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Pathogen profile

The sunflower downy mildew pathogen *Plasmopara halstedii*

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SUMMARY

Downy mildew of sunflower is caused by *Plasmopara halstedii* (Farlow) Berlese & de Toni. *Plasmopara halstedii* is an obligate biotrophic oomycete pathogen that attacks annual *Helianthus* species and cultivated sunflower, *Helianthus annuus*. Depending on the sunflower developmental stage at which infection occurs, the characteristic symptoms range from young seedling death, plant dwarfing, leaf bleaching and sporulation to the production of infertile flowers. Downy mildew attacks can have a great economic impact on sunflower crops, and several *Pl* resistance genes are present in cultivars to protect them against the disease. Nevertheless, some of these resistances have been overcome by the occurrence of novel isolates of the pathogen showing increased virulence. A better characterization of *P. halstedii* infection and dissemination mechanisms, and the identification of the molecular basis of the interaction with sunflower, is a prerequisite to efficiently fight this pathogen. This review summarizes what is currently known about *P. halstedii*, provides new insights into its infection cycle on resistant and susceptible sunflower lines using scanning electron and light microscopy imaging, and sheds light on the pathogenicity factors of *P. halstedii* obtained from recent molecular data.

Taxonomy: Kingdom Stramenopila; Phylum Oomycota; Class Oomycetes; Order Peronosporales; Family Peronosporaceae; Genus *Plasmopara*; Species *Plasmopara halstedii*.

Disease symptoms: Sunflower seedling damping off, dwarfing of the plant, bleaching of leaves, starting from veins, and visible white sporulation, initially on the lower side of cotyledons and leaves. *Plasmopara halstedii* infection may severely impact sunflower seed yield.

Infection process: In spring, germination of overwintered sexual oospores leads to sunflower root infection. Intercellular hyphae are responsible for systemic plant colonization and the induction of disease symptoms. Under humid and fresh conditions, dissemination structures are produced by the pathogen on all plant organs to release asexual zoosporangia. These zoosporangia play an important role in pathogen dissemination, as they release motile zoospores that are responsible for leaf infections on neighbouring plants.

Disease control: Disease control is obtained by both chemical seed treatment (mefenoxam) and the deployment of dominant major resistance genes, denoted *Pl*. However, the pathogen has developed fungicide resistance and has overcome some plant resistance genes. Research for more sustainable strategies based on the identification of the molecular basis of the interaction are in progress.

Useful websites: http://www.heliagene.org/HP, http://lipm-helianthus.toulouse.inra.fr/dokuwiki/doku.php?id=start, https://www.heliagene.org/PlasmoparaSpecies (soon available).

Keywords: effectors, *Helianthus annuus*, infection modes, life cycle, obligate biotroph oomycete, pathogen virulence, *Plasmopara halstedii*.

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INTRODUCTION

Downy mildew (caused by *Plasmopara halstedii*) is one of the major diseases affecting sunflower (*Helianthus annuus*) production. The pathogen has been reported in most countries in which sunflowers are grown. The global impact on yield has been estimated recently to be 3.5% of commercial seed production in the presence of the control methods currently deployed, but loss of yield can be up to 100% in contaminated fields (Centre Technique Interprofessionnel des Oléagineux Métropolitains, http://www.cetiom.fr). In addition, sunflower cultivation has to be abandoned in fields too heavily contaminated by *P. halstedii*.

Sunflower, the fourth most widely grown oil crop in the world after oil palm, soybean and rapeseed, but second in the European Union, produces healthy oil rich in unsaturated fatty acids, and is regarded as a plant able to grow under low water input regimes, and with a limited addition of soil fertilizers and fungicides, in contrast with other oil crops. Total sunflower production has increased worldwide by 32% over the past 20 years, reaching 38 million tons in 2012, with an acreage of 23 million hectares (Food and Agriculture Organization).

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The pathogen *P. halstedii*, first identified in North America by Mr Byron D. Halsted, a botanist at the Bussey Institution (Harvard University) (Nishimura, 1922; Young and Morris, 1927), was later reported in Russia and Western Europe around 1960, where it was introduced probably via infected sunflower seeds (Ioos *et al*., 2007). Since 1992, this pathogen has been submitted to quarantine regulation in the European Union. Thirty-six pathotypes of *P. halstedii*, often known as races, have so far been identified worldwide, mostly in zones in which sunflowers are widely grown: in North America, and in Europe, especially France, Spain, Germany, Ukraine and Russia. *Plasmopara halstedii* pathotypes are defined by an international nomenclature system based on differential virulence profiles on a set of sunflower inbred lines (Gulya *et al*., 1998, Tourvieille de Labrouhe *et al*., 2012).

During the last 20 years, new *P. halstedii* pathotypes showing increased virulence have appeared (Ahmed *et al*., 2012), and several *Pl* resistance genes used in sunflower hybrids have become inefficient: for example, the *Pl2* gene conferring resistance to the most frequent pathotype has been overcome by different emerging pathotypes (Moinard *et al*., 2006; Tourvieille de Labrouhe *et al*., 2000). An understanding of pathotype evolution and sunflower resistance is therefore an agronomic issue.

Nevertheless, despite the economic importance of this crop pathogen, genomic resources for *P. halstedii* and, more generally, for pathogens of the genus *Plasmopara*, are limited. The aims of this review are to summarize our knowledge on the sunflower downy mildew pathogen, with a focus on infection and dissemination mechanisms, and to shed light on *P. halstedii* pathogenicity factors from recent molecular data.

PLASMOPARA HALSTEDII TAXONOMY AND REPRODUCTION

Plasmopara halstedii is a biotrophic oomycete belonging to the Peronosporales, the largest and most devastating group of plant-pathogenic oomycetes, which also includes the hemibiotrophs of the genus *Phytophthora*, causing late blight diseases, and an important group of obligate biotrophs causing downy mildews, such as *Plasmopara viticola* on grapevine and *Bremia lactucae* on lettuce (Gessler *et al*., 2011; Michelmore and Wong, 2008; Thines and Kamoun, 2010). Eight hundred species responsible for downy mildews are grouped into 14 genera subdivided into four morphological and ecological subgroups. In subgroup II are included the genera *Basidiophora*, *Benua*, *Bremia*, *Paraperonospora*, *Plasmopara*, *Plasmoverna* and *Protobremia*, which produce vesicular to pyriform haustoria (Göker *et al*., 2007). Unfortunately, *P. halstedii* was not among the eight *Plasmopara* species included in this study. All downy mildews are obligate biotrophic parasites that require a living plant host to accomplish their life cycle. Most, including *P. halstedii,* have not been successfully cultured *in vitro*.

One hundred and forty-six different *Plasmopara* species are listed in *Index Fungorum*. Few are phylogenetically related on the basis of large nuclear subunit ribosomal DNA sequences and other genes (Göker *et al*., 2007; Riethmüller *et al*., 2002). *Plasmopara* species cause downy mildew disease on various plants: *P. viticola* on grapevine, *P. umbelliferarum* on Umbelliferae, *P. geranii* and *P. pusilla* on geranium and *P. obducens* on impatiens. Seven species pathogenic on Ranunculaceae, originally referred to as *P. pygmaea*, but differing by morphological and molecular traits from all the other *Plasmopara* species, have been placed in the genus *Plasmoverna* (Constantinescu *et al*., 2005). The origins of the species *P. halstedii* remain complex and mostly undetermined (cf. for review, Viranyi and Spring, 2011).

Plasmopara halstedii is a specialized pathogen which attacks several Asteraceae, including annual *Helianthus* species, e.g. *H. argophyllus*, *H. debilis* and *H. petiolaris*, and wild and cultivated forms of *H. annuus*, with strong impacts on sunflower yield. *Plasmopara halstedii* has been reported to infect other Asteraceae (*Bidens*, *Artemisia*, *Xanthium*), which could be potential reservoirs of inoculum.

In oomycetes, two types of sexual reproduction have been described: (i) homothallism, in which sexual reproductive cells (oogonia and antheridia) are produced by the same organism, which could lead to outcrossing or selfing in the absence of self-incompatibility mechanisms; and (ii) heterothallism, in which sexual reproductive cells are produced by two different organisms, leading only to outcrossing. *Plasmopara halstedii* is diploid, homothallic and has been shown to reproduce sexually and asexually in laboratory conditions (Spring, 2000; Spring and Zipper, 2006). Homothallic sexual reproduction has been shown to occur after the inoculation of sunflower seedlings with single zoospores (Spring, 2000; Spring *et al*., 1998; Spring and Zipper, 2000). This is important for epidemiology as a single zoospore in a field can lead to the contamination of soil with oospores, the survival forms of the inoculum. The sexual phase is thus required to produce overwintering oospores, but asexual generations occurring during the sunflower growing season from spring to the end of summer are also important in disease propagation.

Genotypic analysis provided by Ahmed *et al*. (2012) suggested that reproduction is predominantly by selfing in wild *P. halstedii* French populations, but this did not prevent the emergence of new virulence (Intelmann and Spring, 2002). Reduced genetic variation and increased linkage disequilibrium have been shown to occur within French *P. halstedii* populations compared with mostly heterothallic *Phytophthora infestans* and *P. viticola* (Delmotte *et al*., 2006; Delmotte *et al*., 2008; Grünwald and Flier, 2005). These results confirmed the hypothesis that *P. halstedii* wild populations are homothallic, and suggested the absence of a self-incompatibility mechanism, which thus allows selfing and outcrossing mating systems (Ahmed *et al*., 2012). Spring and Zipper (2006) also provided evidence for genetic recombination through parasexual events.

