evaluate the available sources of resistance to most, or at least to most of locally occurring pathotypes of

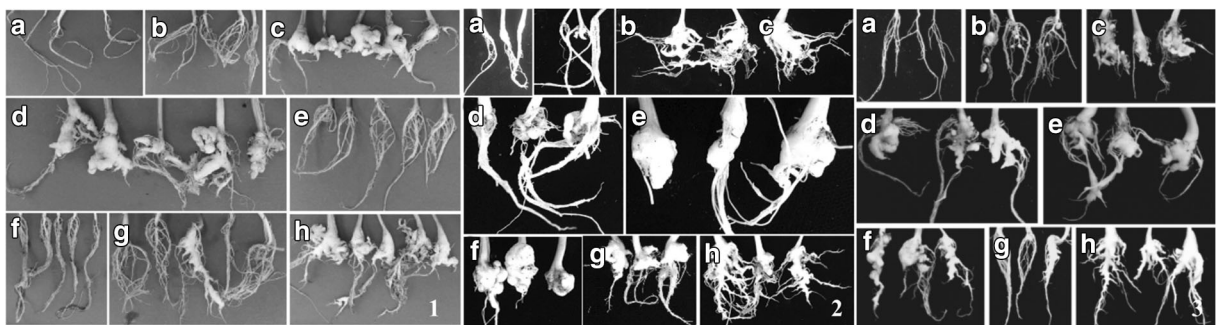


Fig. 1 Root systems of the plant genotypes tested after 35 days after sowing: 1 - pathotype 2; 2 - pathotype 9; 3 - pathotype 3 (a - *B. rapa* line AABBcc, b - *B. napus* cv. Wilhelmsburger, c - *B. oleracea* cv.

Bindsachsener, d - *B. oleracea* cv. Verheul, e - *B. oleracea* cv. Kilaton F₁, f - *B. rapa* cv. Bilko F₁, g - *B. napus* cv. Mendel F₁, h - *B. rapa* ssp. *pekinensis* cv. Granaat)

Table 5 The average spore quantity in roots of *Brassica* genotypes growing in soil infected by *P.brassiccae* pathotypes 2, 3 and 9

Genotype	Average spore quantity in 1 g of roots dry weight											
	Pb2			Pb3			Pb9					
days after sowing	10	20	35	10	20	35	10	20	35	10	20	35
<i>B. rapa</i> var. <i>capitata</i> line AABBcc	8.47 × 10 ⁶ b	6.69 × 10 ⁷ a	7.27 × 10 ⁵ a	3.3 × 10 ⁵ c	8.0 × 10 ⁵ c	2.3 × 10 ⁵ a	3.6 × 10 ⁵ b	7.8 × 10 ³ b	1.3 × 10 ⁵ a			
<i>B. napus</i> var. <i>rapifera</i> cv. Wilhelmsburger	2.34 × 10 ⁷ d	3.55 × 10 ⁸ h	2.32 × 10 ⁶ b	0.9 × 10 ⁶ e	1.3 × 10 ⁵ a	2.1 × 10 ⁵ a	7.1 × 10 ⁵ c	1.0 × 10 ⁴ c	1.6 × 10 ⁸ e			
<i>B. oleracea</i> var. <i>capitata</i> cv. Bindsachsener	1.20 × 10 ⁸ g	2.57 × 10 ⁸ f	1.10 × 10 ⁹ f	1.3 × 10 ⁵ b	0.9 × 10 ⁶ d	2.1 × 10 ⁸ d	2.4 × 10 ⁶ d	3.9 × 10 ³ a	2.0 × 10 ⁷ c			
<i>B. oleracea</i> var. <i>acephala</i> subvar. <i>Lacinata</i> cv. Verheul	2.38 × 10 ⁷ d	1.53 × 10 ⁸ d	2.99 × 10 ⁹ h	1.6 × 10 ⁵ b	6.6 × 10 ⁵ c	8.1 × 10 ⁷ b	2.8 × 10 ⁶ e	1.2 × 10 ⁴ d	3.0 × 10 ⁵ a			
<i>B. oleracea</i> var. <i>capitata</i> cv. Kilaton F ₁	5.69 × 10 ⁷ e	2.91 × 10 ⁸ g	3.35 × 10 ⁷ c	1.2 × 10 ⁵ b	4.6 × 10 ⁵ b	1.9 × 10 ⁸ c	2.0 × 10 ⁷ g	8.5 × 10 ³ b	8.0 × 10 ⁶ b			
<i>B. rapa</i> subsp. <i>pekinensis</i> cv. Bilko F ₁	1.14 × 10 ⁶ a	8.85 × 10 ⁷ b	3.71 × 10 ⁸ e	0.9 × 10 ⁴ a	6.6 × 10 ⁵ c	5.2 × 10 ⁸ e	1.6 × 10 ⁵ a	2.2 × 10 ⁴ e	9.2 × 10 ⁷ d			
<i>B. napus</i> var. <i>napus</i> cv. Mendel F ₁	6.85 × 10 ⁷ f	1.19 × 10 ⁸ c	3.16 × 10 ⁸ d	1.9 × 10 ⁴ a	1.3 × 10 ⁵ a	5.3 × 10 ⁶ a	1.2 × 10 ⁵ a	2.4 × 10 ⁴ f	1.7 × 10 ⁸ f			
<i>B. rapa</i> ssp. <i>pekinensis</i> cv. Granaat	1.95 × 10 ⁷ c	1.64 × 10 ⁸ e	1.12 × 10 ⁹ g	5.2 × 10 ⁵ d	3.6 × 10 ⁷ e	1.8 × 10 ⁸ c	1.5 × 10 ⁷ f	5.0 × 10 ⁴ g	2.1 × 10 ⁸ g			

