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New Highly Aggressive Pathotype 354 of *Plasmopara halstedii* in German Sunflower Fields

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Abstract

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Downy mildew of sunflower is a recurrent threat for crop production and a permanent challenge for resistance breeding. Since 2016, a new pathotype of the oomycete *Plasmopara halstedii* has been found in fields of cutting sunflower in Southern Germany. Infection assays based on sunflower differential lines identified the pathogen as pathotype 354 which has not yet been described anywhere else. The new pathotype readily infected the host lines Ha-304, RHA-265, PM13, 803-1, and Ha-335, whereas RHA-274, PM17, HAR-4, and QHP1 remained uninfected. It is the first isolate of *Plasmopara halstedii* to overcome the Pl-6 resistance of sunflower in Germany.

Keywords: *Helianthus annuus*; oomycetes; sunflower downy mildew; virulence pathotype

Plasmopara halstedii (Farl.) Berl.et de Toni (1888) is known as a highly variable and adaptive oomycete, infecting sunflower in almost all areas of the world. Due to the lack of classifying phenotypic or molecular race markers, the pathogen characterisation has been dependent on infection bioassays with defined host lines baring different resistance genes (Sackston *et al.* 1990). According to the infection results, a triplet code numbering between 100 and 777 is applied to characterise each virulence phenotype (Gulya *et al.* 1991; Tourvieille de Labrouhe *et al.* 2000). This widely accepted system is laborious and required expertise as reviewed recently (Trojanová *et al.* 2017). It has reached its limits as an increasing number of pathotypes have been identified and the extension to a new five digit-code system, though even more laborius, has recently been proposed, recognising the rapid evolution of virulence and the necessity of rigorous, ongoing differentiation (Tourvieille de Labrouhe *et al.* 2012; Gascuel *et al.* 2015).

Since the survey of Gulya (2007), numerous new pathotypes of sunflower downy mildew have been identified in North and South America and Europe, as recently reviewed (Viranyi *et al.* 2015). The intensive survey recorded a total of 45 pathotypes identified world-wide within the period from 2007–2013, almost twice as many as before 2007. Meanwhile, two additional pathotypes, 705 and 715, were identified in the Czech Republic (Sedlářová *et al.* 2016) and the spreading of pathotypes overcoming the Pl-6 resistance gene of sunflower is progressing (Bán *et al.* 2014; Iwebor *et al.* 2016).

The pathotype spectrum of sunflower downy mildew in Germany has also gained diversity, increasing from seven in the year 2000 (Rozynek & Spring 2000) to nine in 2013 (Viranyi *et al.* 2015). However, isolates overcoming the Pl-6 resistance where not known until 2016, when heavy infections were found in a field of cut sunflower planted with a cultivar of unbroken resistance to downy mildew in previous years.

Here we report on the results of infection bioassays with this new isolate which revealed the occurrence of the undescribed pathotype 354.

MATERIAL AND METHODS

Infected leaves of sunflower plants were harvested from a commercial field of cut sunflowers near Tübingen, Baden-Württemberg, in September 2016.

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After careful surface washing with tap water, leaves were kept overnight in a plastic box at 100% humidity and 18°C to induce fresh sporulation. Original leaves from sporulating plants were desiccated and deposited in the herbarium of the University of Hohenheim (collection number HOH 17099). Fresh sporangia were harvested and used for infections as described previously (Rozynek & Spring 2000). Single sporangiophores were selectively used to propagate genetically uniform strains for the subsequent pathotype testing on nine sunflower differential lines: Ha304, Ha265, Ha274, PM13, PM17, Ha803-1, HAR4, QHP1, and Ha335. Infections for the pathotype bioassays were performed with a combined whole seedling inoculation/soil drenching method according to Rozynek and Spring (2000). The inoculum density was adjusted to 10 000 sporangia per ml. Infection monitoring was performed after two weeks of plant cultivation in a climate chamber (80% humidity, 18°C, 14 h light per day) by inducing sporulation through overnight incubation at 100% humidity. Plants were recorded as infected when cotyledons and first true leaves showed sporulation.

