doi: 10.17221/7/2016-PPS

First Report of *Plasmopara halstedii* New Races 705 and 715 on Sunflower from the Czech Republic – Short Communication

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Abstract

Sedlářová M., Pospíchalová R., Drábková Trojanová Z., Bartůšek T., Slobodianová L., Lebeda A. (2016): **First report of *Plasmopara halstedii* new races 705 and 715 on sunflower from the Czech Republic – short communication.** Plant Protect. Sci., 52: 182–187.

Downy mildew caused by *Plasmopara halstedii* significantly reduces annual yields of sunflower. At least 42 races of *P. halstedii* have been identified around the world. For the first time to our knowledge, races 705 and 715 of *P. halstedii* have been isolated, originating from sunflower plants collected at a single site (Podivín, South-East Moravia) in the Czech Republic at the beginning of June 2014. This enlarges the global number of the so far identified and reported races of *P. halstedii* to 44. The increasing complexity of *P. halstedii* pathogenicity led to race identification newly by a five-digit code. According to this new nomenclature, the two races of *P. halstedii* recorded in the Czech Republic are characterised by virulence profiles 705 *71* and 715 *71*.

Keywords: *Helianthus annuus* L.; resistance; sunflower downy mildew; virulence formula

Biotrophic parasite *Plasmopara halstedii* (Farl.) Berl. et de Toni (1888), ranked in the kingdom Chromista, class Oomycetes, family Peronosporaceae, causes downy mildew of sunflower. The mechanisms of plant infection and pathogen reproduction were first studied already a century ago (Nishimura 1922). Systemically infected sunflowers do not produce well ripened achenes which causes high economic losses to the farmers. Moreover, oospores of *P. halstedii* can contaminate soil for several years. Thus sunflower downy mildew has been in the list of quarantine diseases in Europe since 1992, and in the Czech Republic ten years later (Spurný 2005). Global distribution of the pathogen has been reported, with the exception of Australia and New Zealand (Gulya 2007).

Sunflower downy mildew control relies mostly on resistant hybrids and fungicide-treated seed. Currently, ca. 20 downy mildew resistance genes (Pl_1–Pl_{17}, Pl_{21}, and Pl_{ARG}) have been characterised from cultivated and wild species of sunflowers (Gascuel *et al.* 2015; Qi *et al.* 2015). Their utilisation in resistance breeding accelerates microevolution of the oomycete as new races with complex virulence formulas emerge. The existence of *P. halstedii* races was confirmed in the 1970s when a new race was detected in the USA which was able to overcome sunflower resistance gene Pl_1, which ensured resistance to the European race (Zimmer 1974). Changes in virulence of *P. halstedii* in 1980s led to the introduction of a new nomenclature system based on the reaction of nine sunflower differential lines bearing various *Pl* resistance genes and race identification by a triplet code (Sackston *et al.* 1990; Gulya *et al.* 1991; Gulya 1995). Differential lines were produced by USDA (United States Department of Agriculture, USA), INRA (Institut National de la Recherche Agronomique, France), and IFVC (Institute of Field and Vegetable Crops, Novi Sad, Serbia). Until 2007,

Supported by the Internal Grant Agency of the Palacký University in Olomouc, Czech Republic, Grant No. IGA_PrF_2016_001.

doi: 10.17221/7/2016-PPS

18 races had been reported from Europe (Virányi *et al.* 2015) and 20 races from America (Gulya 2007). Over the past 7 years this number increased to a total of 24 races in Europe and 36 in America. The history of the research of *P. halstedii* pathogenic variability has been precisely reviewed by Virányi *et al.* (2015). Permanent changes in pathogen virulence have been reflected by modifications in the sunflower differential set (Tourvieille de Labrouhe *et al.* 2000, 2012). The tentatively adopted set consists of fifteen sunflower differential lines gathered in five triplets and *P. halstedii* races are designated by a five-digit code (Gascuel *et al.* 2015). Despite a long lasting discussion the six additional lines proposed by French researchers have yet to be internationally endorsed, hopefully during the upcoming International Sunflower Association meeting in Turkey (2016).