Means followed by the same letter for time of sampling within a *P. brassiccae* race category do not differ at P < 0.05 based on Newman-Keuls test

Table 6 Impact of *Brassica* genotypes on *P. brassicae* resting spores population in growing media after 35 days of cultivation

Genotype	Average spore quantity in 1 g of dry weight of growing medium					
	Pb2		Pb3		Pb9	
	before sowing	35 days after sowing	before sowing	35 days after sowing	before sowing	35 days after sowing
<i>B. rapa</i> var. <i>capitata</i> line AABBcc	1.20x10 ⁷ a	1.05x10 ⁸ a	2.2x10 ⁷ a	8.5x10 ⁶ c	3.4x10 ⁷ c	4.2x10 ⁷ c
<i>B. napus</i> var. <i>rapiferacv.</i> Wilhelmsburger	1.99x10 ⁷ a	3.80x10 ⁷ a	2.6x10 ⁷ ab	9.2x10 ⁶ d	5.9x10 ⁷ d	1.7x10 ⁸ f
<i>B. oleracea</i> var. <i>capitatacv.</i> Bindsachsener	5.07 × 10 ⁷ b	1.23x10 ⁸ a	3.5x10 ⁷ c	1.0x10 ⁷ e	2.5x10 ⁷ b	1.1x10 ⁷ a
<i>B. oleracea</i> var. <i>acephala</i> subvar. <i>Lacinata</i> cv. Verheul	5.93 × 10 ⁷ b	3.64 × 10 ⁷ a	2.8x10 ⁷ b	6.8x10 ⁶ b	1.5x10 ⁷ a	1.9x10 ⁷ b
<i>B. oleracea</i> var. <i>capitatacv.</i> Kilaton F ₁	8.10x10 ⁶ a	5.59x10 ⁷ a	3.0x10 ⁷ bc	2.4x10 ⁷ g	1.6x10 ⁷ a	4.8x10 ⁷ cd
<i>B. rapa</i> subsp. <i>pekinensis</i> cv. Bilko F ₁	4.57x10 ⁷ b	8.10x10 ⁷ a	2.8x10 ⁷ b	8.2x10 ⁶ c	2.3x10 ⁷ ab	5.3x10 ⁷ d
<i>B. napus</i> var. <i>napusc.</i> Mendel F ₁	4.06x10 ⁷ b	3.60x10 ⁷ a	3.1x10 ⁷ bc	4.1x10 ⁶ a	2.2 × 10 ⁷ ab	1.4x10 ⁸ e
<i>B. rapa</i> ssp. <i>pekinensis</i> cv. Granaat	4.54x10 ⁷ b	2.6x10 ⁸ b	2.8x10 ⁷ b	1.4x10 ⁷ f	1.7x10 ⁷ ab	8.3x10 ⁸ g

Means followed by the same letter for time of sampling within a pathotype category do not differ at $P < 0.05$ based on Newman-Keuls test

P. brassicae and apply gene pyramiding approach. In Poland, research on clubroot resistance was concentrated on vegetable Brassicas and on winter oilseed rape, where disease causes considerable losses (Robak and Gidelska 2009; Kurowski et al. 2009), among others due to high infestation of local soils by *P. brassicae* (Kopecký et al. 2012; Jędrzycka et al. 2014).

