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Identification of *Plasmodiophora brassicae* Wor. isolate suppressing clubroot resistance in 'Kilaxy' F_1 white cabbage

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ABSTRACT

The purpose of this study was the evaluation of the pathogenicity of *Plasmodiophora brassicae* Wor. field isolates gathered from clubroot-infected white cabbage *Brassica oleracea* var. *capitata* L. f. *alba* plants grown in different areas of Poland. The virulence of seven isolates of the pathogen was tested. Breeding materials from white cabbage resistant and susceptible to *P. brassicae* were used as standard objects for isolate virulence screening. Cabbage seeds were infected by submerging them into a resting-spore suspension. A screening of plant resistance using a nine-degree scale based on root symptoms was carried out at the eight-week-old plantlets stage and at harvest time on afield infected by *P. brassicae* spores. Isolates from Pobiednik and Grębałów were defined as more virulent. The P isolate from Pobiednik also infested plants of the clubroot resistant 'Kilaxy' F_1 cultivar, which indicated the suppression of clubroot resistance. Its reaction to the other six isolates confirmed the resistance of this cultivar. The susceptible HTM line was characterised by the highest infestation level. A difference in the susceptibility level was noticed between lines derived from those with resistant genes, i.e. Oregon123 and 'Badger Shipper'. The Oregon123 line was less susceptible to the applied isolates. The effect of the isolates used for seed inoculation on the reaction of the dormant plants to the pathogen on the clubroot field was observed.

Key words: *Brassica oleracea*, disease, susceptibility, resistance screening, virulence

INTRODUCTION

The biotrophic protist *Plasmodiophora brassicae* causes clubroot disease, one of the most damaging diseases for cabbage (*Brassica oleracea* var. *capitata*) and other *Brassica* crops. The pathogen shows a wide biological range – its populations often consist of a mixture of different pathotypes. The pathogen is well fitted for the ecological niche that it occupies. Resting spores of *P. brassicae* have a great ability to survive in the soil for several years. There is evidence that physical, chemical and biological components of the soil environment may also differentially influence the survival of some physiologic races of the pathogen (Kageyama and Asano 2009, Dixon 2009b). *P. brassicae* is an obligate pathogen and cannot be cultured axenically, so isolates for bioassays must be gathered as samples of clubbed plant roots. Galls containing resting spores may be stored at -20°C for

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several years with minimal loss of viability (Dixon 2009a).

European *P. brassicae* field isolates display great variation and show a tendency to overcome different resistance sources from either *B. rapa* or *B. oleracea*. Plant resistance is still the most powerful tool for combating clubroot disease. Future efforts for breeding *P. brassicae* resistance will focus on durability by broadening the genetic basis of clubroot resistance (CR) by using either natural variation or transgenic strategies (Diederichsen et. al 2009). To increase the durability of CR cultivars, the combination of the different CR genes into a single line will be an indispensable means for breeding cultivars with resistance to a broader spectrum of physiological races. Comparative studies of CR genes or their linked markers should provide new insights into these processes (Piao et al. 2009). The breakdown of resistance in CR cultivars has become a serious problem and the relation between CR cultivars and populations of *P. brassicae* needs further monitoring (Osaki et al. 2008). Further advancement in integrated control will rely on cultivar resistance coupled with molecular and serological methods that measure soil inoculum, and the modification of the soil environment to reduce inoculums of *P. brassicae* or to prevent infection (Donald and Porter 2009).

In Poland, specific observations of the occurrence of the protist were conducted in the 1970s and 80s and showed wide biological differentiation and 10 different pathotypes were determined (Robak 1991). Over the years new pathotypes arise according to their dynamic biological changes. Similar changes could be caused by introducing the CR *Brassica* cultivars. Several-year cultivation of those cultivars could attribute to a selection pressure on the *P. brassicae* population and the occurrence of new pathotypes of this pathogen (Robak et al. 2013). Current research carried out by several research centres are focussed on clubroot disease of oilseed rape (*Brassica napus*) and show a significant increase of clubroot incidence in different regions of Poland. Oilseed rape plants infected with the pathogen are found in different regions, mainly observed in the north and northwestern regions. The research has included scientific topics like the composition of the *P. brassicae* population, a study on the pathogenicity and the pathotype recognition, the quest for sources of genetic resistance and the use of molecular markers (Jędryczka et al. 2012, 2013).