In the Czech Republic (formerly Czechoslovakia) the first records of sunflower downy mildew date to 1950s (Bojňanský 1956, 1957). However, a detailed study of the occurrence and pathogenic variability of *P. halstedii* was initiated at our department in 2007. During annual surveys we collected and gradually identified six races, i.e. 700, 704, 710, 714, 730, and 770 (Sedlářová *et al.* 2013; Trojanová *et al.* 2013).

This paper brings information on the occurrence of two new races of *P. halstedii*, 705 and 715, isolated for the first time ever from infected sunflowers in the south-eastern part of the Czech Republic.

MATERIAL AND METHODS

Disease symptoms, specimen collection, and isolate maintenance. In June 2014, during our annual field expedition to the south-east of the Czech Republic, sunflower plants with symptoms of primary infection by *Plasmopara halstedii* were recorded in one wet field near the town of Podivín, South Moravia. Plants at the stage of ca. 4 true leaves were heavily infected with pronounced sporulation of *P. halstedii*. The plants were pulled out, put separately on moisture filter paper in plastic boxes, and transferred to our lab. Eleven bulk isolates (one per plant) were obtained by washing zoosporangia off the leaves with distilled water and subjected to sub-cultivation on susceptible sunflower line, *Helianthus annuus* cv. Giganteus, lacking resistance genes to *P. halstedii*. A short-time storage of *P. halstedii* sporangia on host tissues was performed at –20°C, a long-time storage, if needed prior to pathogenicity/fungicide resistance tests, was held at –80°C. According to our experience, vitality of sporangia decreases rapidly after 2–3 months of storage at –20°C but isolates can be safely maintained up to 6 months at –80°C. Five isolates showed a novel pattern when tested on a set of differential lines of sunflower.

Inoculation procedures for pathogen race identification. To determine the race of *P. halstedii*, isolate seedlings were inoculated according to the soil drenching method (Goosen & Sackston 1968) using a set of 15 differential lines of sunflower (Gascuel *et al.* 2015). An overview of the differential set together with the present resistance genes is given in Table 1. Sunflower seeds originating from INRA, France (lines GB, QHP2, Y7Q, PSC8, XA, PSS2RM, VAQ, RHA419) and from dr. T. Gulya, USDA ARS (lines RHA-265, RHA-274, PMI-3, PM-17, 803-1, HAR-4, Ha-335) were increased by self-pollination in summer 2014 and derived seeds were used for pathotests. Seeds were disinfected by immersion in 0.7% sodium hypochlorite (15% solution of Savo®; Bochemie, Bohumín, Czech Republic) for 10 min, rinsed thoroughly by tap water, and placed on moisture filter paper in Petri dishes at room temperature and darkness for 2–4 days to germinate. Seedlings of sunflower differential lines (20 per line) with an optimal radicle length (1–2 cm), well-developed root hairs, and free from contaminating microorganisms were selected and planted into a tray with perlite in lines. *P. halstedii* isolates, multiplied on seedlings of a susceptible sunflower cv. Giganteus, either fresh or stored frozen on host leaves, were used as a source of inoculum. Zoosporangia were washed off leaves in distilled water and the density of suspension checked using Bürker counting chamber and light microscopy. An appropriate amount of fresh inoculum was pipetted directly to each plantlet to reach the number of ca. 10 000 zoosporangia. Seedlings were covered with a thin layer of perlite and cultivated for the first 12–16 h at 17°C in darkness. Subsequent plant growth proceeded at 21/17°C, 12/12 h (day/night). Whole cultivation was done in wet perlite to ensure optimal conditions for pathogen development. Two-week-old plants were sprayed with distilled water and trays were closed in dark foil for 24 h to stimulate sporulation in humid environment. Semi-quantitative assessment of the disease intensity followed the next day according to standard methods (Oros & Virányi 1987). A week later the whole procedure of water-spraying and evaluation was repeated to observe any late-developing systemic infection. For higher confidence, testing of each isolate was conducted two to three times.