PLASMOPARA HALSTEDII PATHOTYPES AND SUNFLOWER RESISTANCE GENES

Plasmopara halstedii populations have been thoroughly characterized and classified into pathotypes mainly in France (Ahmed *et al*., 2012) and Germany (Rozynek and Spring, 2000). In France, 17 reference pathotypes have been defined according to their virulence profiles when tested for the infection of differential hosts carrying different *Pl* resistance genes (Fig. 1; Gulya *et al*., 1998; Tourvieille de Labrouhe *et al*., 2012). Fourteen of these pathotypes (defined by triplet name, Fig. 1) have been classified into three distinct clusters, according to 12 single nucleotide polymorphism (SNP) markers designed on *P. halstedii* expressed sequence tags (ESTs): cluster 1: 100, 300, 304; cluster 2: 307, 700, 703, 707, 730; and cluster 3: 314, 334, 704, 710, 714, 717 (Ahmed *et al*., 2012; Delmotte *et al*., 2008). These clusters could have resulted from three independent *P. halstedii* introductions into France, and intercluster recombinations could have facilitated the emergence of new pathotypes in response to host resistance (Ahmed *et al*., 2012).

Two pathotypes showing new virulence profiles (304 30 and 774 73) have been recorded more recently and can be differentiated by the addition of six further sunflower lines (Fig. 1a, dark grey rows), extending the pathotype nomenclature to a five-digit code (Tourvieille de Labrouhe *et al*., 2012) (Fig. 1). The addition of these six differential hosts was also a prospective approach aimed to improve the distinction of emerging pathotypes. Assuming a strong correlation between the two codes for a given pathotype, the triplet code is used in the rest of this article.

To avoid downy mildew attacks, modern sunflower cultivars carry one or more dominant resistance genes, denoted *Pl*. *Pl* genes originated mostly from wild *H. annuus*, but also from other *Helianthus* species (*H. argophyllus* and *H. tuberosus*) (Vear *et al*., 2008a). More than 20 *Pl* genes (*Pl1*, *Pl2*, *Pl5–Pl8*, *Pl13–Pl16*, *Pl21*, Pl_{Arg}; Fig. 1), conferring resistance to one or more pathotypes of *P. halstedii,* have been described, and 10 have been mapped in sunflower (Fig. 1b). *Pl* genes are grouped into at least four complex genomic regions on three linkage groups (LGs). Toll/interleukin-1 receptor-nucleotide-binding site-leucine-rich repeat (TIR-NBS-LRR) and coiled-coil (CC)-NBS-LRR resistance gene analogues (RGAs) have been localized genetically to these regions (Fig. 1; Bouzidi *et al*., 2002; Gentzbittel *et al*., 1998; Radwan *et al*., 2003, 2004, 2008), but no *Pl* gene has yet been cloned. Quantitative resistance to pathotypes 710 and 703 of *P. halstedii* has been described in the sunflower line XRQ (Tourvieille de Labrouhe *et al*., 2008) and is conferred by three quantitative trait loci (QTLs) (Vear *et al*., 2008b; Vincourt *et al*., 2012). These QTLs have been mapped on LG10, LG8 and LG7 (Vincourt *et al*., 2012). The major QTL, QRM1 (Fig. 1), explaining 65% of the variability, was mapped on LG10 in a genetic region of 0.8 cM (Vincourt *et al*., 2012). A map-based cloning approach based on recombinant lines segregating only for QRM1 is underway in our group (As-Sadi, 2011; S. Muños, LIPM, Castanet-Tolosan, France, personal communication).

MILDEW SYMPTOMS CAUSED BY *P. HALSTEDII* ON SUNFLOWER

Contrary to the related *P. viticola*, which attacks only green parts and fruits of grapevine (Musetti *et al*., 2005), field infections of *P. halstedii* in sunflower usually result from root attacks by zoospores released at the beginning of spring from zoosporangia derived from oospores (primary infections, Fig. 3). Root infection leads to either seedling damping off, or to severe symptoms, in particular plant dwarfing (Fig. 2a), leaf discoloration (Fig. 2b) and sporulation (Fig. 2f,g), ultimately resulting in important yield losses caused by the production of infertile flowers (Sackston, 1981). Dwarfing of sunflower plants is currently not explained, although it might be the result of hormonal manipulation or nutrient uptake by *P. halstedii*.

During sunflower growth, under humid and cool conditions, zoosporangia produced mainly on the surfaces of leaves and cotyledons are responsible for the contamination of the shoots of neighbouring plants, where they release zoospores (secondary infections, Fig. 3). Symptoms induced by secondary infections are often less severe than those induced by primary infections (Gulya *et al*., 1998). In contrast, in grapevine, yield losses by *P. viticola* are mostly attributed to leaf infections (Schruft and Kassemeyer, 2000).

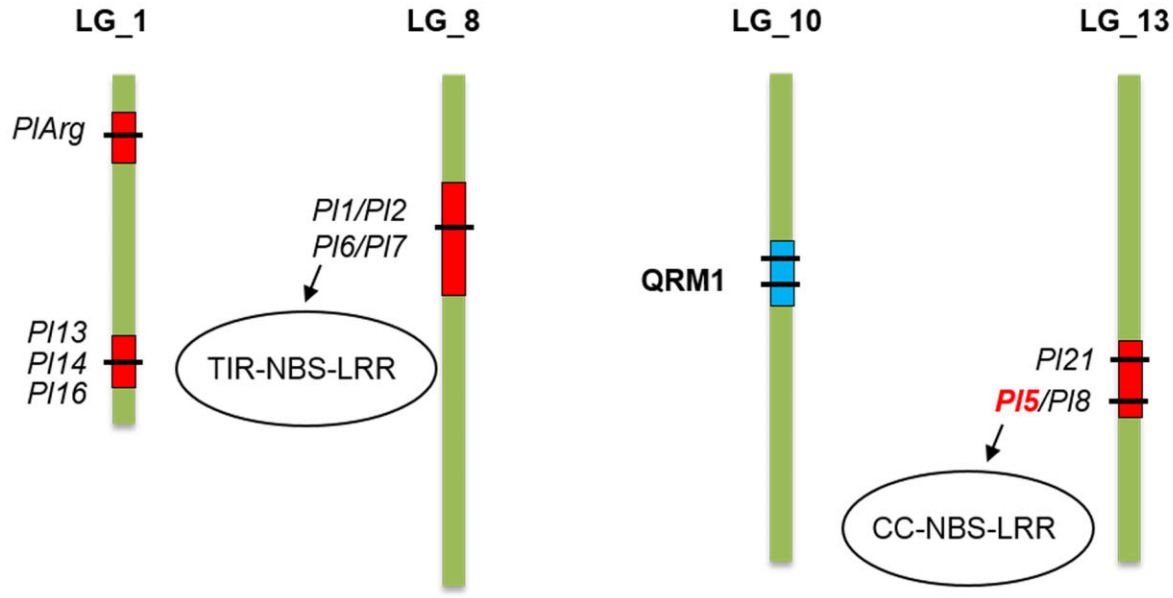
Two types of resistance to downy mildew conferred by *Pl* genes have been described in sunflower: type I resistance is characterized by the absence of symptoms on shoots and the absence of the pathogen above the base of hypocotyls; type II resistance is characterized by weak sporulation symptoms restricted to cotyledons, and the absence of symptoms in the upper part of the plant with the pathogen never reaching the true leaves (Mouzeyar *et al*., 1994). Resistant sunflower genotypes present either type I or type II resistance depending on their *Pl* resistance genes (for example, the *Pl5* allele from the inbred line XRQ confers type II resistance, whereas *Pl6* confers type I resistance). These resistances have been used to protect crops against *P. halstedii* pathotypes, but, except for Pl_{Arg} (Fig. 1a), they have all been overcome during the last 15 years. For example, the *Pl2* and *Pl6* genes have been overcome by eight pathotypes (Delmotte *et al*., 2008; Moinard *et al*., 2006).