The genotypes selected for the study have been found in other researches as resistant to a number of *P. brassicae* pathotypes, but our results indicate that in most of them resistance was broken down by the Polish isolates of the pathogen. Only genotypes with ID of less than 20 are considered as highly resistant (Chytilová and Dušek 2007). In our experiments only *B. rapa* var. *capitata* line AABBcc (ECD 03) fulfilled this criterion in reference to all three *P. brassicae* pathotypes isolated in Poland. High resistance of this genotype confirms earlier results of Kuginuki et al. (1999), Donald et al. (2006) and Hasan et al. (2012).

Some of the other genotypes tested were resistant only to selected pathotypes, e.g. *B. napus* var. *rapifera* ‘Wilhelmsburger’ to Pb2 and Pb3, *B. oleracea* var. *capitata* ‘Kilaton F₁’ and *B. rapa* subsp. *pekinensis* ‘Bilko F₁’ to Pb2. High resistance of *B. napus* var. *rapifera* ‘Wilhelmsburger’ to *P. brassicae* pathotype 3 found in our experiments confirms earlier results of Kuginuki et al. (1999), Donald et al. (2006) and Hasan et al. (2012). But research partially contradicts results of other studies. Headed cabbage ‘Kilaton F₁’ in our experiments was fully resistant to Pb2 infection, but was highly susceptible to Pb3 and Pb9 (IDs 68 and 72, respectively), but in the three-year field studies done by in Polish conditions by Cebula et al. (2010) this cultivar was fully resistant to clubroot. However, they have not determined the pathotype(s) of *P. brassicae* infesting the experimental plots. *B. oleracea* var. *acephala* ‘Verheul’, which according to Cao et al. (2014) was highly to moderately resistant to Pb3, in our experiments was highly to moderately susceptible to all the pathotypes tested (IDs 71, 46 and 40 in reference to Pb2, Pb3 and Pb9, respectively). Also *B. napus* var. *napus* ‘Mendel F₁’, which carries a major dominant gene and two recessive genes conferring clubroot resistance and, according to Diederichsen et al. (2006) and Rahman et al. (2014), is fully resistant to majority of *P. brassicae* pathotypes dominant in Europe, in our research it was moderately or highly susceptible to Polish isolates of Pb3 and Pb9 (IDs 32 and 100, respectively). This shows that the pathotype of *P. brassicae* isolates collected in various areas and determined using differential sets does not

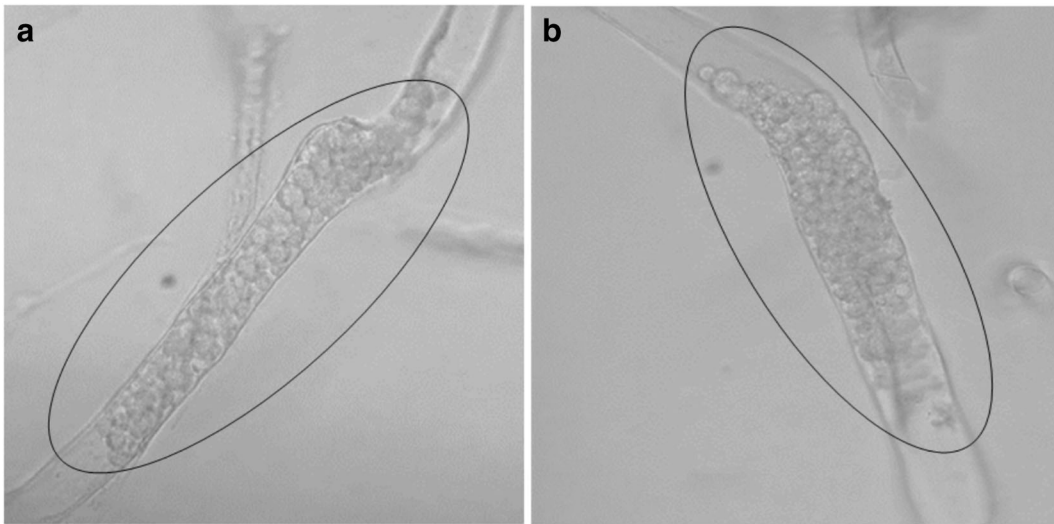


Fig. 2 Exemplary picture of mature plasmodia (zoosporangia) in root hairs of *B. oleracea* cv. Kilaton F₁ (a) and *B. napus* cv. Mendel F₁ (b) grown in the medium inoculated with Pb2

necessarily reflect its actual level of virulence toward various *Brassica* genotypes. Thus, prior to the introduction for cultivation of new resistant *Brassica* species/cultivars or selecting them as a source of resistance traits in breeding programmes their resistance/susceptibility to local *Plasmodiophora brassicae* isolates shall be tested before hand, irrespective of the pathotypes of the pathogen determined elsewhere with differential set.