doi: 10.17221/7/2016-PPS

Table 1. Sunflower differential lines with corresponding resistance genes (modified according to that proposed by Gascuel *et al.* 2015) and virulence phenotype of new races 705 *71* and 715 *71* (+ = virulent; – = avirulent)

Set of differentials	Number	Virulence value	Sunflower line	Known *Pl* (*R* genes)	Race/virulence profile	
					705 *71*	715 *71*
	D1	1	Ha-304	–	+	+
1	D2	2	RHA-265	Pl_1	+	+
	D3	4	RHA-274	Pl_2/Pl_{21}	+	+
	D4	1	PMI-3	Pl_{PMI3}	–	+
2	D5	2	PM-17	*Pl5*	–	–
	D6	4	803-1	*Pl5* +	–	–
	D7	1	HA-R4	Pl_{15}[1]/Pl_{16}[2]	+	+
3	D8	2	QHP-2	?	–	–
	D9	4	Ha-335	Pl_6	+	+
	D10	1	Y7Q	Pl_6 –	+	+
4	D11	2	PSC8	Pl_2	+	+
	D12	4	XA	Pl_4	+	+
	D13	1	PSS2RM	Pl_6/Pl_{21}	+	+
5	D14	2	VAQ	Pl_5	–	–
	D15	4	RHA-419	Pl_{ARG}	–	–

[1]Gascuel *et al.* (2014); [2]Liu *et al.* (2012)

Degree of infection and race determination. In sunflowers two types of resistance to downy mildew conferred by *Pl* genes have been recognised: type I resistance characterised by the absence of symptoms on shoots and the absence of the pathogen above the base of hypocotyls, and type II resistance characterised by weak sporulation symptoms restricted to cotyledons, and the absence of symptoms in the upper parts of plant with the pathogen never reaching true leaves (Mouzeyar *et al.* 1994). For each plant infection degree was evaluated as the area of cotyledons/leaves covered with zoosporangiophores using the scale ranging from 0 to 3 (Oros & Virányi 1987) where 0 = no sporulation, 1 = sparse sporulation, 2 = less than 50% of cotyledon/leaf area covered, and 3 = more than 50% of cotyledon/leaf area covered with zoosporangiophores. The total degree of infection (P) was calculated for each differential line and expressed as a percentage of the maximum scores according to equation:

$$P = \frac{\sum n \times v}{x \times N} \times 100$$

where: P – total degree of infection; n – number of seedlings in each infection degree; v – infection degree (0–3 for seedlings); x – scale range = 3; N – total number of assessed seedlings

Subsequently the susceptibility/resistance of an isolate was recognised (degree of infection ≥ 50% → susceptibility; ≤ 10 → resistance; 10–50% → repetition of the experiment) and corresponding race of each isolate determined according to virulence phenotype under the rules of the proposed five-digit coding system (Gascuel *et al.* 2015).

Fungicide test. A leaf disc immersion test (Rozynek & Spring 2001) was applied to assess susceptibility/resistance of all isolates to metalaxyl. In brief, leaf discs 6 mm in diameter were cut off cotyledons of susceptible *H. annuus* cv. Giganteus plantlets with a cork borer bathed in distilled water for a short time and dried. Distilled water or 10 mg/l solution of metalaxyl (*N*-(2,6-dimethylphenyl)-*N*-(methoxyacetyl)-D-alanine methyl ester; FLUKA Metalaxyl-M, Sigma-Aldrich spol. s.r.o., Prague, Czech Republic) was pipetted into wells of Nunc 24-well multidish (Thermo Fisher Scientific, Prague, Czech Republic). Sunflower cotyledon discs were floated on the surface by adaxial side and the centre of the upper side was inoculated by pipetting 10–15 µl of suspension containing ca. 10.000 of *P. halstedii* zoosporagia (Figure 1; inoculum preparation see above). The multi-well plate was covered and placed at 18–19°C in dark for 12 h, later in day/night