To study *P. halstedii* resistance in laboratory conditions, we infected sunflower seedlings and grew them in hydroponic cultures (Fig. 2c), such that they showed the same symptoms as plants in the field. In our experiments, we compared a pair of downy mildew-susceptible and downy mildew-resistant near-isogenic sunflower lines (NILs), called TS and TSRM, respectively. The inbred line TS has no identified *Pl* gene and is susceptible to all *P. halstedii* pathotypes. TSRM was obtained by backcrossing of the *Pl*5 gene from the inbred line XRQ into TS (six back-crosses and two selfings

a

Nomenclature		Sunflower differential line			17 *P. halstedii* races														
Digit	Virulence value	Number	Sunflower line	Known *PI* R genes	100 10	300 10	304 10	304 30	307 51	314 30	330 42	334 53	700 60	703 60	704 71	707 71	710 60	714 71	717 71	730 60	774 73
1st	1	D1	Ha-304	No *PI* gene	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
	2	D2	RHA-265	*PI1*	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
	4	D3	RHA-274	*PI2/PI21*	R	R	R	R	R	R	R	R	S	S	S	S	S	S	S	S	S
2nd	1	D4	PMI3	PI_{PMI3}	R	R	R	R	R	S	S	S	R	R	R	R	S	S	S	S	S
	2	D5	PM17	*PI5*	R	R	R	R	R	R	S	S	R	R	R	R	R	R	R	S	S
	4	D6	803-1	*PI5* +	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S
3rd	1	D7	HAR-4	PI_{15}	R	R	R	R	S	R	R	R	R	S	R	S	R	R	S	R	R
	2	D8	QHP1	$PI1/PI_{15}$	R	R	R	R	S	R	R	R	R	S	R	S	R	R	S	R	R
	4	D9	Ha-335	*PI6*	R	R	S	S	S	S	R	S	R	R	S	S	R	S	S	R	S
4th	1	D10	Y7Q	*PI6* –	S	S	S	S	S	S	R	S	R	R	S	S	R	S	S	R	S
	2	D11	PSC8	*PI2*	R	R	R	S	R	S	R	R	S	S	S	S	S	S	S	S	S
	4	D12	XA	*PI4*	R	R	R	R	S	R	S	S	S	S	S	S	S	S	S	S	S
5th	1	D13	PSS2RM	*PI6/PI21*	R	R	R	R	S	R	R	S	R	R	S	S	R	S	S	R	S
	2	D14	VAQ	*PI5*	R	R	R	R	R	R	S	S	R	R	R	R	R	R	R	R	S
	4	D15	RHA419	PI_{Arg}	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R

b



to obtain homozygous *PI*5 resistance in a TS genetic background). TSRM presents type II resistance to *P. halstedii* pathotypes 100, 304, 703 and 710, as shown for *PI*5 in other genetic backgrounds.

Two weeks after infection of TS and TSRM by pathotype 710, infected TS plants showed dwarfing (Fig. 2d), leaf discoloration (Fig. 2g) and sporulation on cotyledons and leaves (Fig. 2f,g). In contrast, infected TSRM plants grew as well as non-infected plants (Fig. 2e), showed no leaf discoloration and only rare sporulation on cotyledons, but never on leaves, as described previously for type II resistance (Mouzeyar *et al*., 1993, 1994). In addition, hydroponic culture revealed root phenotypes (Fig. 2d,e). In infected

Fig. 1 Nomenclature of *Plasmopara halstedii* pathotypes and genetic map of sunflower resistance genes. (a) *Plasmopara halstedii* pathotypes were defined according to an international nomenclature based on the virulence profile of a given isolate on 15 sunflower differential lines (D1–D15) selected according to their resistance patterns (Gulya *et al*., 1998; Tourvieille de Labrouhe *et al*., 2012). Susceptibility (S) and resistance (R) were defined by the presence or absence of disease symptoms and sporulation on leaves 2–3 weeks after inoculation of sunflower root seedlings, grown in soil (Mouzeyar *et al*., 1993). A triplet coding system was initially set up on nine sunflower lines (D1–D9, Gulya *et al*., 1998), but the occurrence of new *P. halstedii* pathotypes led to the addition of six other sunflower lines (in dark grey) and to a five-digit coding system (D10–D15, Tourvieille de Labrouhe *et al*., 2012). The phenotyping results on each triplet of sunflower differential lines give the pathotype digit values. If the first differential line of a set of three is susceptible, a value of '1' is assigned to the pathotype. If the second line is S, a value of '2', and, for the third line, a value of '4'. When the line is resistant, a value of '0' is assigned to the pathotype. The virulence code is additive within each set. For example, virulence code 710 is explained by '7' (S for D1–D3, 1 + 2 + 4 = 7), '1' (S for D4) and '0' (R for D7–D9). Dominant genes giving resistance to *P. halstedii* are called *Pl* genes. The known functional R genes are indicated in the sunflower differential lines. '+' or '–' after the *Pl* gene indicates the strength of resistance alleles. (b) Eleven sunflower *Pl* genes have been mapped on three of 17 sunflower linkage groups (LGs) (Bachlava *et al*., 2011; Bouzidi *et al*., 2002; Franchel *et al*., 2013; Liu *et al*., 2012; Mulpuri *et al*., 2009; Radwan *et al*., 2003; Vincourt *et al*., 2012; Wieckhorst *et al*., 2010). *Pl1*/*Pl2*/*Pl6* and *Pl7* are localized in a Toll/interleukin-1 receptor-nucleotide-binding site-leucine-rich repeat (TIR-NBS-LRR)-rich region. *Pl5*/*Pl8* and *Pl21* are localized in a coiled-coil (CC)-NBS-LRR-rich region. QRM1, the major quantitative trait locus (QTL), conferring a quantitative resistance to pathotype 710 of *P. halstedii*, was mapped on LG10 (Vear *et al*., 2008b; Vincourt *et al*., 2012).

Fig. 2 Disease symptoms caused by *Plasmopara halstedii* on sunflower in field (a, b) and hydroponic (c–g) conditions. Plant dwarfing (a, arrow) and leaf discoloration (b) of sunflower plants. (c–g) Two-day-old sunflower seedlings were root inoculated by *P. halstedii*, pathotype 710 (Mouzeyar *et al*., 1993) and grown in oxygenated liquid growth medium under hydroponic conditions, shown in (c). A saturated atmosphere, obtained by placing for 2 days plastic covers over the trays, 12 days after inoculation, induced sporulation on cotyledons of the susceptible line TS (c, f, g), but not on the resistant near-isogenic line TSRM (c), as for inoculation tests performed in soil (Mouzeyar *et al*., 1993). Dwarfing symptoms are visible on infected (inf.) TS plants, but not on TSRM (c), compared with uninfected (ctrl) TS and TSRM plants (d, e). Infection of TSRM did not affect shoot growth compared with non infected TSRM plants (e). (d, e) Note the brownish coloration on infected roots of both lines. (f, g) Typical 'downy' appearance on abaxial and adaxial cotyledon surfaces caused by sporulation on TS. (g) Leaf discoloration on 14-day-old plants. (d, e) Scale bars: 5 cm.

plants of both TS and TSRM, roots turned brown and showed reduced growth, especially in the case of TS, compared with uninfected roots (Fig. 2d,e). It is not clear whether the brown root phenotype could be attributed to a general plant defence reaction to *P. halstedii* infection or to disease symptoms of roots. In TSRM plants, the root phenotype was less marked compared with TS plants, and probably not sufficiently strong to impact TSRM shoot development (Fig. 2d,e).

LIFE CYCLE OF *P. HALSTEDII*

Two modes of spore germination preceding plant infection have been described for obligate biotroph oomycetes: (i) indirect germination occurring by the release of motile zoospores from mature sporangia, their encystment on the plant surface and spore germination; (ii) direct germination: production of a germ tube by the sporangium itself. Although direct germination is the only

