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Clubroot Caused by *Plasmodiophora brassicae* Wor.: a Review of Emerging Serious Disease of Oilseed Rape in the Czech Republic

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Abstract

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Winter oilseed rape is the second most frequently grown crop in the Czech Republic. Clubroot, caused by *Plasmodiophora brassicae* (Wor.), was previously a problem in production of vegetable. The disease has been spreading on winter rape (*Brassica napus* L.) over the past four years. Infected stands were reported throughout the country in autumn 2011. The pathogen is probably widely spread in the whole country, its incidence depends on suitable weather conditions. Every field with clubroot is considered as contaminated for many years. The amounts of inoculum and its distribution are not sufficient yet, but this is just the initial stage of colonisation. There is a similar situation resembling experiences from other countries (Canada, Germany) where clubroot has gradually expanded in the last 15 years.

Keywords: winter oilseed rape; *Brassica napus* L.; clubroot disease; biology; pathopypes; resistance

Introduction

Winter oilseed rape (*Brassica napus* cv. *napus,* the family Brassicaceae) is one of the most widely grown crops in the world (USDA, GOV; http://www.fas.usda.gov/oilseeds/Current) and also in the Czech Republic, where its production ranks second only after cereal crops. In 2013 the oilseed rape was grown on a record production area (418 808 ha) followed by a slight decrease (389 297 ha) in 2014. However, the 2014 yield was high – 3.95 t/ha – the highest in the history of oilseed rape production in the Czech Republic and former Czechoslovakia (CSO – Czech Statistical Office; https://www.czso.cz/csu/czso/zem_ts).

Together with the increasing concentration of oilseed rape in the country more problems with pests and pathogens occur. One of them, the soilborne plant pathogen *Plasmodiophora brassicae* Wor., causes the clubroot disease of crucifer crops including oilseed rape (Hwang *et al*. 2012). *P. brassicae* occurs in more than 60 countries including the Czech Republic and it causes 10–15% loss in the production of oilseed rape worldwide (Dixon 2009a). The potential hosts of *P. brassicae* could be all species from the family Brassicaceae, including weeds (Dixon 2009a). Until the season 2011 symptoms appeared regularly on oilseed rape in northern Moravia and southern Bohemia, where the clubroot was first reported by Rod (1996) three decades ago. In those areas with the long tradition of cruciferous vegetable production, because in the past the clubroot was a problem of vegetable production in the Czech Republic (Rod 1996). Over the past four years, the disease has been spreading on oilseed rape. The first strongly infected stands were reported throughout the country in autumn 2011 (Figure 1 – red marks). The serious infestation was found on 44 farms, mainly in Bohemia, northern Moravia and in the Czech-Moravian Highlands (Kazda *et al*. 2013). It is known

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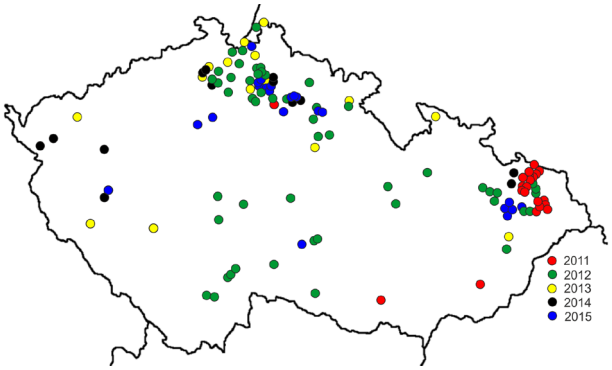

Figure 1. Development of clubroot infestation in the Czech Republic in 2011–2015

that *Plasmodiophora brassicae* occurs on a large area of arable land in the Czech Republic where winter or spring rape is grown regularly. The incidence in autumn 2012 showed new localities (Figure 1 – green marks) mainly in northern Bohemia and some in eastern Bohemia and northern Moravia again. However, small outbreaks of individual plants need not be detected at all. In 2013 the incidence was weaker than in 2012, but many new outbreaks were reported in autumn 2014. In spring 2015 some fields were infested so heavily that they were resown with another crop, because winter rape was completely destroyed (UOGP – Union of Oil seed Growers and Processors – pers. commun.). 130 infested stands have been reported in the country and the number will likely grow, because lots of places have not been revealed yet and the infection is still spreading.

The situation for the farmers is serious because there is currently only one economical and environmentally friendly management practice for the clubroot control. Resistant oilseed rape cultivars, which are bred to be resistant against the most prevalent pathotypes, are currently the only way of protection (Hwang *et al.* 2012). Populations of *P. brassicae* are very heterogeneous and there can even be more pathotypes in one field (Diederichsen *et al.* 2009). Therefore resistant cultivars have to be used very carefully together with proper management practices and crop rotation to maintain the disease resistance of these cultivars (Diederichsen *et al.* 2009, 2014).

Taxonomy, life cycle and symptoms

Recent systematic classification

The taxonomical position of the pathogen is still changing. According to the database Index Fungorum (Kirk 2014) and other authors (Cavalier-Smith & Chao 2003) this organism belongs to the kingdom *Protozoa*, phylum *Cercozoa*, class *Phytomyxea*, order *Plasmodiophorida* (in zoological taxonomy) and family *Plasmodiophoridae*. On the other hand, other authors (Neuhauser *et al.* 2011; Burki & Keeling 2014) rank the pathogen to the supergroup (kingdom) *Rhizaria*, phylum *Cercozoa*, class *Phytomyxea,* order *Plasmodiophorida.*

Life cycle

The life cycle of *Plasmodiophora brassicae* can be divided into three parts – primary and secondary phase of infection and spores surviving in the soil (Ayers 1944; Ingram & Tommerup 1972) (Figure 2). Resting spores (size 2–3 µm) are released to the soil from rotting clubs. Most spores can be found at the depth of 15 cm, maximum depth of 40 cm (Kim *et al.* 2000; Dixon 2009b). The spore vitality is preserved for a long time with the half-life of spores ranging between 3 and 6 years (Wallenhammar 2010). Different authors report different vitality, e.g. 15–20 years (Rod 1996) or up to 20 years (Wallenhammar 1996). The germination of resting spores is probably triggered by root exudates from different plants (not just crucifers), acid pH values of soil, or even some other microorganisms (Dixon 2009b). The moving zoospores are released from resting spores and they seek the root hairs, where primary infection takes place (Ayers 1944). Water film is needed for their movement (Hwang *et al.* 2012). The period when zoospores are free in the soil is the most vulnerable