The aim of this study was the recognition of the pathogenicity level of *P. brassicae* populations in Poland. The especially aggressive isolates of the pathogen were used for screening white cabbage breeding materials in a clubroot resistance project.

MATERIAL AND METHODS

The assessment of the virulence of seven *Plasmodiophora brassicae* isolates was the subject of the research carried out in 2010 (Tab. 1). Four isolates from Miechów (D), Pobiednik (P), Grębałów (E) and Goźlice (G) obtained in 2009 from a clubroot-infested field of white cabbage *Brassica oleracea* var. *capitata* L. f. *alba* in the southern region of Poland were used. Additionally, three isolates from Szczecin-Dąbie (Sz), Ostromęczyn (Os) and Krojczyn (K) that were recognised as more virulent in previous research conducted in 2008-2009 were added (Wesołowska 2011). The naturally infected plants were collected from large commercial plantations of white cabbage. The samples of clubbed roots were washed in tap water, dried on paper towels and stored frozen at -20°C for future use.

Four standard objects of white cabbage with known reactions to the pathogen were used for the virulence screening of the collected isolates. They consisted of white cabbage inbred lines from cultivars containing CR genes, 'Badger Shipper' and Oregon123, susceptible HTM inbred line from 'Hitoma' F_1 as a susceptible standard and CR

Table 1. Origins of *Plasmodiophora brassicae* isolates

No.	Area	Geographical coordinates	Isolate symbol
1	Grębałów	50°05′32″N 20°04′03″E	E
2	Miechów	50°21′28″N 20°01′57″E	D
3	Pobiednik	50°04′16″N 20°12′31″E	P
4	Krojczyn	52°41′00″N 19°12′00″E	K
5	Szczecin Dąbie	53°24′04″N 14°40′40″E	Sz
6	Ostromęczyn	52°16′11″N 22°50′17″E	Os
7	Goźlice	50°41′57″N 21°28′27″E	G

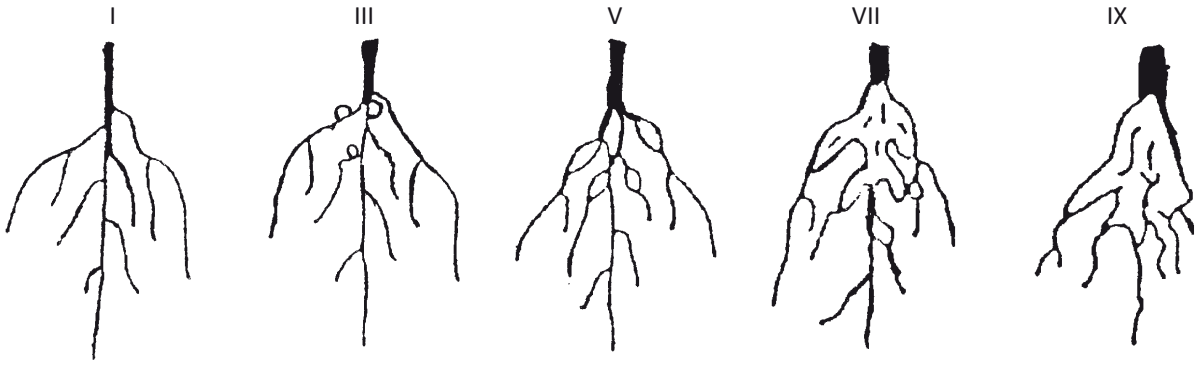

Figure 1. Nine-grade scale based on clubroot symptoms (Wesołowska 2011)