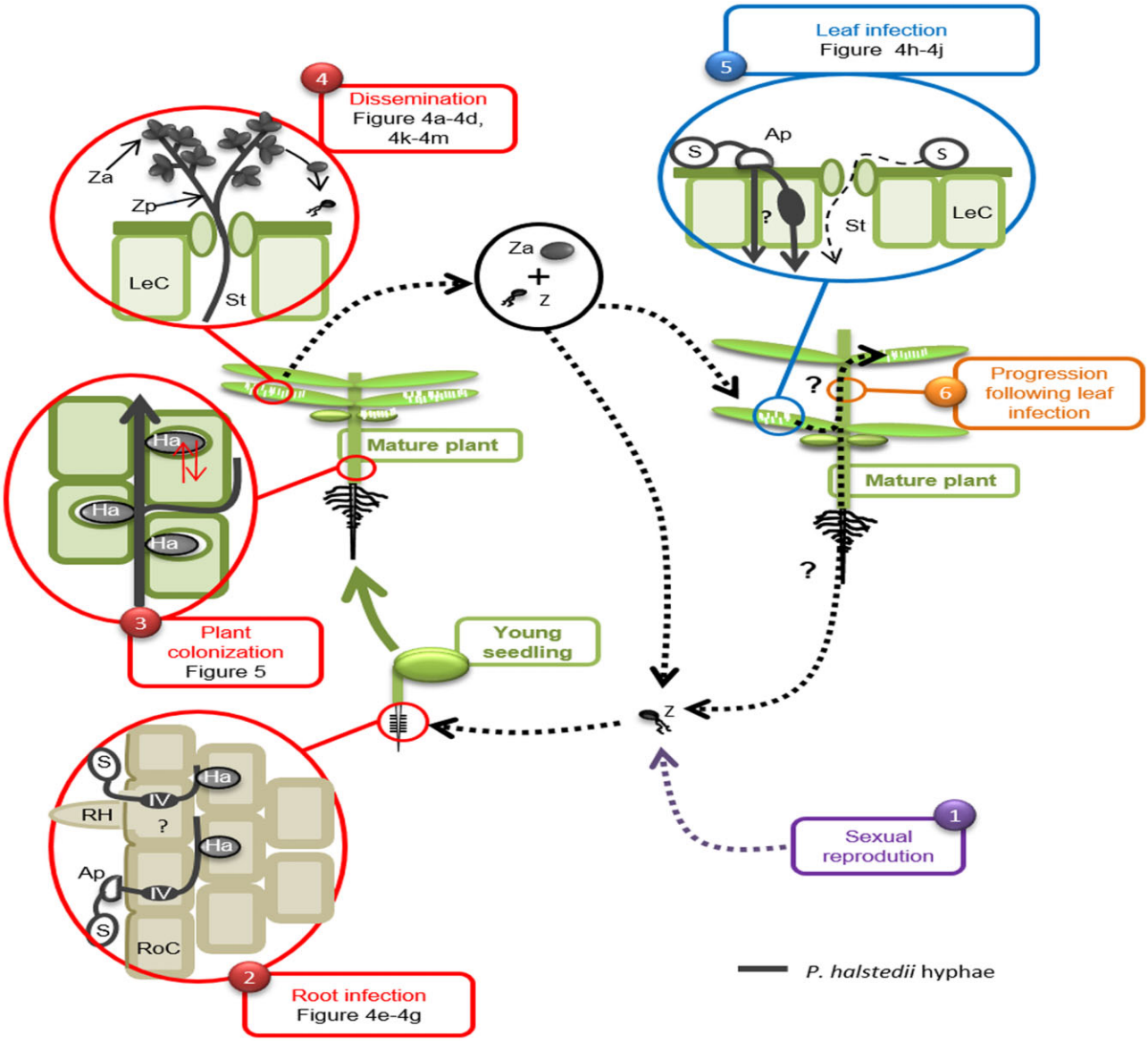


Fig. 3 Life cycle of *Plasmopara halstedii*. (1) In spring, biflagellate zoospores (Z) originating from zoosporangia produced by overwintering sexual oospores are released in soil free water. Spore (S) encystment occurs in contact with a sunflower root. (2) Zoospores germinate in a few hours and enter the root by two methods: (i) by direct penetration into a root cell (RoC) with or without the formation of an appressorium (Ap), a bulge in which the pathogen increases osmotic pressure and drills root epidermal cells; and (ii) through injuries frequently present at the base of root hairs (RH). The formation of infection vesicles (IV) then occurs in plant epidermal cells infected with appressoria (Wehtje *et al*., 1979). (3) Pathogen hyphae (grey thick line) progress further through the intercellular spaces between cortical root cells to reach and colonize shoot tissues. At this stage of infection, *P. halstedii* establishes a large number of infection/nutrition structures, named haustoria (Ha). (4) Under high humidity conditions and moderate temperature (late spring and summer), *P. halstedii* will emerge first on the abaxial surface of leaves and cotyledons via stomata (St), close to a leaf cell (LeC). The branched structures that are produced are dissemination structures, named zoosporangiophores (Zp), carrying zoosporangia (Za). Zoosporangia can be disseminated and contaminate the leaves of other plants. They release up to 20 zoospores (Z). (5) Spores grouped around leaf trichomes or near veins encyst and germinate in a few hours to penetrate leaf tissues at intercellular junctions by forming appressoria (thick gray line). Rare events of penetration via stomata are also observed (thin dotted line). (6) Pathogen progression in plant tissue following leaf infection remains poorly characterized.

method of germination for the genera *Hyaloperonospora* and *Peronospora*, which never produce mobile zoospores, the genus *Plasmopara* mainly uses the indirect germination of zoospores (Grenville-Briggs and Van West, 2005).

Root infection

Although *P. halstedii* can be dispersed by wind, water and via contaminated seeds (see section on Pathogen sporulation and

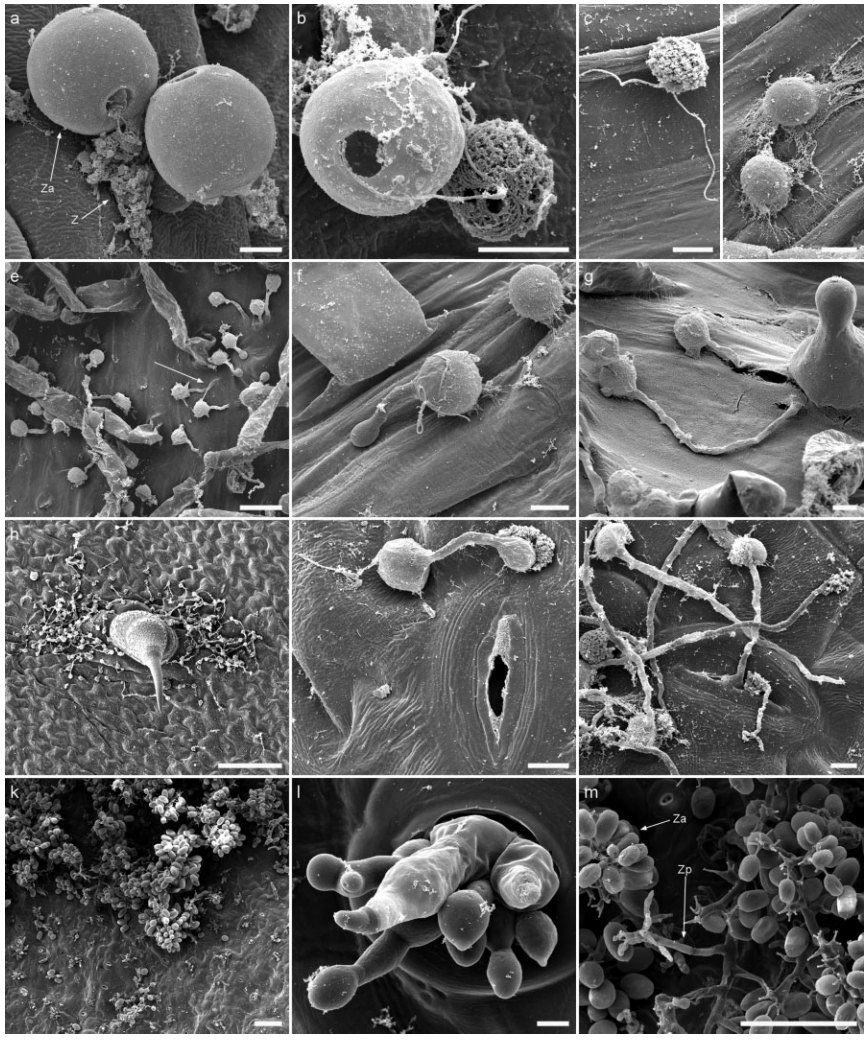


Fig. 4 Scanning electron microscopy (SEM) images illustrating key steps of the *Plasmopara halstedii* life cycle. (a–d) Release and encystment of *P. halstedii* zoospores from zoosporangia. (a) Zoospores (Z) released from zoosporangia (Za) opercula. (b) A swollen zoospore on the right of a zoosporangium shows a lumpy surface and uses two flagella to move towards plant tissues (c). (d) Encysted spores lose their flagella and show a smooth surface. Scale bars: (a–d) 5 μm. Germination of spores into sunflower roots (e–g) and leaves (h–j). (e) Germinating spores in the root hair emerging zone. White arrow indicates penetration of a germ tube directly through the epidermis without appressorium formation. (f) Spore with a germ tube forming an appressorium. (g) Spore penetrating at the base of a root hair without a visible appressorium. (h) Spores germinating on leaves, grouped around a trichome. (i) Spore germination with appressorium formation near a stomata. (j) Rare entry of *P. halstedii* in leaves through stomata. Scale bars: 20 μm (e); 5 μm (f, g, i, j); 100 μm (h). (k–m) Dissemination structures of *P. halstedii*. (k) Sporulation of *P. halstedii* on the lower face of a cotyledon. (l) Enlargement of a zone from (k) showing zoosporangiophores exiting the plant through stomata. (m) Zoosporangia (Za) carried by a zoosporangiophore (Zp). Scale bars: 100 μm (k, m), 5 μm (l).

dissemination), it is mostly a soil-borne pathogen (Ioos *et al*., 2007). In the field, root infections of young sunflower plants are responsible for the most severe symptoms and have a strong impact on yield. Infections have been shown to have a maximum efficiency during the first 5 days after seed germination, but can still occur until sunflower plants reach the four-leaf stage (2–3 weeks; Zimmer, 1971, 1975). *Plasmopara halstedii* zoospores released either from oospore sporangia (in the spring) or from asexual sporangia (from spring to summer) (Fig. 4a) can infect young sunflower plants (Cohen and Sackston, 1973; Nishimura, 1922; Spring and Zipper, 2000).

To decipher the different strategies deployed by the pathogen to colonize sunflower roots, scanning electronic microscopy (SEM) was used to follow root infection on TS and TSRM plants inoculated by pathotype 710 and grown in hydroponic conditions. *Plasmopara halstedii* zoosporangia release up to 20 mobile zoospores each (Fig. 4a,b). These mobile zoospores of *P. halstedii* possess two flagella (Fig. 4b,c) used to swim in soil free water and to reach epidermal root cells. In our infection conditions, all germinated zoospores were randomly dispersed on epidermal cells without apparent chemoattraction or electrotaxis, as reported for other root oomycete pathogens, such as *Phytophthora sojae* and *Phytophthora palmivora* (Morris *et al*., 1998; Tyler *et al*., 1996), and for *P. halstedii* by Delanoë (1972), who described zoospore attraction to sunflower root surfaces by unidentified chemical compounds secreted by the plant.

Contact with the root surface induced *P. halstedii* zoospore encystment involving ultrastructural changes, including the loss of flagella and the appearance of a smooth surface (Fig. 4d). Other changes, not checked in our experiments, including the synthesis of a cell wall and deposition on the spore surface of an amorphous electron-dense material, were described by Gray *et al*. (1985) in *P. halstedii* as in other oomycetes. Twenty-four hours after infection, the majority of spores were encysted and then germinated in the root hair emerging zone (Fig. 4e). *Plasmopara halstedii* germ tubes can enter root tissues by three different ways: mostly by the formation of an appressorium structure (Fig. 4f), but also through injuries, especially at the

bases of root hairs (Fig. 4g), or by direct penetration without an appressorium through the wall of epidermal cells (Fig. 4e). These three penetration modes have been described previously for pathotypes other than 710 (Nishimura, 1922; Wehtje *et al*., 1979; Wehtje and Zimmer, 1978). Furthermore, it has been shown that the penetration of *P. halstedii* pathotype 300 via an appressorium leads to the formation of an infection vesicle in the root epidermal cell, suggesting a successful infection (Wehtje *et al*., 1979). Subsequently, the pathogen may grow through one or two cortical cells before it becomes intercellular (Wehtje *et al*., 1979).