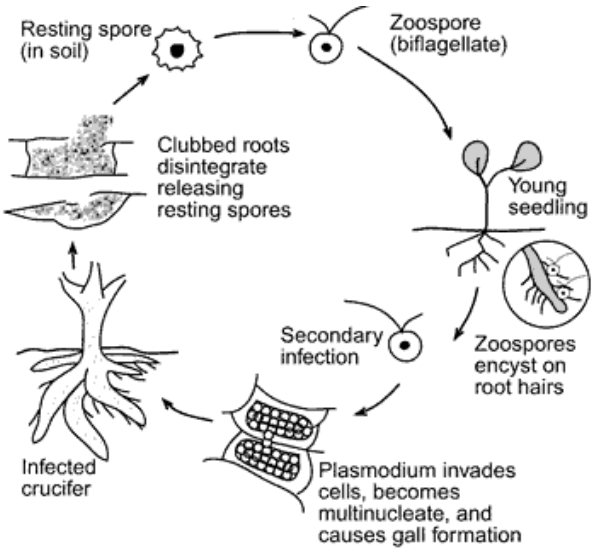

Figure 2. The life cycle of *Plasmodiophora brassicae* (modified according to Ohio State University)

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part of the life cycle (Dixon 2009b). In the root hairs zoospores are changed to primary plasmodia, which are divided into paraplasmodia, and finally to sporangia with 4–8 spores (Ayers 1944; Ingram & Tommerup 1972). This process (phase) lasts for 5–7 days (Dixon 2009b).

The secondary phase of infection begins in the soil. Secondary zoospores penetrate the root cortex of the host and secondary plasmodia are established. After karyogamy and meiosis, the paraplasmodium is changed to haploid resting spores which cause the multiplication of the root cells (Ayers 1944; Ingram & Tommerup 1972; Naiki & Dixon 1987). The pathogen affects the host phytohormones, mainly auxins and cytokinins, which are upregulated and cause hypertrophy and hyperplasy of the infected root cells (Siemens *et al*. 2006; Ludwig-Müller *et al.* 2009).

The clubs with spores are not formed in resistant cultivars of oilseed rape. However, the primary phase of the infection does occur in the root hairs (Deora *et al*. 2013).

The hosts of *P. brassicae* are all 330 genera and 3700 species of Brassicaceae (Dixon 2009a), possibly with one exception – horse radish (*Armoratia rusticana* L.) (Rod 1996).

The primary phase of the infection occurs also in non-host families like Papaveraceae, Poaceae, and Rosaceae (Colhoun 1958; Karling 1968). The aromatic plants like mint, thyme, cumin, summer savory or basil can provoke resting spore germination and can be used in management like 'bait crops' (Rod 1994; Rod & Robak 1994).

Symptoms of infection

Symptoms – the clubs – begin to appear on lateral roots and heavily infected plants may have a totally

Figure 3. Symptoms caused by *P. brassicae* on susceptible oilseed rape

Figure 4. Clubroot symptoms on oilseed rape in the field

destroyed main root (Figure 3), which causes damage to xylem cells and water transport is interrupted. This leads to aboveground symptoms like stunting, wilting, and yellowing or anthocyanin coloured leaves and premature ripening (Hwang *et al*. 2012). The young clubs are small and pearl shaped; the mature clubs have different shapes and weight of up to 1 kg (Rod 1996). Initially, clubs are light coloured and white in cross-section. Maturing clubs become darker as they lignify and the surface becomes rougher (Rod 1996; Dixon 2009b) (Figure 4). The clubbed root could be confused with galls created by the cabbage gall weevil (*Ceutorhynchus pleurostigma*) or symptoms caused by wrong application of herbicides (dinitroaniline group) (Rod 1996).

Biology and ecology

Symptom occurrence depends on specific moisture and temperature conditions, especially in the oilseed rape seeding period. Infection is supported by higher temperatures and soil moisture (Gossen *et al*. 2012). In many fields in the Czech Republic *P. brassicae* spore levels are low and these are not homogeneously distributed. As soon as the inoculum level is sufficiently increased by spore dissemination with machinery, there will arise a potential, in interaction with weather conditions, for nationwide infection. It is important to know that once the field has been infested, it remains infested for a very long time. Every infected plant has a potential to return 8×10^8 resting spores back to the soil (Hwang *et al*. 2011a). When the conditions are favourable, 1 resting spore is enough to infect a plant (Rennie *et al*. 2011).

Spores

The spore load in the soil is not important when the other conditions are favourable and support

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spore germination and viability of zoospores. Even a low concentration of zoospores in opportune conditions can lead to successful infection and colonisation (Webster 1986). The presence of spores in the proximity of root hairs is also important, because the mobility of zoospores is just within 10–20 mm (Dixon 2009b). Antagonism between zoospores can work, if more pathotypes occur together, they can compete for place in root hairs (Jones *et al*. 1982a). The number of germinating spores grows with an increasing amount of root exudates of the host plant (MacFarlane & Last 1957). Soil microbiota can also play a role, the bacteria *Bacillus* spp. and *Pseudomonas* spp. can stimulate spore germination (Einhorn *et al*. 1991) whereas the soil fungus *Heteroconium chaetospira* can suppress it (Narisawa *et al*. 2005).

A larger inoculum load is necessary for infection in unsuitable conditions: 50 viable spores in 1 g of soil may be necessary for infection, whereas in ideal conditions just 1 spore in 10 g of soil is needed (Rod 1996). The age of the club is also important; spores from old degraded clubs germinate better than spores from 'young' immature clubs.

The structure of spores influences their survival in the soil and their tolerance to chemicals (Wallenhammar 2010). Cell walls of spores are composed of five layers, which contain 25.1% chitin, 33.6% protein, 17.5% fats, and 2.5% are carbohydrates (the cellulose was not confirmed). The protein components are important in a germination process (Moxham & Buczacki 1983).