Fungicide tests were carried out in leaf disk experiments as described by Rozynek and Spring (2001). Metalaxyl M and dimetomorph were tested in concentrations of 1–10 ppm and 0.1–1 ppm, respectively. Sporulation on leaf disks was monitored at 10 days post-infection (dpi).

Release of zoospores was recorded microscopically (Wilovert S inverse microscope; Hund, Wetzlar, Germany) with fresh sporangia spread on 0.75% water agar after incubating the Petri dishes for 3 h in darkness at 18°C. Nuclei of sporangia were stained with 4',6-diamidino-2-phenylindole (DAPI) and analysed with a Zeiss Axioplan microscope (Zeiss, Göttingen, Germany) coupled to a digital camera (Powershot 650; Canon) and equipped with a blue violet filter combination with UV 395-440 excitation/FT 460/LP 470 barrier for fluorescence observation. Brightness and contrast of images were adjusted using Photoshop CS2 (Adobe).

RESULTS AND DISCUSSION

Field observations. As is typical for cropping of cut sunflower in Germany, sowing is done ca. every 2 weeks starting in early May. When visiting the field in August, scattered infections were observed in some dwarfed plants remaining from the first two sowings, from which the majority of putatively healthy plants had been harvested. The systemic infection in these early sown plants suggested that primary infection was soil-borne, most likely from oospores of unrecognised infections in the previous season. In later sowings, the ratio of infected plants gradually increased to about 80% in the sixth planting. Infections ranged from systemic to late systemic (lower plant parts showing no symptoms) and local, indicating intensive secondary infections through sporangia from older neighbouring plants during phases of cool and humid weather conditions. Transitions from local leaf lesions to systemic infections of upper plant parts, as described previously (Spring 2009), could frequently be observed.

Pathotyping. Infection tests were carried out with sunflower differential lines of the three common sets (Table 1). The pathogen originally harvested from the field succeeded to infect Ha304 and Ha265 of set 1, PM13 and Ha803-1 of set 2 as well as Ha335 of set 3, hence it was classified as virulence type 354. Repetitions with strains generated from Ha335 infections and from single sporangiophore propagation resulted in identically uniform infection patterns, thus confirming that the isolate was not a mixture of different pathotypes. Pathotype 354, to the best of our knowledge, has not been described in any previous investigations.

Table 1. Sunflower differential lines used in pathotype bioassay (information on resistance genes (Pl) as reported by Gascuel *et al.* 2015)

Ha304	Ha265	Ha274	PM13	PM17	Ha803-1	HAR4	QHP1	Ha335
No Pl gene	Pl_1	$Pl_{2/21}$	Pl_{PMI13}	Pl_5	Pl_{5+}	Pl_{15}	$Pl_{1/15}$	Pl_6
S	S	R	S	R	S	R	R	S
	Set 1			Set 2			Set 3	
Pathotype	**3**			5			4	

Pathotype numbering follows the triplet code system in the three test sets (set 1–3) counting susceptibility in first place with 1, second with 2, and third with 3; S – susceptible; R – resistant

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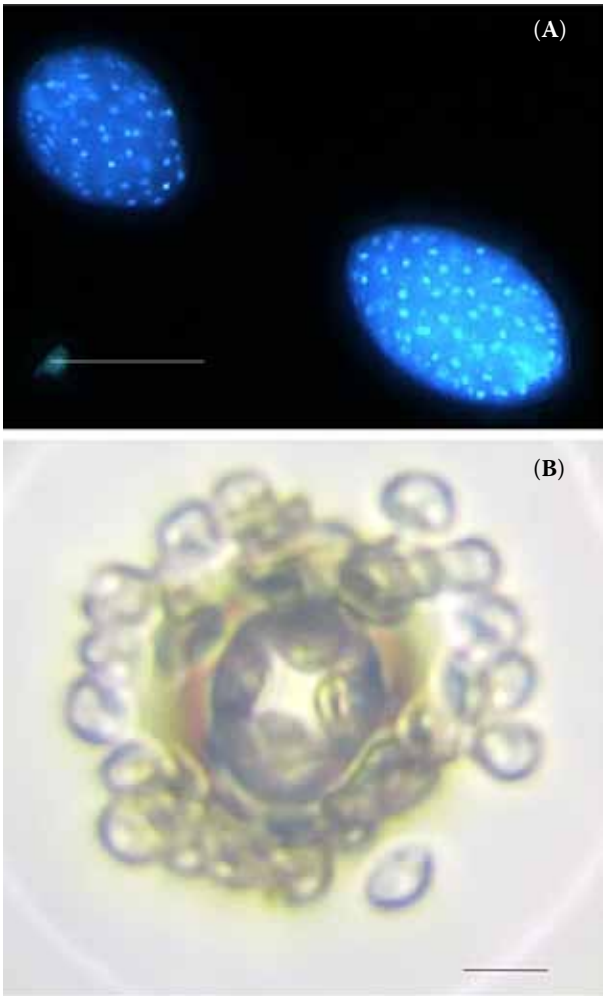