'Kilaxy' F_1 from Syngenta Seeds as a resistance standard. The cabbage seeds were inoculated by submerging -them into a pathogen spore suspension. The inoculum was prepared by macerating gall tissue obtained from diseased cabbage roots in a blender with distilled water. Later, the resting spores were separated by filtration through eight layers of cheesecloth and centrifugation. The final spore concentration was adjusted with distilled water to 10^8 spore 1 ml^{-1}. Thirty seed samples per one object were overflowed with 5 ml inoculum of the pathogen and kept in 19°C temperature. After a 36-hour-long incubation period, seeds were sown individually in multi-pot packs filled with substrate and grown in a phytotron chamber at 20°C under a light-dark cycle of 16:8 hours. The plants were examined for clubroot development for the first time at eight weeks after inoculation and for the second time at harvest time in a field contaminated by *P. brassicae* spores. The nine-grade scale based on root symptoms established by Williams (1985) and modified by Reby and Michalik (2005) (Fig. 1) was used. The extent and severity of the clubroot symptoms were assessed visually according to a nine-grade scale: I – without visible disease symptoms; III – singular galls on lateral secondary roots; V – larger galls on lateral roots; VII – enlarging gall on the main and lateral roots; IX – large singular gall on root neck without root.

The disease index (DI) was calculated using the nine-grade scale according to the formula:

$$DI = \frac{\sum(n \times p)}{\sum n}$$

where n is the number of plants in the indicated class, *p* - class I – IX.

The disease indexes of the tested objects were calculated according to plant susceptibility at the eight-week seedling stage and evaluated once more during harvest, on the clubroot-infected field. The clubroot field was established eight years ago. Harvest remains and the soil from the field near Krakow where heavily clubbed plants of white cabbage were observed were gathered and scattered on the surface of the area. Next, oilseed rape, which is susceptible to clubroot, was sown and plants of different cabbage cultivars artificially inoculated by *P. brassicae* were cultivated. Each year, clubbed plant roots were mixed with the soil for extra infection with the pathogen in the field, which was carried out after the autumn disease evaluation and harvests of tested plants. As a result of the procedure, the field was contaminated by a mixture of different *P. brassicae* pathotypes.

The procedures concerning the seed inoculation method and screening tests were the same as in the research conducted in 2008-2009 and are described in detail by Wesołowska (2011).

RESULTS AND DISCUSSION

A wide range of different methods of plant clubroot susceptibility screening have been developed. The soil inoculation method and dipping method seem to be the most popular. In such methods plant materials were inoculated either by sowing seeds in an infected substrate or by dipping the roots of approximately 10-day-old seedlings in a resting spore suspension (Robak 1991). In the current investigation, a double-stage evaluation of the clubroot resistance was used. This procedure was established by Reby and Michalik (2005). Firstly, cabbage seed samples were inoculated by submerging them into the water pathogen spore suspension. This method was a modification of the one described by Lewis and Brokenshire (1978). The seed inoculation technique was found to be simple and not time-consuming. Additionally, after the plantlet stage screening, the resistant plants were transplanted into the clubroot-infested field for a second evaluation. This way of testing on

Table 2. Percentage of plants without disease symptoms and disease index (DI) of standard objects evaluated in the seedling test

Cultivars	Percentage of plants without disease symptoms							Disease Index						
	Sz*	Os	K	D	P	E	G	Sz	Os	K	D	P	E	G
Badger Shipper	88	100	82	68	0	100	19.5	1.3	1.0	1.6	2.0	4.0	1.0	6.2
Oregon123	100	100	75	94	65	97	91	1.0	1.0	1.9	1.1	2.3	1.0	1.2
HTM	13	48	4	13	0	18	7	6.1	3.3	8.7	7.3	8.0	5.4	5.2
Kilaxy F_1	100	100	100	97	9	100	100	10.	1.0	1.0	1.1	6.5	1.0	1.0
Mean	75	87	65	68	19	79	54	2.3	1.6	3.3	2.9	5.2	2.1	3.4

*Abbreviations *P. brassicae* isolates: see Table 1

adult plants in field conditions was described by Manzanares-Dauleux et al. (2000).