Soil moisture, temperature and soil reaction (pH)

One of the most important factors for infection is moisture, which affects the movement of zoospores (Karling 1968). Low-lying soils which are poorly drained are the most suitable for pathogen development, especially after a rainy period (Dixon 2009b). Rod (1996) stated that minimum soil moisture should be 50% of maximum water capacity (MWC) and ideal is 70–90% MWC. However, higher soil-moisture levels are detrimental because the pathogen needs aerobic conditions in order to germinate.

Temperature affects mainly the root hair colonisation and zoosporangia growth during the second week after infection (Rod 1996). The effect of temperature is mainly regulatory and does not directly block infection, unlike pH. The ideal temperature for the pathogen's development varies due to other conditions, geographic region and infected host (Dixon 2009b). Colhoun (1953) stated that the ideal temperature is 23°C, according to Wellman (1930) the ideal temperature is in the range of 18–24°C and infection is inhibited if the temperature is below 12°C or higher than 27°C. These results have been proven in experiments with oilseed rape, where ideal temperatures for infection were 23–26°C (Gossen *et al*. 2012). Clubroot development was greatest in this temperature range and in temperatures below 17°C or above 26°C clubroot development was significantly lower. These results show that oilseed rape seeding into a colder soil can delay infection and clubroot development, which can have a positive effect on yield.

The pH value of soil is one of the important factors for infection progress, which is significantly lower in an alkaline environment because of the root hair infection reduction, retarding the plasmodia maturation and gall formation (Palm 1958, 1963; Webster & Dixon 1991a). The effect of pH on the root hair infection and maturation is activated by exposure to alkaline pH within 3 days from penetration (Webster & Dixon 1991a). Alkaline environment affects primary and secondary invasion, cortical migration and cell hypertrophy. The presence of calcium ions during the *P. brassicae* infection leads to induced cell death or hypersensitivity (Takahashi 2002, 2006).

Elements

Calcium itself has also a major role in *P. brassicae* infection. High concentrations of calcium ions at pH 6.2 or 7.2 reduce total numbers of root hair infection and the rate of maturation (Dixon 2009b). Higher concentrations completely inhibit later stages of *P. brassicae* development in the root hair, even when more inoculum is applied (Dixon 2009b). Dixon and Page (2001) showed that the germination of resting spores and the zoospore motility are altered in the presence of calcium.

There is also a strong correlation between the infection by *P. brassicae* and the concentration of **boron** and calcium in the soil (Palm 1963). Boron interacts with calcium in infection inhibition, they affect mainly the zoospores. If there is a boron deficiency in the soil, the calcium ions could not regulate the clubroot infection. In the environment with an elevated concentration of boron, inhibition functions of calcium are visible in both phases – root hair infection and cortical infection (Webster & Dixon 1991b). Boron slows down the sporangia development in the phase of cortical infection (Dixon 1991). It also affects the host immune system and symptoms

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of *P. brassicae* infection are milder (Dixon 2009b). An increasing amount of boron in the soil can help limit the zoospore penetration into the root system. The boron application should therefore precede the sowing of oilseed rape in the soil (Dixon 2009b).

A high concentration of **nitrogen** in the soil can lead to the suppression of clubroot symptoms; and on the contrary, a low concentration can help the disease development and consequently stronger symptoms (Webster 1986). The compounds which are produced during the calcium cyanamide degradation can reduce the club formation (Page 2001; Bhattacharya & Mandal 2006). These compounds lower the viability of zoospores and also change the microbial flora around them and support the growth of microbial antagonists of *P. brassicae* (Manolii *et al*. 2005; Dixon 2009b). The calcium cyanamide application is economically challenging and its use is more reasonable in smaller fields with vegetable production than on large areas with oilseed rape (Dixon 2009b).

Other products which contain nitrogen and calcium compounds are from seaweeds and corals. These products bear organic and nonorganic parts and growth regulators which affect the growth and reproduction of *P. brassicae* (Tilston *et al*. 2002).

The impact of **phosphorus** on the secondary phase of *P. brassicae* infection has been investigated (Abbasi & Lazarovits 2006a,b). **Molybdenum** also has some effects in interaction with boron and calcium (Sen 2005).

Suppressive soils

Suppressive soils can cut down the pathogen settlement, growth, and development, they weaken the pathogen and thus there is no or just low infection. The pathogen inability to settle is probably based on a complex of abiotic and biotic soil characteristics (Dixon 2009b). Extensive studies have been performed in Japan in Honshu, Fukushima region (Dixon 2009b). There are areas with high-humic Andosols which suppress the *P. brassicae* growth even at a high spore concentration. Suppression is probably caused by the unique composition of microflora, which is in natural antagonism to *P. brassicae* (Murakami 2000). In the Czech Republic suppressive soils were found in Bolehošť in the Rychnov nad Kněžnou region and in Nahořany (Náchod region) (Rod 1996). However, the suppressive nature of soil from these localities remains after heat treatment, therefore it cannot be of biological origin (Rod 1996).

Pathogenesis related genes

In order to develop new sources of resistance and control measures a systematic understanding of the mechanisms of *P. brassicae* is crucial. However, the obligate nature of the pathogen does not allow using the majority of techniques for the study of molecular mechanisms of pathogenesis. So far, there have been only a few reports on the molecular characterisation of *P. brassicae* candidate genes expressed during the pathogen growth in a host plant (Bulman *et al*. 2006, 2007; Siemens *et al.* 2009; Feng *et al*. 2010).

A few genes have been shown to express themselves during the pathogenesis of *P. brassicae*, viz. *Y10*, *PbTPS*, *PbSTKL1*, *PnBrip9*, *PbCC249*, *PRO1* etc. (Hwang *et al*. 2012). *Y10*: expression is exclusively correlated with the vegetative plasmodial stage (Ito *et al*. 1999), *PbTPS* is a trehalose-6-phosphate synthase gene with expression corresponding with the accumulation of trehalose in resting spores (Brodmann *et al*. 2002), *PbSTK1*: expression is strongly increased beginning 30 days after inoculation and coincides with the resting spore formation (Ando *et al*. 2006), *PbBrip9*: is strongly expressed during disease stages corresponding to the occurrence of sporulating plasmodia (Siemens *et al*. 2009), and *PRO1* is a serine protease (Feng *et al*. 2010). So far, *PRO1* has been proven experimentally to be important for the resting spore germination. *PRO1* exhibited high activity at the temperature and pH that are almost optimal for the germination of resting spores as indicated by Dixon (2009a,b). In addition, *PRO1* protein enhanced the stimulating effect of root exudates on the resting spore germination. This indicates that *PRO1* may play a role in clubroot pathogenesis by stimulating the resting spore germination through its proteolytic activity. *PRO1* is a potential target for clubroot management due to its role in pathogenesis (Feng *et al*. 2010).