In our conditions, no differences were observed in the establishment of infection structures between interactions that were incompatible or compatible as a result of the presence/absence of the *Pl5* resistance gene. In contrast, Wehtje and Zimmer (1978) reported that resistance conditioned by the *Pl2* gene (type I resistance) could inhibit the penetration or pre-penetration processes during or soon after zoospore encystment.

Plant colonization and defence reactions

Following root penetration, *P. halstedii* grows intercellularly through plant tissues by coenocytic hyphae (Fig. 5a–c), producing infection/nutrition invaginations, named haustoria, in root, hypocotyl and cotyledon cells (Fig. 5a–c). During the compatible interaction between TS and pathotype 710, *P. halstedii* hyphae reach the top of the plant and infection becomes systemic (Fig. 2g), which is not observed for the resistant line TSRM.

However, in roots of both susceptible and resistant sunflower lines, a few hyphae and haustoria were observed, suggesting that, in type II resistance, the growth of the pathogen is not completely blocked at the root level (data not shown). However, from 6 days post-inoculation (dpi) in hypocotyl sections, large numbers of hyphae could be observed between cortical cells of TS plants (Fig. 5d, arrow), whereas no hyphae were detected in its NIL counterpart TSRM (Fig. 5e). This trend was confirmed at 10 dpi. In TS, all hypocotyl tissues, except vascular bundles, were fully infected by *P. halstedii* (Fig. 5f), whereas, in TSRM, far fewer hyphae were observed and none were seen in the central cylinder (Fig. 5g). Moreover, defence reactions, visualized by groups of brown collapsed cells formed in contact with the pathogen, were only observed in TSRM cortex at 10 dpi (Fig. 5g, thick arrow). These reactions resembled the hypersensitive response (HR)-like reactions of hypocotyls and cotyledons described for type II (*Pl1*, *Pl5* and *Pl8*) and type I (*Pl2*) resistances (Mouzeyar *et al*., 1993; Radwan *et al*., 2005, 2011). Early HR-like reactions (before 6 dpi), resulting from contact with the pathogen, could explain the difference in *P. halstedii* colonization levels between TS and TSRM plants.

HR-like reactions described in type I and type II resistances were accompanied by callose deposition, phenolic compound accumulation, and cell divisions around necrotic cells in several studies (Mouzeyar *et al*., 1993; Radwan *et al*., 2005, 2011). HR-like reactions observed in the hypocotyl might increase sunflower resistance by limiting pathogen growth. The induction of sunflower defence genes during *P. halstedii* interactions is, however, poorly documented (Radwan *et al*., 2005, 2011; As-Sadi, 2011).

The presence of HR-like reactions does not completely stop pathogen progression in hypocotyls and cotyledons of TSRM (as shown by weak sporulation on cotyledons). However, the absence of *P. halstedii* in TSRM leaves suggests that the region of the cotyledon node may serve as a mechanical or physiological barrier to oomycete spread, as hypothesized by Delanoë (1972) and Allard (1978).

Defence gene activation, leading to the production of antimicrobial compounds, HR-like reactions and other unknown mechanisms, is probably sufficient in resistant plants to impair mycelium growth in leaves and to stop systemic spread that would result in the formation of dissemination structures.

Pathogen sporulation and dissemination

At about 14 dpi in the growth chamber at a cool temperature (18–20 °C), after 2 days under saturating humidity conditions, sporulation occurred; a dense white down, visible to the naked eye, was produced on the cotyledon and/or leaf surfaces (Figs 2f,g and 3). This down was composed of zoosporangiophores (or conidiophores) (Fig. 4k–m). In the field, *P. halstedii* sporulation is only observed under favourable environmental conditions and notably high humidity.

Plasmopara halstedii hyphae were seen to exit the plant mainly through the stomata of leaves and cotyledons (Fig. 4l). At these sites, the hyphae developed branched zoosporangiophores, carrying the dissemination organs, zoosporangia, at the ends of the branches [Figs 4m and S1 (see Supporting Information)]. Zoosporangiophores were also formed on hypocotyl tissues and on roots (Fig. S1), but we cannot exclude the possibility that their formation on roots was facilitated by the hydroponic conditions used. It should be noted that *P. halstedii* zoosporangiophores and zoosporangia that formed on sunflower cotyledons, hypocotyls or roots showed different shapes (Fig. S1), as already described by Zahka and Viranyi (1991). Nishimura (1922) observed zoosporangia already formed by the pathogen in the substomatal cavity before the leaf emergence of zoosporangiophores. In addition, Bouterige *et al*. (2003) studied zoosporangia maturation *in vitro* leading to the formation and liberation of zoospores by the addition of 1% sucrose. At the end of the maturation, an apical papilla was formed at the top of zoosporangia through which the zoospores would be released (Figs 4a,b and S1).

The same types of dissemination structure have been observed for other phytopathogenic oomycetes, such as *P. viticola* on grapevine (Gessler *et al*., 2011). The shape of zoosporangiophores

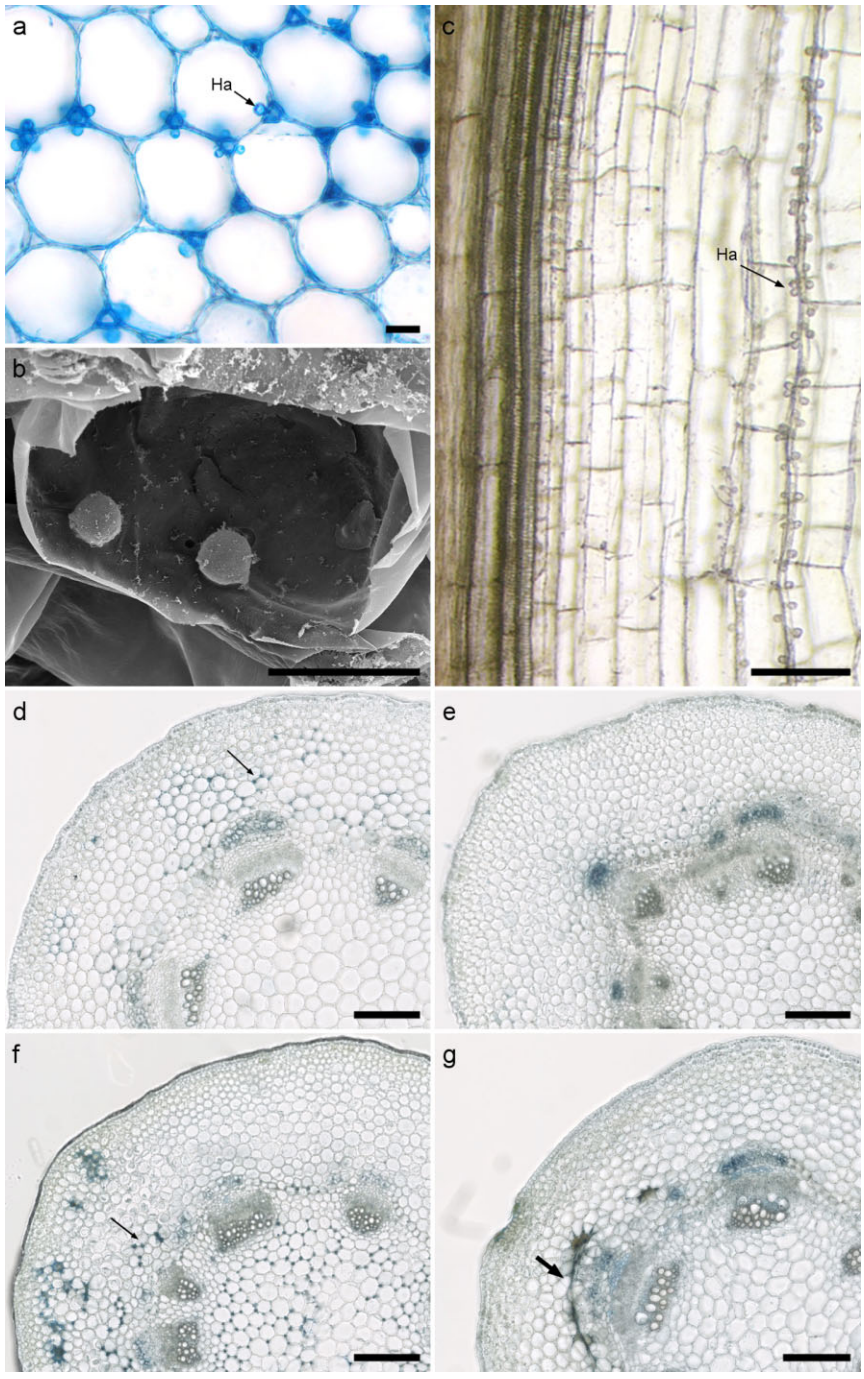


Fig. 5 *Plasmopara halstedii* hyphae and haustoria in hypocotyl and cotyledon tissues of susceptible and resistant sunflower lines. (a) Transverse sections of hypocotyls stained with 'cotton blue' in a lactophenol blue solution. Intercellular hyphae form haustoria (Ha) in adjacent cortical cells (black arrow). (b) Several haustoria were frequently observed in single hypocotyl and cotyledon cells [scanning electron microscopy (SEM) observations]. (c) Longitudinal section of a hypocotyl showing hyphal growth parallel to vascular bundles as a single track from the bottom to the top, and haustoria (Ha) in adjacent cells. (d–g) Time course of *P. halstedii* (pathotype 710) in a hypocotyl of susceptible (d, f) and resistant (e, g) near-isogenic lines of sunflower. Transverse section at 6 (d, e) and 10 (f, g) days post-inoculation stained with lactophenol blue. In the susceptible line TS, *P. halstedii* grew in both cortical and medullar cells of the hypocotyl (see arrows in d and f), whereas, in the resistant line TSRM, no (e) or very few (g) hyphae were visible and spider web hypersensitive response (HR)-like necroses (thick arrow, g) developed within the cortical parenchyma. Scale bars: 20 μm (a, b); 200 μm (c–g).

has been used to classify oomycetes, but this method has been overemphasized and is controversial (Thines, 2014; Voglmayr *et al*., 2004).