doi: 10.17221/7/2016-PPS

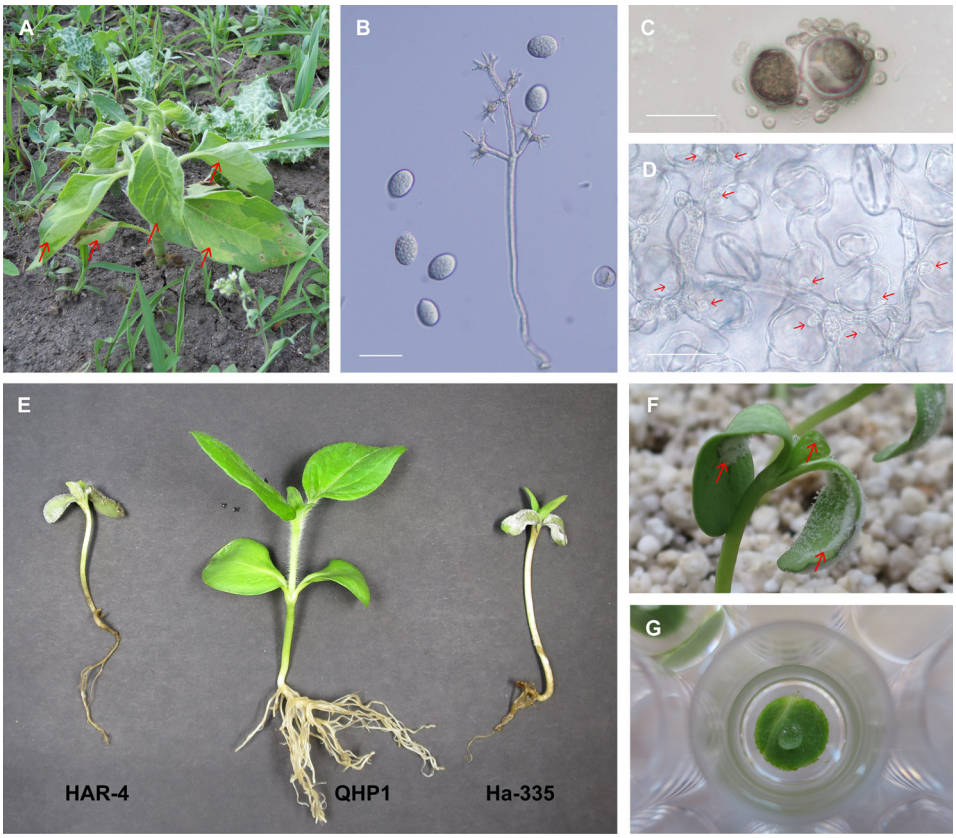

Figure 1. *Plasmopara halstedii* infected plant in the field near Podivín with typical symptoms (see arrows) of chlorotic spots and sporulation (**A**), zoosporangiophore (**B**), and zoosporangia releasing zoospores (**C**); sunflower leaf tissue with downy mildew intercellular hyphae and haustoria – marked by arrows (**D**). Reaction phenotypes of sunflower lines from the third triplet of differential set to infection by *P. halstedii* race 705 *71* (**E**) and detail of sporulation on cotyledons and first true leaves of line HAR-4 (**F**). Absence of sporulation in fungicide test (**G**). Bar in microphotographs corresponds to 50 µm (Photos (A–B) and (D–G) by M. Sedlářová and (C) Z. Drábková Trojanová)

12/12 hours. Presence of sporulation was thoroughly checked using stereomicroscope two weeks post inoculation. The presence of intensive sporulation on > 75% of inoculated control discs was the measure of a successful test.