With the availability of the newly sequenced genome of *P. brassicae* single-spore isolate e3, new and emerging aspects can be explored to determine the candidate genes responsible for pathogenicity. Since the genome is de novo thus restricts the chances to find the genes/proteins, however, with the help of advanced bioinformatics tools and approaches, mining and annotation can be done efficiently. Henceforth, in our lab we have examined a few gene families including an important gene family *Immunophilins* (IMMs) in *P. brassicae* (Singh, unpublished results). This gene family is a subgroup

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of Cyclophilins, FKBP, and Parvulins (Wang & Heitman 2005) and is shown to implicate in various abiotic, biotic stresses and auxin signalising (Marivet *et al*. 1992; Viaud *et al*. 2002, 2003; Sharma & Singh 2003; Aviezer-Hagai *et al*. 2007; Ahn *et al*. 2010; Meiri *et al*. 2010; Singh *et al*. 2014; Jing *et al*. 2015; Kumari *et al*. 2015).

Pathotypes

The terminology is quite complicated; the term 'pathotype' of *P. brassicae* is used instead of the term 'race' (Crute *et al*. 1980; Voorrips 1995). The term 'population' is then used for a collection of spores obtained from infested soil or infected plant material, which is used for the inoculation of differential plants (Buczacki *et al*. 1975). 'Differential plant' means a plant species with specific reaction to *P. brassicae* infection used for pathotype identification (Buczacki *et al.* 1975). The term 'race' of *P. brassicae* cannot be used because it designates a genetically uniform population which does not occur in *P. brassicae* populations (Parlevliet 1985).

P. brassicae populations are combinations of several pathotypes (Diederichsen *et al*. 2009). More than one pathotype can even occur in one gall (Jones 1982b; Fähling *et al*. 2003). Pathotypes can be distinguished in biological tests – differential systems/sets.

Differential sets

Differential sets can separate pathotypes according to disease symptoms on roots of differential hosts (Table 1) (Hwang *et al*. 2012). A differential set according to Williams (1966) uses 4 differential plants (hosts) which can identify 16 pathotypes. It was created mainly for North America, but this technique is used worldwide.

A differential set according to Buczacki *et al*. (1975) is called ECD set (European Clubroot Differential set) and is used mainly in Europe. It encloses 15 differential plant species which are divided into 3 genomic subsets. The first group of 5 hosts covers the genotype of *Brassica rapa*; the second group contains the genotype of *Brassica napus*; and, the third subset includes hosts from the *Brassica oleracea* genotype. In the case of infection each host gets a special score. The pathotype designation is arranged in triplets (e.g. 16/30/24), each genotype is counted separately (Table 1). This set can theoretically identify 48 pathotypes.

Later, a differential system according to Somé *et al*. (1996) was created. It contains just 3 differentials with two of them from ECD classification – ECD 06 and ECD 10. This system can distinguish 8 pathotypes. Somé *et al*. (1996) worked with an isolated single spore, which means that just one spore was used for the hosts' inoculation and results bring information

Table 1. Differential sets for designation of *P. brassicae* pathotypes

Name of set	ECD genotype	ECD No./score	Differentials name
ECD – European Clubroot Differential set (Buczacki *et al.* 1975)	*Brassica rapa* ssp. *rapifera* Polish rape	ECD 01/1	line aaBBCC
		ECD 02/2	line AAbbCC
		ECD 03/4	line AABBcc
		ECD 04/8	line AABBCC
	B. rapa var. *pekinensis*	ECD 05/16	Chinese cabbage cv. Granaat
	B. napus var. *napus* (fodder rape)	ECD 06/1	Dc 101 Nevin
		ECD 07/2	Dc 119 Giant Rape
		ECD 08/4	Dc 128 selection from Dc 119
		ECD 09/8	New Zealand resistant rape
	B. napus var. *rapifera*	ECD 10/16	Dc 130 Wilhelmsburger
	B. oleracea var. *capitata* (cabbage)	ECD 11/1	Badger Shipper
		ECD 12/2	Bindsachsener
		ECD 13/4	Jersey Queen
		ECD 14/8	Septa
		ECD 15/16	Verheul (Kale)
Williams (1966)		4 plants	Laurentian (*B. napus* var. *napobrassic*) ECD 10, 11, 13
Somé et al. (1996)		3 plants	Brutor (*B. napus* var. *napus*), ECD 06, 10

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about a genetically uniform pathotype. This method demands lots of time and work, because single-spore inoculation is successful in 10% of cases only.

Molecular designation of pathotypes is not possible yet. Just one pair of primers was designed, which can identify pathotype P1 according to Williams (1966) and Faggian *et al*. (1999).