Figure 1. Nuclei in sporangia of *Plasmopara halstedii* pathotype 354 and release of zoospores: (**A**) DAPI stained sporangia showing high numbers of fluorescent nuclei, bar equals 50 µm and (**B**) release of zoospores from a single sporangium, bar equals 10 µm

The new pathotype overcomes the resistance mechanisms contributed by the Pl genes Pl_1, Pl_{PM13}, Pl_{5+}, and Pl_6, but is controlled by $Pl_{2/21}$, Pl_5, and $Pl_{1/15}$. It is the first time that an isolate breaking the Pl-6 resistance has been reported to occur in Germany. In contrast, pathogens infecting Ha335 have been found in French (304, 307, 314, 334, 704, 707, 714, 717, 774; reviewed by Viranyi *et al.* 2015), Czechian (705, 715; Sedlářová *et al.* 2016), Hungarian (704, 714; Bán *et al.* 2014), and Russian (334; Iwebor *et al.* 2016) sunflower fields for several years. It is noteworthy that our isolate of pathotype 354 was extremely aggressive and that many plants died from damping off within the first two weeks post inoculation. This was particularly observed with Ha335, where significantly more seedlings were killed than in the nonresistant Ha 304.

An explanation for the high infection pressure could be the unusually high number of zoospores produced per sporangium. Staining of mature sporangia with DAPI revealed an unexpectedly high number of nuclei (Figure 1A). When sporangia were placed on water agar, many of them released more than 40 zoospores (Figure 1B). This is about twice as much as was observed from other isolates in our previous studies (Spring *et al.* 1998). A detailed comparison of sporangial characteristics between *P. halstedii* pathotypes is planned.

Fungicide sensitivity of the new *P. halstedii* isolate was tested against the systemic compound metalaxyl M (1–10 ppm) and the local systemic dimetomorph (0.1–1 ppm). In both cases the lower concentration was sufficient to inhibit sporulation completely, whereas leaf disks in the water control had sporulated 10 dpi. Previous tests reported that the normal range of sensitivity in *P. halstedii* for metalaxyl starts at 0.02 ppm of active ingredient and resistant strains tolerate 10–100 ppm (Rozynek & Spring 2001). This indicates that the isolate of pathotype 354 could be controlled chemically (e.g. by seed coating) in the field as long as no resistant sunflower host is available.

CONCLUSIONS

Comparison of the pathotype situation in Europe and America between the periods before and after 2007 have shown an ongoing increase in the diversity of *P. halstedii* (Viranyi *et al.* 2015). Although this may be partially due to punctually intensified monitoring, it certainly also reflects the genetic plasticity of the pathogen (Viranyi & Spring 2011; Gascuel *et al.* 2015). This is uncommon for a pathogen with homothallic sexual reproduction (Spring 2000) and contrasts the situation found in other downy mildews such as *Peronospora tabacina* where the European and American populations revealed very low genetic variability (Trigiano *et al.* 2012). Parasexual genetic recombination in *P. halstedii* has recently been suggested as an additional option to overcome genetic uniformity and to enforce adaptation to infection barriers (Spring & Zipper 2006, 2017). A comparison of the genetic background of the new patothype with respect to effector genes, numerously identified in the *P. halstedii* genome (Sharma *et al.* 2015) and recently investigated as possible markers for future pathotyping (Gascuel *et al.* 2016) could provide useful insights in this regard.

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