Shoot infection

Following sporulation, the dissemination of zoosporangia can lead to the secondary spread of infections (Fig. 3). This step in the life cycle of *P. halstedii* is responsible for late infections of neighbouring plants and contributes to pathogen dissemination (Spring, 2009).

In order to mimic shoot contaminations by zoosporangia in the field, a drop infection method, by the deposition of a suspension of zoosporangia on sunflower TS and TSRM leaves, was used. Grouped around leaf trichomes (Fig. 4h) and main veins (data not shown), zoospore encystment involved similar ultrastructural changes as in root infections (Bouterige *et al*., 2003; Nishimura, 1922). Encysted zoospores germinated rapidly [starting from

4 h post-inoculation (hpi)] and subsequently penetrated plant tissues. Generally, the penetration of leaf tissues was enabled by appressoria formation close to intercellular junctions (Fig. 4i). Although Nishimura (1922) reported that *P. halstedii* enters leaves by stomatal openings, we rarely observed this on TS and TSRM leaves inoculated by pathotype 710 (Fig. 4j). These results contrast with *P. viticola* grapevine leaf infections, in which zoospores germinate on stomata (Dièsez-Navajas *et al*., 2007; Kiefer *et al*., 2002) or differentiate a germ tube towards stomatal openings (Burruano, 2000). In contrast with root infections by *P. halstedii*, direct entry in leaf tissues was not observed, probably because of the cuticle present on the sunflower leaf surface. Following appressorium formation, it is still unclear how *P. halstedii* invades leaf tissues: does it establish an infection vesicle in the epidermal cell as in roots, and how does it develop in leaf parenchyma?

More generally, following leaf infection, little is known about further *P. halstedii* colonization of the entire plant and how it impacts sunflower yield. However, leaf infections leading to systemic infection in upper plant parts and, conversely, basipetal growth of *P. halstedii* hyphae in the shoot, have been reported (Meliala *et al*., 2000; Spring, 2009).

MOLECULAR RESOURCES AND PATHOGENICITY EFFECTORS

Plant-pathogenic oomycetes, either obligate biotrophs or hemibiotrophs, rely for their developmental cycle on pathogenicity factors, called effectors, that modify the metabolism of host plants to their benefit and thus enable pathogenicity (Bozkurt *et al*., 2012). Repertoires of hundreds of effector proteins that can be localized in either the apoplasm or cytoplasm of plant cells have been described to be encoded in oomycete genomes (Stassen and Van den Ackerveken, 2011). Within the class of cytoplasmic effectors, the RXLR and Crinkler (CRN) families have been shown to be secreted by the pathogen from haustoria and translocated into host cells (Schornack *et al*., 2010; Whisson *et al*., 2007). In addition to a signal peptide, RXLR proteins exhibit a characteristic RXLR amino acid motif that is sometimes associated with a (D)EER motif, whereas CRN proteins show a characteristic LXLFLAK motif (Rehmany *et al*., 2005; Torto *et al*., 2003). The different virulence profiles shown by pathotypes of *P. halstedii* (Fig. 1) could be explained by an intricate repertoire of effectors, and this has motivated our effort to develop *P. halstedii* genomics.

Despite the economic importance of downy mildew diseases caused by *Plasmopara* species, genomic resources were very limited until recently, even for *P. viticola*, the agent of grapevine downy mildew (As-Sadi *et al*., 2011; Bouzidi *et al*., 2007; Mestre *et al*., 2012). The obligate biotrophic requirements of these organisms, and our consequent inability to grow them *in vitro*, could explain the lack of genomic resources. The genome size of *P. halstedii* was estimated at 100 Mbp (Q. Gascuel, O. Catrice, L. Godiard LIPM, Castanet-Tolosan, France, unpublished data), close to the genome sizes of other Peronosporales, such as *Hyaloperonospora arabidopsidis* (100 Mbp) and *Phytophthora sojae* (95 Mbp) (Baxter *et al*., 2010; Tyler *et al*., 2006). In contrast with these model oomycetes that can be transformed, genetic tools must be improved for *P. halstedii*. For example, transient expression of green fluorescent protein (GFP) was shown to be possible in *P. halstedii* using electroporation of sporangia, but GFP expression was lost during subsequent infections (Hammer *et al*., 2007).

The first characterization of *P. halstedii* ESTs used a subtractive hybridization method applied to infected sunflower seedlings, and led to the identification of 230 non-redundant ESTs, 145 of which were assigned by polymerase chain reaction (PCR) tests on genomic DNA to be from *P. halstedii* (Bouzidi *et al*., 2007). Among the predicted proteins, three shared significant homology to a *Phytophthora infestans* elicitor and to protease inhibitors related to pathogenicity, and five corresponded to unknown proteins having a signal peptide suggesting that they could be secreted proteins, but no RXLR or CRN effector was identified (Bouzidi *et al*., 2007).

Extensive *P. halstedii* EST sequencing and search for RXLR and CRN effectors have been initiated by our group (As-Sadi *et al*., 2011). 454® pyrosequencing of cDNA was performed using two sunflower genotypes, XRQ and PSC8, infected by pathotype 710. These genotypes are resistant and susceptible, respectively (As-Sadi *et al*., 2011). About 45 000 non-redundant clusters, that may be from either sunflower or pathogen origin, were assembled and made available through a web-based portal http://www.heliagene.org/cgi/HP.cgi. This resource was screened on dedicated plant or oomycete libraries using *in silico* BLAST filtering, and 577 clusters predicted to have an oomycete origin were identified, including 389 new *P. halstedii* genes (As-Sadi *et al*., 2011). Most of the genes encode predicted proteins with unknown functions (only 32 had an Interpro motif), but the highest BLAST scores were obtained with proteins of hemibiotroph *Phytophthora* species (73% of the genes) and, to a lesser extent (17%), with the obligate biotroph *Hyaloperonospora arabidopsidis*, suggesting that *P. halstedii* does not share closer sequence homology with a pathogen causing downy mildew than with other Peronosporales oomycetes causing late blight disease. Homology searches based on known sequences of oomycete cytoplasmic effectors led to the characterization of 20 potential effectors having the conserved motifs found in RXLR and CRN effectors, showing, for the first time, that *P. halstedii* probably has the same kind of cytoplasmic effectors as found in other oomycetes (As-Sadi *et al*., 2011).

To increase the number of *P. halstedii* expressed sequences, we performed a deeper transcriptome 454® sequencing based on

the following materials: hypocotyls from infected susceptible sunflower plants of the inbred GB line and *in vitro*-germinated spores corresponding to a pre-infectious stage (the last stage at which it is possible to obtain *P. halstedii* material free of plant tissue). Four pathotypes of *P. halstedii* (100, 304-10, 703 and 710), representatives of the three groups of pathotypes described by Delmotte *et al*. (2008), were used; 80 354 non-redundant EST clusters were assembled and compared with draft genomic assemblies from *P. halstedii* in order to select only the oomycete clusters (S. Carrère, J. Gouzy, L. Godiard, LIPM, Castanet-Tolosan, France, unpublished data). The selected 24 597 *P. halstedii* clusters (mean size of 621 nucleotides) and 17 417 predicted peptides (mean size of 163 amino acids) will soon be made available in a new database, named PlasmoparaSp. This database was screened to identify the repertoire of expressed RXLR and CRN effectors from *P. halstedii*. Forty-nine expressed putative RXLRs and 54 CRNs were identified (L. Godiard, LIPM, Castanet-Tolosan, France, unpublished data); these numbers are consistent with data from other oomycetes (Cabral *et al*., 2011; Haas *et al*., 2009). All the selected RXLRs have a predicted signal peptide, suggesting that they could be secreted from the pathogen. In contrast, 80% of *P. halstedii* CRNs were not predicted to have a canonical signal peptide, as reported for CRNs from other oomycete species (Stam *et al*., 2013), suggesting that the highly conserved N-terminal sequences detected in *P. halstedii* CRN effectors could not be recognized by the prediction programs used. Nevertheless, the role of signal peptides in effector secretion must be shown experimentally. The putative translocation motifs of *P. halstedii* RXLR and CRN effectors were very similar to the published consensus sequences. Forty per cent of the RXLRs showed the exact RXLR consensus followed, or not, by the EER motif, and 29% had only the (D)EER motif. *Plasmopara halstedii* CRN effectors presented the characteristic modular structure composed of an N-terminal conserved region including the LXLFLAK and HVLVVVP motifs, followed by a variable C-terminal domain. Variant motifs of LXL*F*LAK (28%) were observed, including LXL*S*LAK (35%) and LXL*Y*LAK (20%), as observed for other oomycetes (Gaulin *et al*., 2008). Our analyses revealed the presence in *P. halstedii* CRN effectors of nine different CRN C-terminal domains (DBE, DBF, DFB, DN5, DN17, DO, DXZ, newD2 and SN6) already described in *P. infestans* (Haas *et al*., 2009). Comparing the putative effectors of *P. halstedii* with those of other oomycetes identified both conserved and *P. halstedii*-specific effectors, and revealed that the effector repertoire of *P. halstedii* is overall closely related to that of *Phytophthora* species (P. Mestre, INRA, Colmar, France and L. Godiard, LIPM, Castanet-Tolosan, France, unpublished results).