An evaluation of clubroot development in eight-week-old cabbage plants from the standard objects showed an influence of the pathogenicity level of the *P. brassicae* isolates used for seed inoculation on plant reaction. All of them were virulent against the susceptible HTM line plants. Heavily infested root systems were observed in plants from seeds inoculated by the K isolate from Krojczyn (DI - 8.7) and the P isolate from Pobiednik (DI - 8.0) (Tab. 2). All of the plants from the HTM line and 'Badger Shipper' derived from the P isolate combinations were completely infested. The isolate from Pobiednik was found to be particularly aggressive not only to the susceptible HTM and 'Badger Shipper' but also to the clubroot resistant 'Kilaxy' F_1. The cultivar disease index calculated for that was 6.5 and only 9% of the plants were classified as healthy. Plants derived from Oregon123 were less susceptible to the P isolate (DI - 2.3) and characterised by 65% of the plants without clubroot galls.

The unique susceptibility reaction of 'Kilaxy' F_1 to the P isolate contrary to its resistance to the other six isolates was not noticed in tests carried out in 2008-2009, when the CR cultivar confirmed its resistance reaction to all eight isolates used for seed inoculation (Wesołowska 2011). The reaction of 'Kilaxy' F_1 to the P isolate from Pobiednik was an example of the resistance decline of a CR cultivar. The 'Kilaxy' F_1, as one of CR cultivars form Syngenta Seeds B.V., appeared on the market in 2005. The breeders decided to use the *B. rapa* CR genes from European fodder turnip and introduced it into *B. oleracea* varieties. In turnip, CR has been found to be controlled by three independent dominant genes, each conferring resistance to different *P. brassicae* pathotypes. The resistance will not work against all pathotypes and a small number of isolate infections was observed (Diederichsen et al. 2009, Piao et al. 2009).

Dixon (2012) mentioned that CR cultivars available from several seed houses strengthen clubroot control. But it is essential that resistant cultivars be used together with other controls, as they are not an answer in themselves. Hirai (2006) noticed that field populations of the pathogen have a wide variation of virulence, which causes the infection of CR cabbage cultivars.

A difference in the reaction to the isolate from Goźlice and from Pobiednik between lines derived from 'Badger Shipper' and Oregon123 with CR genes was observed. That dependence was observed in 2008-2009, but it referred to isolates from Szczecin-Dąbie, Krojczyn and Ostromęczyn. In both series of research the reactions of 'Badger Shipper' and Oregon123 to the pathogen were different, especially when more virulent isolates were used for seed inoculation. The other reaction in relation to some isolates observed between 'Badger Shipper' and Oregon123 might be caused by the interaction of CR genes and these isolates probably coming from distinct populations of the pathogen. Such an influence of the isolates used on 'Badger Shipper' and Oregon123 plant reactions was reported by Reby and Michalik (2005), Laszczak et. al. (2006) and Wesołowska (2011).

Eight-week-old plants of standard objects without visible disease symptoms were planted on the clubroot-infested field. This area was characterised by a high level of *P. brassicae* resting spore contamination, which featured a mixture of many populations or pathotypes. The gradual death of clubroot susceptible plants was observed during the growing period on the area. By autumn all plants from the P isolate combination shad died due to clubroot development. In that group plants not only from the susceptible HTM line but also from 'Badger Shipper' and even CR 'Kilaxy' F_1 were found. The isolate from Pobiednik was noted as being particularly aggressive to these plants according to the seedling test results. The high

Table 3. Percentage of plants without disease symptoms and disease index (DI) of standard objects evaluated in the field test