Pathotypes in the Czech Republic

In 1987–1988 a pathotyping project was conducted by the former Research Centre for Vegetables in Olomouc (Rod 1991, 1994, 1996). Isolates collected from vegetable-producing areas were tested. In total 92 isolates from the Czech Republic and Slovakia were

Table 2. List of localities used for pathotype classification (vegetable isolates) by Rod (1994) in years 1987–1988. Showed just localities from Czech Republic

Isolate No.	Locality	Williams	ECD	Isolate No.	Locality	Williams	ECD
1	Ruzyně	7, 10	16/15/31	36	Rychnov n. Kněžnou	7	16/13/31
2	Rapotín	7	16/12/29	37	Luže	6, 7	16/10/31
3	Dlouhá Lhota	7, 6, 10	16/15/31	38	Řepníky	7, 4	16/15/31
4	Drahenice	6	16/02/28	39	Zámrsk	6, 4	16/14/28
5	Kačina	7	16/15/31	40	Česká Třebová	6	16/00/14
6	Sezimovo Ústí	6	16/02/28	41	Kunčina	7	16/15/31
7	Dražice	7, 2	16/14/31	42	Sádek	7	16/15/15
8	Borkovice	6, 7	16/02/28	43	Velký Beranov	7	16/15/31
9	Větrov	6	16/00/30	44	Babice	4	31/31/31
10	Čimelice	7, 4	16/31/31	45	Hrotovice	6	16/02/28
11	Písek-Hradiště	6, 7	16/04/14	46	Vranovice	6	16/02/14
12	Klatovy	6, 7	16/03/30	47	Brno	6	16/14/10
13	Radnice	7, 10, 4	16/14/31	48	Brno-Tuřany	7	16/15/31
14	Tachov	6	16/01/30	49	Brno-Jundrov	6, 7	16/00/30
15	Velká Hleďsebe	7	16/07/31	50	Brno-Komárov	7, 4	16/14/12
16	Horní Slavkov	6	16/02/28	51	Blansko	7	16/15/31
17	Sokolov I	10, 4	19/31/31	52	Rohozec	6	16/10/28
18	Sokolov II	7, 4	29/31/25	53	Spytihněv	7, 4	16/14/31
19	Chomutov	6	16/03/28	54	Prostějov	6, 7	16/15/31
20	Litvínov	7	16/00/31	55	Vrbátky I	6, 7, 2	16/15/31
21	Trávčice	7, 10	16/15/31	56	Vrbátky II	7	16/06/13
22	Polepy	7, 10	16/15/31	57	Troubky	7	16/14/31
23	Pertoltice p. R.	6	16/03/28	58	Výmyslov	6, 2	16/14/31
24	Hrádek n. Nisou	6, 7	16/09/15	59	Olomouc -Holice	6, 2	16/01/31
25	Liberec	7, 2	16/15/15	60	Červenka	7	16/13/31
26	Hodkovice u L.	7, 10	16/15/31	61	Dlouhá Loučka	7	16/15/31
27	Turnov	10, 4	16/31/13	62	Libina	6, 2	16/15/30
28	Benecko	6	16/03/28	63	Žulová	6, 7	16/07/30
29	Jilemnice	6	16/02/28	64	Krnov	6	16/03/28
30	Podhorní Újezd	7	16/14/31	65	Světlá Hora u Bruntálu	10, 4	17/31/31
31	Nový Bydžov	7, 4	16/15/31	66	Bolatice	6, 10	16/15/31
32	Předměřice n. L.	6	16/00/12	67	Karviná	7, 10	16/14/31
33	Nahořany	7	16/15/31	68	Nošovice	6, 2	16/08/28
34	Červený Kostelec	6	16/02/28	69	Frýdlant n. O.	1, 4	16/31/31
35	Broumov	6	16/03/30				

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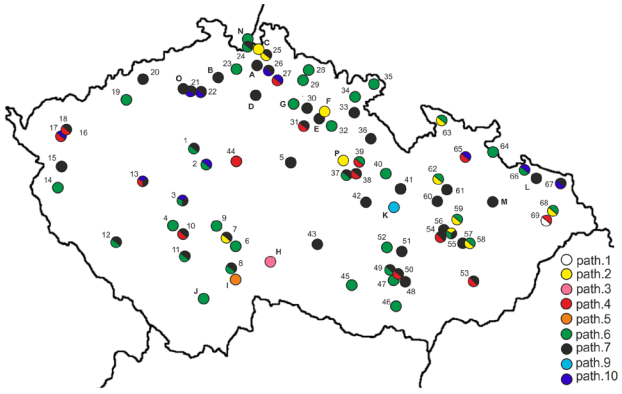

Figure 5. Pathotype screening of vegetable isolates (Rod 1991, 1994, 1996) – numbers (without Slovakia), and oilseed rape isolates (Ricarova *et al.* submitted) – letters. All pathotypes were identified according to Williams (1996)

tested (Table 2). The project revealed 6 pathotypes according to Williams (1966) with the most prevailing pathotype 7 (almost 50% of samples) (Figure 5). Other revealed pathotypes were 6, 4, 10, 2, 3 and pathotype 1 in one isolate. In some isolates pathotypes 2 and 3 were detected together. Pathotype 3 was revealed only in Slovakia. According to ECD classification, 35 pathotypes were identified, but 22 of them occurred only once. The most common was the pathotype ECD 16/15/13 (28% cases) as well as ECD 16/02/28, and 16/14/31 (9 % cases).

Table 3. The list of localities used for pathotype classification (OSL isolates) by Ricarova *et al.* (in submission)

Isolate	Locality	Williams (1996)	Somé *et al.* (1996)	ECD
A	Modlibohov	7	P3	16/14/31
B	Holany	7	P3	16/14/15
C	Bílý Kostel	2	P3	16/14/13
D	Horka u Bakova	7	P3	16/14/15
E	Třebnouševes	7	P3	16/14/31
F	Miletín	2	P3	16/14/15
G	Kbelnice	6	P3	16/2/14
H	Žirovnice	3	P3	16/2/14
I	Horusice	5	P4	16/18/15
J	Hrdějovice Ves	6	P3	16/14/12
K	Pohledy	9	P1	16/31/8
L	Kozmice	7	P3	16/14/15
M	Klokočov	7	P3	16/2/15
N	Hrádek n. Nisou	6	P4	16/10/4
O	Terezín	7	P3	16/14/13
P	Horní Ředice	2	P3	16/14/13

Pathotyping was also conducted in 2014 using isolates from an oilseed rape study with all differential sets (Ricarova *et al.* in submission) (Table 3). Out of 17 samples, 6 pathotypes according to Williams (1966) were detected with the most prevalent pathotype 7 (41% of cases) (Figure 5). Other identified pathotypes were 6, 2, 3, 5, and 9. According to Somé *et al.* (1996) 3 pathotypes were identified with the most frequent pathotype P3 (82% of cases). Pathotyping also revealed P4 and P1. The ECD set identified 10 pathotypes and the most common was ECD 16/14/15 (24% cases), other pathotypes, which occurred more than once, were 16/14/13, 16/14/31, and 16/2/14.