FUTURE PROSPECTS

Plasmopara halstedii, an obligate biotroph oomycete causing downy mildew on the important crop plant sunflower, offers puzzling traits, such as diversified infection modes on plant roots and shoots, and a still enigmatic reproduction cycle. The adaptable characteristics of *P. halstedii* might favour the rapid evolution of its virulence, illustrated by the occurrence of novel pathotypes overcoming the resistance used in sunflower cultivars. In addition, evolutionary questions raised about its increasing virulence make plant genetic control of this pathogen a major stake and render this pathogen particularly interesting to study. Moreover, a global population genetic study should provide information on the origin of this invasive pathogen from North America and elucidate its worldwide dispersion and co-evolution with sunflower.

Pathogen effectors are probably major players in the interaction between *P. halstedii* and sunflower, as illustrated in other oomycete–plant interactions. An essential issue will be to define *P. halstedii* effectors that may have an impact on its capacity to infect the plant, on host specificity and on the pathogen developmental cycle *in planta*. The establishment of *in vitro* growth conditions and the development of genetic tools for *P. halstedii* would be good assets to solve these challenges.

To circumvent these difficulties, we developed genomic data on several *P. halstedii* pathotypes, and aimed to describe the complete set of effectors. A comprehensive study of effector polymorphism will be performed in *P. halstedii* pathotypes in order to find key determinants of pathogen virulence and host specialization. The identification of effectors with large variability between *P. halstedii* isolates and under positive selection should constitute a starting point to understand pathogen evolution. In contrast, the identification of the effectors most conserved between *P. halstedii* pathotype isolates, corresponding to essential pathogen genes, could be used to screen sunflower genetic resources to find more sustainable resistance genes.

In parallel, functional studies should be used to specify the roles of these proteins in sunflower cells and how they condition pathogen virulence. For this purpose, agroinfection methods have been set up in sunflower plants, allowing the transient expression of *P. halstedii* effectors (Q. Gascuel, unpublished data). Ultimately, these studies should unveil molecular and physiological mechanisms, and hence lead to a better understanding of sunflower–*P. halstedii* interactions.