Cultivars	Percentage of plants without disease symptoms							Disease Index						
	Sz*	Os	K	D	P	E	G	Sz	Os	K	D	P	E	G
Badger Shipper	67	65	24	21	0	33	0	3.2	2.9	5.6	6.0	9.0	5.5	9.0
Oregon123	90	35	35	51	4	50	74	1.8	4.2	2.3	2.9	5.0	2.5	1.5
HTM	0	0	0	0	0	0	0	9.0	9.0	9.0	9.0	9.0	9.0	9.0
Kilaxy F1	78	90	57	81	0	93	91	1.7	1.8	2.4	1.6	9.0	1.1	1.3
Mean	59	48	29	38	2	44	41	3.9	4.5	4.8	4.9	8.0	4.5	5.2

*Abbreviations *P. brassicae* isolates: see Table 1

susceptibility of the HTM line was confirmed. All of the combinations of that line were characterised by a DI- 9.0, which means a complete lack of plants with healthy roots (Tab. 3). In the field, the CR 'Kilaxy' F_1 plants were the least invaded of all objects apart from plants of the P isolate combination, in that the entire population of plants died because of the very high clubroot infestation. Plants from cultivars from seeds inoculated by all of the other isolates had from 57 to 93 percent of plants without disease symptoms. The dormant plants of 'Badger Shipper' showed a wide range of DI from 2.9 to 9.0 (Tab. 3). Plant roots from the G and the P isolate combinations were heavily clubroot infested with a DI of 9.0. Both of the isolates are known for being more virulent according to the seedling test. These studies concluded that the isolates used for seed inoculation influenced the dormant plants' reaction to the pathogen. Plants from the Oregon123 line were also less susceptible to the pathogens in the field. The disease indexes calculated for those sub-lines were from 1.5 to 5.0 and the most infested plants were observed in the P isolate combination. The varying reactions of the pathogen for 'Badger Shipper' and Oregon123 were more visible in the autumn in the field.

CONCLUSION

1. The *P. brassicae* isolate from Pobiednik caused the breakdown of 'Kilaxy' F_1 clubroot resistance.
2. There was an observed effect of *P. brassicae* isolates used for seed inoculation on the reaction of dormant plants to the pathogen on the clubroot field.

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OKREŚLENIE PATOGENICZNOŚCI IZOLATÓW *PLASMODIOPHORA BRASSICAE* WOR. WYSTĘPUJĄCYCH NA TERENIE POLSKI

Streszczenie: Celem badań była ocena patogeniczności izolatów *Plasmodiophora brassicae* Wor. pobranych z porażonych kiłą roślin kapusty głowiastej białej pochodzących z różnych rejonów Polski. Oceniano wirulencję siedmiu izolatów tego patogena. Do określenia wirulencji izolatów *P. brassicae* użyte zostały obiekty wzorcowe, były to materiały hodowlane kapusty głowiastej białej odporne i podatne na kiłę. Nasiona kapusty inokulowano poprzez moczenie ich w zawiesinie zarodników przetrwalnikowych patogena. Ocenę podatności roślin na kiłę prowadzono w oparciu o 9-cio stopniową skalę porażenia korzeni roślin w stadium ośmiotygodniowej rozsady i podczas jesiennego zbioru roślin na polu zainfekowanym *P. brassicae*. Izolaty pochodzące z Pobiednika i Goźlic były bardziej wirulentne. Izolat P z Pobiednika spowodował porażenie roślin odmiany ‘Kilaxy’ F_1 co oznaczało przełamanie cechującej tę odmianę odporności na kiłę. Odmiana ta w stosunku do pozostałych izolatów potwierdziła swoją odporność. Wrażliwa linia HTM charakteryzowała się najwyższym stopniem porażenia. Odnotowano różny poziom wrażliwości na zastosowane izolaty pomiędzy liniami pochodzącymi z mających geny odporności odmian ‘Oregon123’ i ‘Badger Shipper’. Mniej podatna na zastosowane izolaty była linia ‘Oregon123’. Obserwowano wpływ zastosowanych do inokulacji nasion izolatów na reakcję na patogena dojrzałych roślin kapusty na polu kiłowym.

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