Comparison of the pathotyping results of Czech vegetable isolates and Czech oilseed rape isolates brings information about the "preference" of *P. brassicae* strains to these species. Pathotypes 9 and 3 according to Williams (1966) seem to be unique for the infection of oilseed rape, because none of these pathotypes was recorded on vegetables during screenings performed by Rod (1991, 1994, and 1996) in the Czech Republic. This is an interesting finding, because pathotype 3 is one of the most widespread pathotypes on oilseed rape especially in Canada (Strelkov *et al.* 2006, 2007). On the other hand, pathotypes 1 and 4 seem to be unique for vegetables.

The geographic pattern of *P. brassicae* distribution is not probably connected with any special environmental feature like soil type or climate conditions. The spread of clubroot is mainly historical, because of a long tradition of cabbage cultivation, which was dominant in all infested localities (Chytilová & Dušek 2007). The oilseed rape cultivation does not have such a long continuance as the first fields were recorded in the 20s of the last century (5900 ha), after Second World War there were 32 605 ha and cropping on a larger area came only in the 80s of the last century (102 160 ha). Since then the cropped area was growing and a real breakthrough came with the new millennium, when the production areas of oilseed rape reached 344 117 ha and the peak was in 2013 (418 808 ha) (Czech Statistical Office). As the area of vegetable cultivation was gradually decreasing in the Czech Republic, fields used before for vegetable production started to be cropped with oilseed rape and further spreading of clubroot infection was therefore easier.

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Diagnostics

Plasmodiophora brassicae is an obligate parasite which cannot be cultivated on artificial media and this complicates its detection (Hwang *et al*. 2012). Detection methods can generally be divided into: biological/symptomatic, microscopic, serologic, and molecular ones (Faggian *et al*. 2009; Hwang *et al*. 2012).

Biological method (bioassay)

Using a susceptible species to detect the *P. brassicae* presence in a soil sample is one of the reliable methods (Faggian & Strelkov 2009). Susceptible plants are grown in the soil suspected of the *P. brassicae* presence in controlled conditions for 5–6 weeks. After this time plants can be examined for the presence of disease symptoms on roots (Figure 4) (Faggian & Strelkov 2009). Chinese cabbage cv. Granaat is often used as susceptible plant, as it is a universally susceptible host of all *P. brassicae* pathotypes (Hwang *et al*. 2012). This method is time-consuming as well as laborious and can be used only if the inoculum reaches a concentration of at least 1000 spores per 1 g of soil (Faggian & Strelkov 2009). The limitation can also be a lack of space in greenhouses. The bioassay results bring only information about the pathogen presence in the soil, but not about its quantity (Hwang *et al*. 2012).

Microscopy

Another diagnostic tool includes the microscopic examination of root hairs and root cortex for the presence of *P. brassicae* infection sites (MacFarlane 1952; Hwang *et al*. 2011b). The soil can also be examined microscopically with a fluorescent dye being used to aid the spore detection (Takahashi & Yamaguchi 1988). The authors evolved the protocol for microscopic detection with two fluorophores, which can even detect the viability of spores. The first fluorophore – Calcoflor White M2R – binds to chitin in the cell walls of undamaged spores. The second fluorophore – ethidium bromide – penetrates damaged and nonviable spores. Then viable spores are fluorescing with blue colour and nonviable ones with red. A correlation between the blue light intensity and index of disease (ID) was also confirmed (Takahashi & Yamaguchi 1989). This method is time-consuming and laborious again and also requires well-trained personnel.

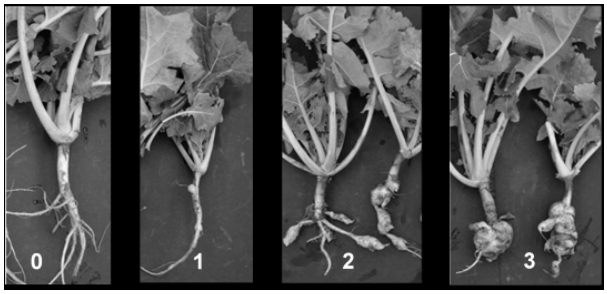

Figure 6. Disease rating scale (0 – no symptoms, 1 – minor clubs, 2 – clubs on lateral roots, 3 – severe clubbing)

Staining can also be performed in clubs or on the whole tissue suspected of infection by *P. brassicae*. Different chemicals which bind to chitin are used. For viability determination, staining with Evans blue (Rennie *et al*. 2011) or orcein (Rod 1996) may be used. Nonviable spores absorb dye inside and viable spores have stained just cell walls (Naiki & Dixon 1987).

Serology

Serological tests are based on polyclonal antisera (Lange *et al.* 1989; Wakeham & White 1996). Tests based on dip-stick method, indirect ELISA, and immunofluorescences have achieved the sensitivity of 100 spores per 1 g of soil (Wakeham & White 1996). The methods based on polyclonal antisera bring results which can be affected by variability in antiserum specificity and sensitivity. Therefore the test based on monoclonal antiserum should be evolved, because it would be more specific and also more accessible (Faggian & Strelkov 2009)

Molecular methods

Primers and **PCR** (polymerase chain reaction) technique are well developed to amplify specific sections of the *P. brassicae* genome (Hwang *et al*. 2012). PCR brings quick and reliable results about pathogen presence or absence in different kinds of samples (Hwang *et al*. 2012). Designed primers amplify the ITS regions (section on ribosomes) (Chee *et al.* 1998; Faggian *et al.* 1999; Wallenhammar & Ardwidsson 2001; Cao *et al*. 2007), one primer pair is based on isopentyl transferase amplification (Ito *et al*. 1999). PCR protocol developed by Cao *et al*. (2007) is commonly used for commercial purposes for *P. brassicae* detection from plant tissue or from soil (Hwang *et al*. 2012). This is the first 'one step' PCR protocol which can identify as little as 100 fg of pathogen DNA or even less in a sample, which corresponds to 1000 spores per 1 g of soil or 11% of disease index. Common PCR assay provides information about the presence/absence of pathogen in a sample (Hwang *et al*. 2012).