In the available literature there is no information about resistance of the genotypes tested in our study to *P. brassicae* pathotype 9. According to our best knowledge this is the first such a report.

Our research shows that of seven clubroot-resistant genotypes of *Brassica* evaluated, only *B. rapa* var.

capitata line AABBcc, *B. napus* var. *rapifera* ‘Wilhelmsburger’, *B. oleracea* var. *capitata* ‘Kilaton F₁’ and *B. rapa* subsp. *pekinensis* ‘Bilko F₁’ can be recommended as a source of clubroot resistance in *Brassica* breeding programmes or for cultivation in low- to moderately-infested soils in Poland.

In our study, the primary root infection was not correlated with plant’s resistance to clubroot. In fact, the root hairs of *B. rapa* var. *capitata* line AABBcc, which was resistant to all the pathotypes tested, had the highest population of *P. brassica* plasmodia of all the genotypes tested. This confirms the findings of Kageyama and Asano (2009) who suggested that susceptibility of root hairs to

Table 7 The average and maximum number of plasmodia containing mature, spherical zoosporangia observed in the hair roots of seven *Brassica* genotypes growing in medium infested with different *P. brassicae* pathotypes

Genotype	Average number of plasmodia in root hairs per 1 cm of root*			Maximum number of plasmodia in root hairs per 1 cm of root		
	Pb2	Pb3	Pb9	Pb2	Pb3	Pb9
<i>B. rapa</i> var. <i>capitata</i> line AABBcc	17.6	6.1	15.0	48	23	40
<i>B. napus</i> var. <i>rapifera</i> cv. Wilhelmsburger	5.8	2.3	5.7	11	5	19
<i>B. oleracea</i> var. <i>capitata</i> cv. Bindsachsener	6.4	6.1	2.1	17	18	11
<i>B. oleracea</i> var. <i>acephala</i> subvar. <i>lacinata</i> cv. Verheul	5.2	14.6	1.3	28	38	5
<i>B. oleracea</i> var. <i>capitata</i> cv. Kilaton F ₁	16.8	10.0	2.8	44	31	8
<i>B. rapa</i> subsp. <i>pekinensis</i> cv. Bilko F ₁	9.1	12.6	4.0	35	39	18
<i>B. napus</i> var. <i>napus</i> cv. Mendel F ₁	7.2	14.0	25.0	20	15	63

*average number of plasmodia in 18 pieces of root hairs for each genotype

infection cannot be correlated with susceptibility of cortical cells and resultant galls formation. As reported by McDonald et al. (2014), primary infection can occur in both susceptible and resistant host cultivar, and even in non-host plants, however secondary plasmodia frequently would not mature in resistant genotypes and are unable to produce resting spores. This hypothesis is confirmed by our findings that only in the resistant cultivar *B. rapa* var. *capitata* line AABBcc the number of spores in the roots decreased in the course of infection with all the tested pathotypes of pathogen. Our microscopic observation showed that the emergence of the first mature plasmodia in root hairs depends on the genotype of the tested plants. It was noted that the average number of plasmodia in the root depended both on the plant genotype and on the pathotype *P. brassicae*. In the case of pathotype 2 the appearance of mature plasmodia containing spherical sporangia was observed earlier than in the case of Pb3 and Pb9. Morgner and Sacristán (1995) also reported colonization of clubroot-resistant *Brassica* genotypes by *P. brassicae*. In their quantitative study pathogen structures were observed 12 days after inoculation of *B. napus* and *B. oleracea*. Voorrips (1992) demonstrated large differences in the levels of primary root hair infection among thirteen host genotypes with varying levels of resistance, however did not observe correlation between resistance to root hair infection and resistance to clubroot. According to this author, only complete resistance to root hair infection would confer resistance to clubroot.

It is widely believed that the clubroot incidence can be limited by planting the bait, decoy or catch plants, which can stimulate germination of resting spores and thus reduce their quantity of in the soil (Yamagishi et al. 1986; Murakami et al. 2000). According to Ikegami (1985), cultivation of clubroot-resistant Japanese radish (*Raphanus sativus*) during 5 years resulted in a considerable decrease of resting spore populations in the soil. Resting spores population was also slightly reduced after two cycles of other cruciferous non-host crops (Ahmed et al. 2011). However, in our research, cultivation of the clubroot-resistant genotypes contributed to the increase of the number of spores in the growing media inoculated with pathotypes 2 and 9. These results suggest that

the cultivation of resistant cultivars in heavily infected areas does not guarantee a reduction of the soil infestation with *P. brassicae* resting spores shall not be recommended as a measure to control clubroot.

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Conflict of interest All authors listed have contributed to the work, have read the manuscript and declare that there are no potential conflicts of interest.

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