RESULTS AND DISCUSSION

Races of *Plasmopara halstedii* isolates collected in June 2014 were specified according to a proposed nomenclature based on the virulence profile of a given isolate on 15 sunflower differential lines selected according to their resistance/susceptibility patterns (Tourvieille de Labrouhe *et al.* 2012; Gascuel *et al.* 2015). This differential set of sunflowers is specified in Table 1. The seedling inoculation pathotests repeatedly proved the presence of race 705 *71* in three, and race 715 *71* in two bulk isolates of *P. halstedii* originating from sunflower plants collected at a locality near the town of Podivín, in the south-eastern part of the Czech Republic (Figure 1). In this waterlogged field, sunflower plants with symptoms of *P. halstedii* primary infection had been repeatedly recorded in previous years (Sedlářová *et al.* 2013). New combinations of the infection phenotype of sunflower differentials which were recorded during recent testing are shown in Table 1.

Leaf disc immersion tests (Rozynek & Spring 2001) revealed susceptibility of all the tested isolates to metalaxyl, used for sunflower seed dressing. No recorded fungicide resistance is a good message for local farmers. On the other hand, they should keep agronomic practices not to sustain the pathogen life cycle and microevolution.

The survey of the *P. halstedii* research history has been nicely reviewed thanks to the cooperation of Prof. F. Virányi from Hungary, Dr. T.J. Gulya from the

doi: 10.17221/7/2016-PPS

USA, and Dr. D. Tourvieille de Labrouhe from France (Virányi *et al.* 2015). The authors summarise that until 2015 at least 42 races of *P. halstedii* have been reported, out of which 24 races came from European countries and 36 races from America. However, the virulence pattern observed in our tests has not been published yet. Several factors must be taken into account when comparing results published during the last 40 years. The increasing knowledge of *P. halstedii* intraspecific variability has been tightly linked with cultivars/genotypes of host plants used for testing. Gradual changes in the differential set of sunflower lines make the comparison of different research teams over the time rather difficult. Recently, several modifications have been made in the third triplet, e.g. at the position of D8 the USDA's line HA-R5 (Gulya *et al.* 1991) has been replaced by INRA's selection QHP-1 (Tourvieille de Labrouhe *et al.* 2000, 2012) and newly by a further reselection named QHP-2 (Pierre Desray, INRA, pers. comm.). In our tests line QHP-1 in addition to QHP-2 was used in the position of D8 and resulted in the same reaction pattern (data not shown). Other issue to be discussed is the insufficiency of information what resistance genes are present in the individual differential lines. Unfortunately, data available for several lines are confusing, e.g. Liu *et al.* (2012) mapped Pl_{16} in sunflower line HA-R4 (used at the position of D7) and Pl_{13} in HA-R5 (D8) whereas French colleagues, especially Gascuel *et al.* (2015), have recently report genes Pl_{15} in HA-R4 (D7) and Pl_1/Pl_{15} in QHP-1, a selection used instead of HA-R5 (D8). Nevertheless, such situation is not unique in plant pathology. Similar ambiguity occurs in race specific interactions where a pathogen's virulence pattern is determined on a set of host lines. Resistance genes and/or quantitative trait loci in sunflower differential lines remain to be reliably characterised by genetic methods in the future.

In the Czech Republic the occurrence of *P. halstedii* is rather limited (Sedlářová *et al.* 2013) and the incidence of the quarantine disease varies greatly depending on the weather. Detailed research of these isolated populations of *P. halstedii* is still in process but races detected so far have been similar to those reported from central European countries, especially Hungary (compare within Table 1 in Virányi *et al.* 2015). Similar to Hungarian populations of *P. halstedii*, the races 704 and 714 have recently been recorded also in the Czech Republic (Trojanová *et al.* 2013; Bán 2014a,b). To our knowledge, this paper is the first report of *P. halstedii* races 705 and 715 ever in the Czech Republic, Europe, and also worldwide. Outbreaks of *P. halstedii* races bearing new combinations of virulence overcoming resistance genes utilised in sunflower breeding are warning. However, further increasing complexity of *P. halstedii* virulence is very probable as in populations of other downy mildews infecting crops and vegetables which are intensively grown in monocultures.

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Received: 2016–01–08
Accepted after corrections: 2016–04–13
Published online: 2016–05–10

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