On the other hand, the **qPCR** method (Q-RT PCR; quantitative polymerase chain reaction) can detect not

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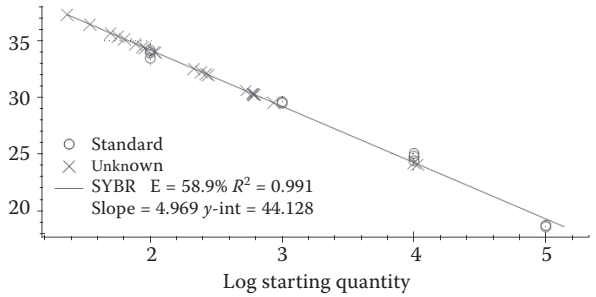

Figure 7. Quantitative PCR standard curve of *P. brassicae* detection

x-axis: amount of DNA, *y*-axis: Cq – quantification cycle; standards (circles): known amount of spores, daggers: unknown samples (Ricarova, unpublished)

only the presence but also the amount of *P. brassicae* in a sample (Hwang *et al*. 2012). Several protocols were developed (Sundelin *et al.* 2010; Rennie *et al*. 2011; Wallenhammar *et al*. 2012). The qPCR method is based on detection of fluorescent signal during the amplification of pathogen DNA. The protocol of Rennie *et al*. (2011) was developed for SYBR green signal. The number of spores is counted as the amount of DNA reported by machine in proportion to the amount of sample entering the reaction (Figure 7). This method can detect 1000 spores and more per 1 g of soil with the level of precision in thousands (Rennie *et al.* 2011).

Management

Sanitation

Chemical protection against *P. brassicae* is complicated and impossible on large areas. Therefore one of the protective measures may be to avoid spreading of spores. The machinery sanitation is one of the methods. The equipment like ploughs or seeders can carry 10 kg of infested soil. Therefore machinery cleaning is an important preventive measure together with the cleaning of tools and vehicles or control of plant material, seed, and water transport (Howard *et al*. 2010; Hwang *et al*. 2014). The methodology for cleaning and sanitation was developed by Howard *et al.* (2010). All the procedures were laboratory tested and verified to be effective. The cleaned machines are set on special grids, which retain used water and chemicals. The sanitation process consists of **rough cleaning** – scraping off the soil with brushes or air flow, **fine cleaning** with pressure washers and **disinfection** of cleaned surfaces for at least 20 min to remove all remaining spores. The key steps are rough and fine cleaning which should remove 99% of all spores. Disinfection is the final step. Some disinfectants were tested and the best results were obtained with sodium hypochlorite, hydrogen peroxide, acetic acid or potassium peroxymonosulphate. Cleaning by high temperature was also tested, but it proved to be ineffective, because spores were still viable even after 3 h at temperatures above 100°C (Howard *et al*. 2010).

Seeding date

Another form of preventive measures is to delay the seeding date of winter rape, because *P. brassicae* infection is supported by soil moisture and higher temperature above 25°C (Feng *et al*. 2010; Sharma *et al*. 2011). Low temperatures inhibit the pathogen infection and development (Hwang *et al*. 2011a). Older host plants show lower susceptibility to infection in a colder soil and if infection occurs, it does not have such a negative effect on yield (Hwang *et al*. 2011a). The Canadian trials with shifting the seeding date of spring rape towards earlier dates showed lowering the disease index and higher yield, but the germination rate was decreased too (Hwang *et al*. 2011a). Gossen *et al*. (2012) reported a reduction of the disease index by 10–15% and a yield increase of 30–58%. Similar results were obtained in trials with vegetables (Gossen *et al*. 2009). Younger plants are more vulnerable to infection than plants aged 10–25 days. A decrease in susceptibility is probably related to the cell wall thickening of older plants (Mellano *et al*. 1970) and formation of defence structures (Hwang *et al*. 2014). The seeding date shifting should not be the only protective measure and it is necessary to combine it with other interventions (Hwang *et al*. 2014).

Chemical control

Fungicides. Chemical products for clubroot treatment are divided into soil fumigants and soil fungicides (Hwang *et al*. 2014). The application of these preparations is technically and economically challenging. The most frequently used soil fumigant is metam sodium; sodium *N*-methyldithiocarbamate, the active ingredient in the soil fumigant Vapam®, which is commonly used in the USA and Canada. Metam sodium is changed by contact with the soil to methyl isothiocyanate in gas form that diffuses through the soil and has both fungicidal and nematicidal effects. The fumigation is performed mainly at the entry into a field or in newly infested areas to stop the spreading of the spores (Howard *et al*. 2010; Hwang *et al*. 2014). The Vapam application in a dose of 0.4–1.6 ml/l lowered 12–16 times the primary and secondary infection

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and club formation on oilseed rape plants (Hwang *et al*. 2014). The side effect was an improvement of the plant health. The Vapam efficacy is decreased by soil moisture; the ideal moisture should be around 10% of the soil capacity (Hwang *et al*. 2014). The soil should be covered after application to minimize evaporation and air pollution (Hwang *et al*. 2014). In the Czech Republic the soil fumigant Basamid® is registered (active substance dazomet 97%). It is applied in granular form (20–60 g/m^2) (eagri.cz), watering and covering the soil with foil are recommended after application (Rod 1996).

The soil fungicides are economically demanding again, and also very environmentally unfriendly, so the application is possible on small plots only (Hwang *et al*. 2014). Peng *et al*. (2014) tested the application of fungicides Terraclor® (PCNB, pentachloronitrobenzene 75%), and Ranman® (160 g/l cyazofamid) prior to seeding. All fungicides showed effects against *P. brassicae*, but none of the fungicides worked on plots with a very high concentration of spores.

Biofungicides, Biofungicides containing bacterial or fungal antagonists were also tested (Serenade® with *Bacillus subtilis* and Prestope® with *Gliocladium catenulatum*). These biofungicides have antibiosis effects and also stimulate the immune response of infected plants (Peng *et al*. 2014). The biofungicides were applied in water after seeding and they were effective at 85–100%, but only in soils with a low inoculum level (Peng *et al*. 2014).

Another management practice, which is between prevention and protection, is liming for raising the soil pH. Liming can be done with normal calcium or calcium cyanamide.