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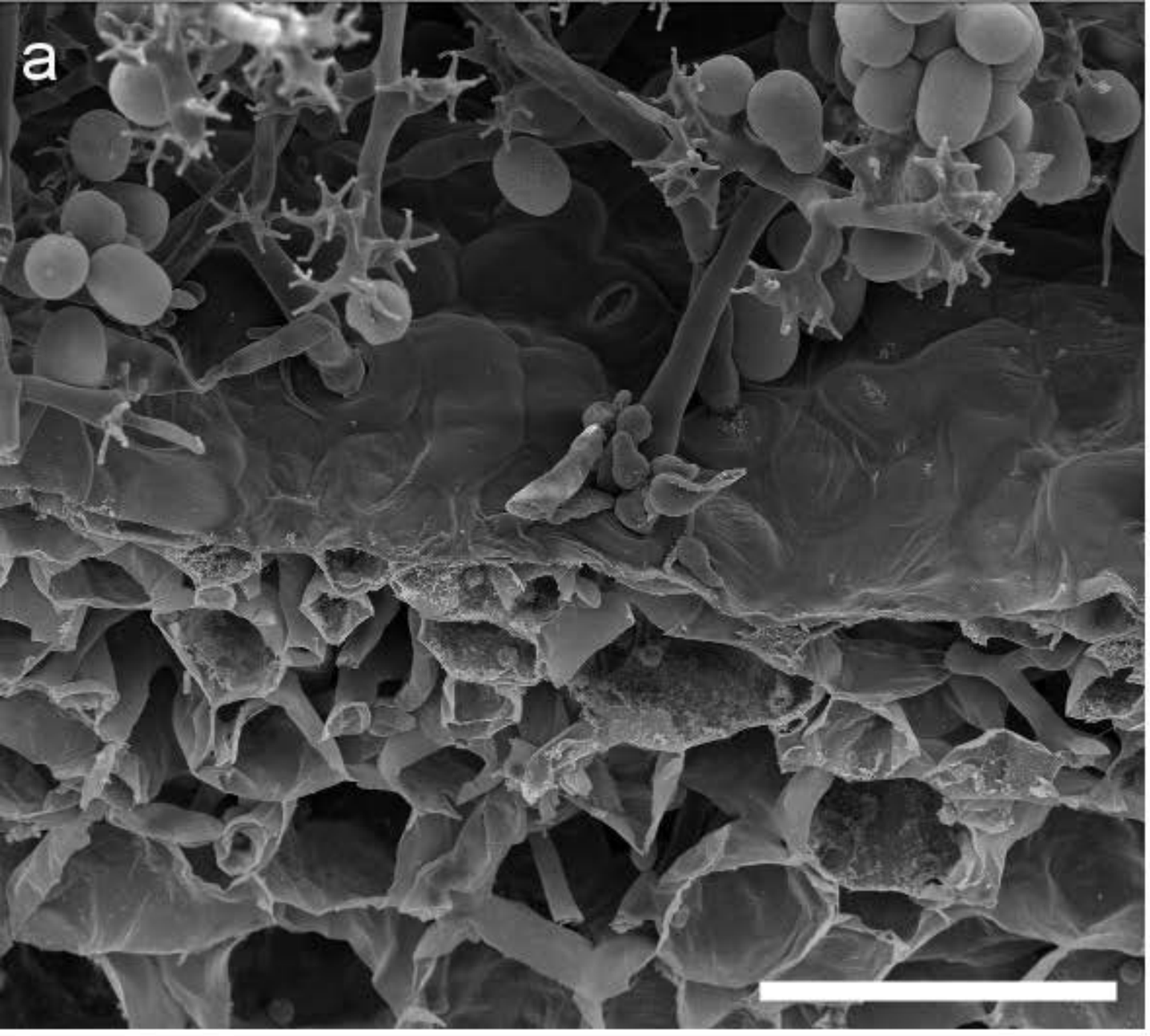
SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1 Various *Plasmopara halstedii* zoosporangiophores formed on sunflower tissues. Zoosporangiophore (Zp) carrying zoosporangia (Za) on cotyledon (a), hypocotyl tissue (b), emerging probably through a lenticel. (c, d) Zoosporangiophores formed on roots, near root hairs. Scale bars: 100 μm (a, b); 50 μm (c, d).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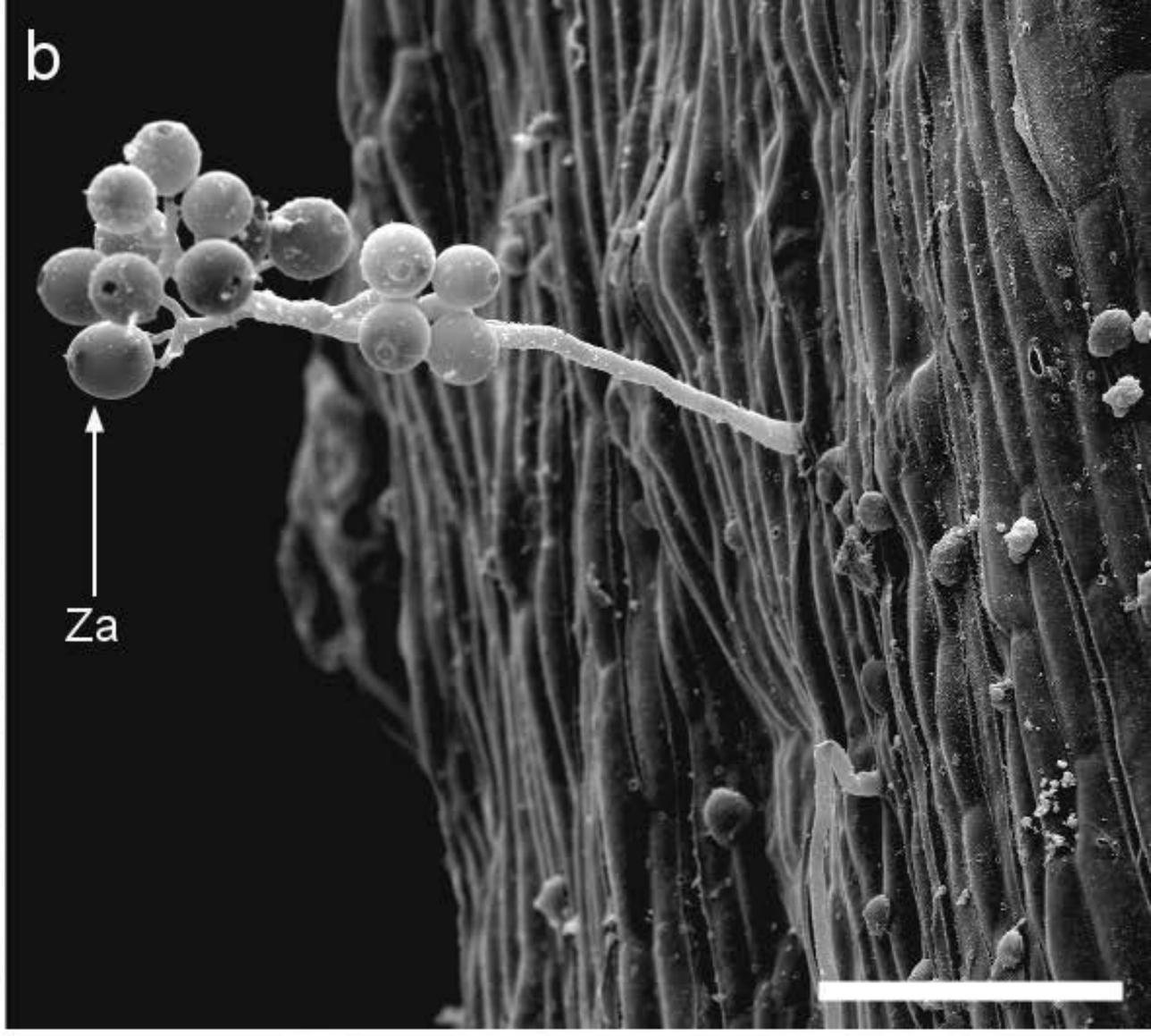
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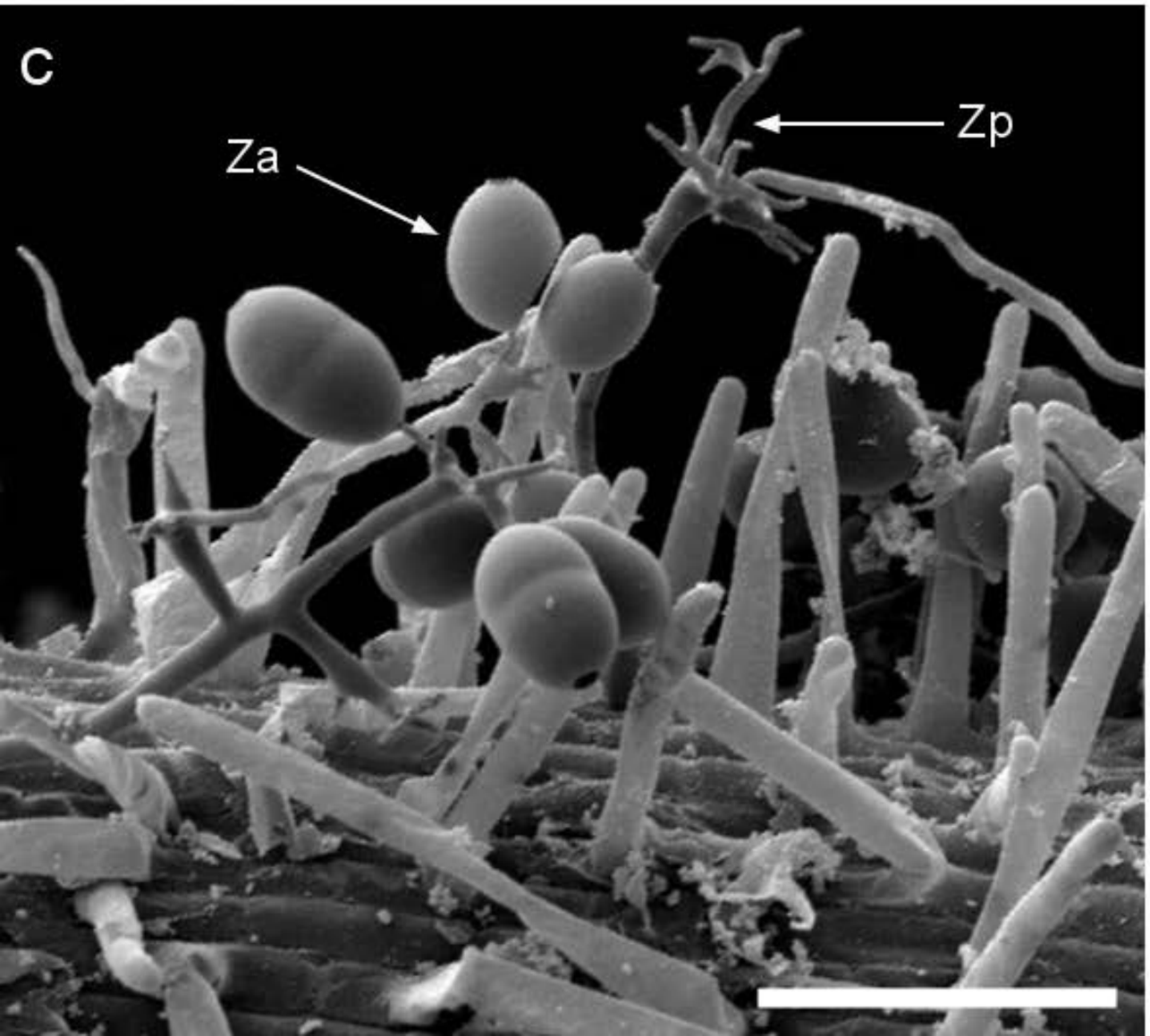
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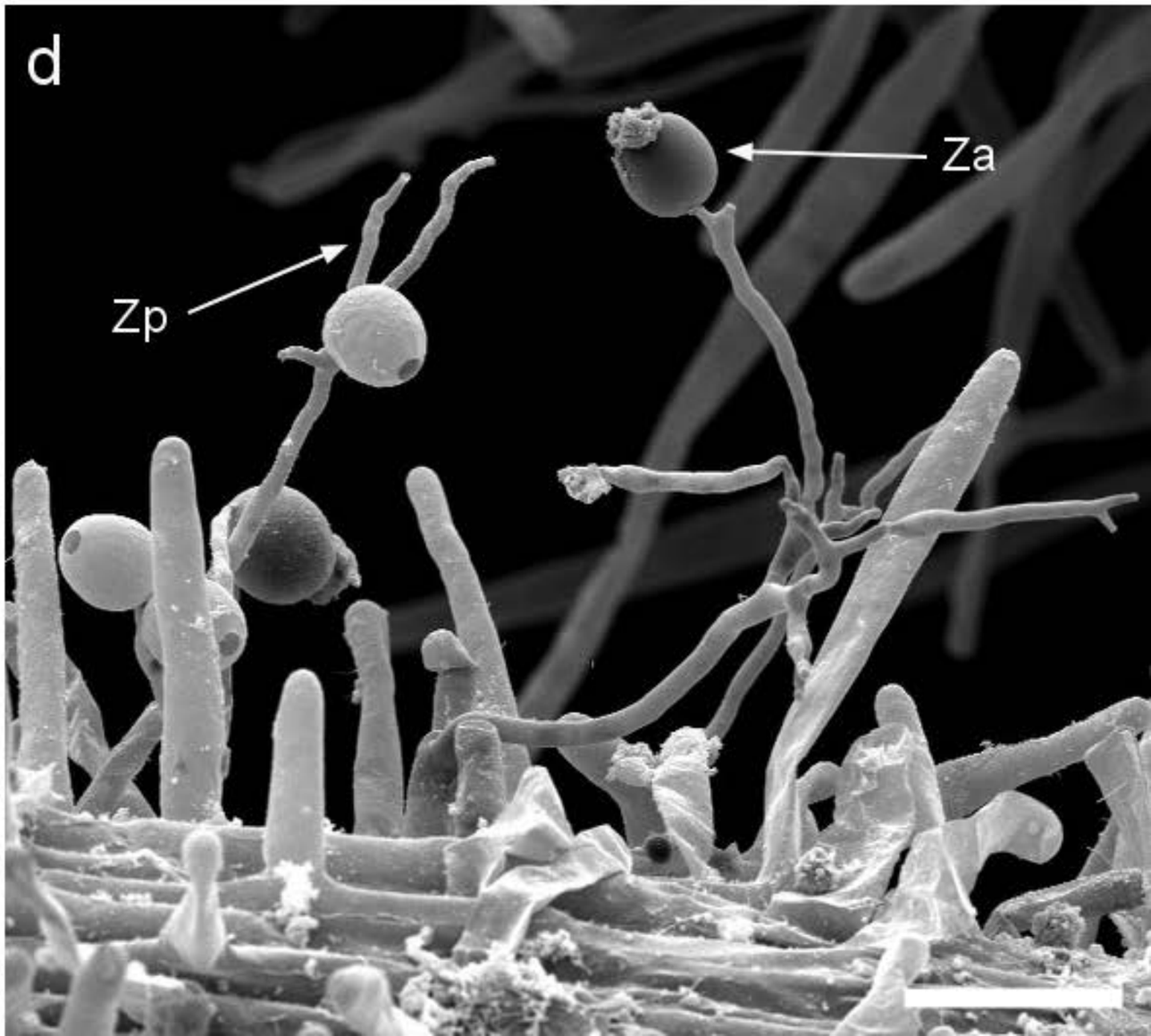
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