Bait crop

One of the further management strategies in *P. brassicae* protection is using 'bait crops', which induce the resting spore germination in the absence of a susceptible host (Rod & Robak 1994; Friberg *et al*. 2005; Hwang *et al*. 2011b, 2014). Plants from the families Papaveraceae, Poaceae and Rosaceae provoke the spore germination, but only the primary phase of the life cycle occurs and resting spores are not developed (Colhoun 1958; Karling 1968). Rod and Robak (1994) evaluated many different species; for example aromatic herbs, which lowered the inoculum level by 50% in three years. Other evaluated 'bait crops' are: grasses like velvet-grass (*Holcus lanatus*), perennial ryegrass (*Lolium perenne*), creeping bentgrass (*Agrostis stolonifera*), orchard grass (*Dactylis glomerata*) or broadleaf crops like poppy (*Papaver rhoeas*), clover (*Trifolium repens*), sorrel (*Rumex* spp.) or strawberry (*Fragaria* spp.) (Rod 1996; Dixon 2009b; Ahmed *et al*. 2010).

Resistant cultivars

Currently, resistant cultivars are the only working protection against *P. brassicae* in combination with suitable crop rotation and management strategies (Peng *et al*. 2014).

Genetics and breeding. The clubroot host-plant resistance can be found in main cruciferous crops (except *Brassica juncea* and *B. carinata*) (Diederichsen *et al*. 2009). The resistance against *P. brassicae* is polygenic (quantitative) and also monogenic (qualitative) (Crute *et al*. 1980; Voorrips 1996). Many of the resistance types are pathotype specific and do not cover all pathotypes of *P. brassicae* (Diederichsen *et al*. 2009). The studies proved the existence of 8 resistance loci, some of them are pathotype specific major genes, and some are quantitative loci (QTL) with specific or nonspecific resistance responses (Voorrips 1996; Hwang *et al*. 2012). Some studies also proved that resistance is qualitative and is controlled by dominant and recessive genes (Chiang & Crête 1983; Yoshikawa 1993).

The oilseed rape resistant cultivar Mendel was bred from ECD hosts – resistant turnip (*B. rapa*, ECD-04) and kale (*B. oleracea* ECD-15) through backcross with oilseed rape (Diederichsen *et al*. 2009). The F1 backcrosses were inoculated with *P. brassicae* and the most resistant ones were selected for further breeding and genetic analysis (Diederichsen *et al*. 2009). Later the presence of resistant genes was confirmed. There is one major locus and two recessive genes in the cultivar Mendel whereas other genes were lost in the breeding process and negative selection (Diederichsen *et al*. 2009). This is why cv. Mendel is recommended to use wisely together with other management strategies (Diederichsen *et al*. 2009; Dixon 2009b). The same situation exists in other resistant cultivars.

Mechanism of resistance. The resistance mechanisms are based on suppressing the pathogen development in a host rather than on infection avoidance. The term resistance is used because the infection inhibition is based on resistance genes, not on tolerance genes. The zoospore penetration occurs in the root hairs of a resistant host (the primary phase of the pathogen life) (Hwang *et al*. 2012). The secondary phase of infection in the root cortex also follows, but

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formed plasmodia do not mature and resting spores are not created (Hwang *et al.* 2011b; Deora *et al.* 2013). Slight suppression of primary infection and significant suppression of secondary infection occur during the resistance response, the disruption of the cell walls is lesser and the secondary thickening of the stem is not stopped (Donald *et al.* 2008). The immune response includes the activation of metabolic pathways for synthesis of phytoalexins, phenolic compounds, lignin and other substances that block the pathogen invasion (Siemens *et al.* 2006).

Resistant cultivars in the field. The first resistant cultivars were Mendel and Tosca, marketed at the beginning of the new millennium. These cultivars are heterozygous hybrids with one gene of pathotype specific resistance. Unfortunately, the Mendel host-plant resistance was broken in some parts of Germany and France, where it was grown since 2000, when it was put on the market (Diederichsen *et al.* 2014). Also in Great Britain resistance was broken quite rapidly in the field, where cultivars were grown under strong selection pressure (Diederichsen *et al.* 2003). These cultivars were also tested on local pathotypes in Canada. Cultivar Mendel showed resistance against pathotypes 3, 5, 6, and 8 (Williams set) (Rahman *et al.* 2014). Cultivar Tosca was susceptible to pathotype 3 and resistant to pathotype 2 (Williams set) (Peng *et al.* 2013). These results showed that the host-plant resistance is quite variable and some genes may be ineffective in some regions with specific pathotypes (Peng *et al.* 2014). New resistant cultivars of spring rape with genes from European cultivars were introduced into Canada in 2009 (Peng *et al.* 2014).

Currently, on the European market, there are available resistant cultivars which are derived from the first resistant cultivar Mendel (Deiderichsen *et al.* 2014). It is expected that a wider range of resistant varieties will come to the market in the next years (Diederichsen *et al.* 2014). However, these varieties cannot be grown without suitable agronomic measures and pathogen-resistance management strategies (Diederichsen *et al.* 2014).

Field experiments with resistant cultivars in infested fields were also conducted in the Czech Republic (Říčanová, unpublished). Eight resistant cultivars were tested, some of them are normally available on the market and some were test cultivars in the breeding process (new breeds). Normally traded cultivars had stable performance in resistance results and good yield. Cultivars from new breeding showed some variations in resistance performance and yield.

CONCLUSION

The pathogen *P. brassicae* has been spreading in the fields in the Czech Republic and its importance has steadily grown. The disease development strongly depends on weather, so it does not result in severe symptom expression every year. Clubroot thrives in seasons with warm and moist autumn. If followed by a cold winter with frosts, then the biggest yield losses are incurred – plants with the broken vascular system cannot compensate it. Therefore, it is very important to apply suitable clubroot management practices consisting in later sowing, use of resistant cultivars, liming, and correct crop rotation. Resistant cultivar stewardship is also necessary to avoid resistance erosion and selection of new *P. brassicae* pathotypes. Sanitation is a good additional protective measure, but in the high-season it is often complicated to implement. New molecular techniques are useful tools for clubroot detection in the soil and may help to avoid yield losses with early use of resistant cultivars.

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