Arabidopsis thaliana, A New Host for *Polymyxa* Species

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Abstract

Polymyxa species are obligate biotrophs belonging to the Plasmodiophorid group, responsible for transmitting a large number of plant viruses to many crop species. Their obligate nature makes them difficult to study. Controlled environment experiments were used to investigate the potential of infection of *Arabidopsis thaliana* by *Polymyxa* spp. to provide a more tractable system. Two ecotypes of *Arabidopsis*, Columbia and Landsberg *erecta* were grown in soils known to be infested with *Polymyxa*. At the end of a two month growth period, both ecotypes were found to harbour *Polymyxa*-like structures or spores. These findings were confirmed by *Polymyxa*-specific PCR tests and rDNA sequencing which positively identified the presence of *Polymyxa* in the roots of both ecotypes of *Arabidopsis*. Both *Polymyxa graminis* and *Polymyxa betae* were identified. This is the first report of infection of *Arabidopsis* by *Polymyxa* spp. and shows the possibility of using this system for studies of infection biology and host-parasite interactions.

Introduction

Polymyxa species are a group of obligate root infecting organisms belonging to the Plasmodiophorid group that are important plant-virus vectors (Kanyuka *et al*., 2003). *Polymyxa graminis* transmits viruses such as *Soil-borne cereal mosaic virus (*SBCMV*), Soil borne wheat mosaic virus* and *Wheat spindle streak mosaic virus* to cereals. *P. betae* transmits *Beet necrotic yellow vein virus*, the cause of Rhizomania, to sugar beet.

A number of subgroups (ribotypes) of *Polymyxa* spp. have been identified according to rDNA sequence data (Ward *et al*., 1994; Ward & Adams, 1998; Legrève *et al*., 2002; Ward *et al*., 2005). Some of these ribotypes appear to differ in host range and temperature requirements leading to the suggestion that they should be classified as *formae speciales* (Legrève *et al*., 1998; 2002). Two groups of *Polymyxa graminis* isolates are found in temperate regions, ribotype I (f. sp. *temperata*) and ribotype II (f. sp. *tepida*). All ITS rDNA sequences for *P. betae* reported to date fall into two types which differ by only one base pair (Ward & Adams, 1998; Legrève *et al*., 2002).

Due to their obligate nature and relatively long life-cycle, *Polymyxa* species have been difficult to study. The development of a model system for studying *Polymyxa*-plant interactions would be extremely useful. *Arabidopsis thaliana* is an invaluable model system for several reasons: 1) short generation time, 2) the ability to grow large numbers in a relatively small space, 3) its ability to self-fertilise, 4) the large number of progeny that can be produced from a single plant, 5) its small haploid genome containing a relatively small number of repetitive genetic elements, 6) the availability of a fully-sequenced genome, 7) the availability of mutagenised lines, 8)

ease of transformation and, 9) the large number of ecotypes exhibiting natural variation available (Meyerwitz, 1989). These features are in contrast to many crop species such as cereals where genetic resources are less well advanced.

Arabidopsis has already been used very successfully to study the interactions of another plasmodiophorid, *Plasmodiophora brassicae* (Koch *et al*., 1991). The ability to separate host sequences from those of *Plasmodiophora* by bioinformatics analysis has simplified the interpretation of data e.g. from suppressive subtractive hybridisation experiments to study gene structure and expression (Bulman *et al*., 2006; Bulman *et al*., 2007). Sources of resistance and factors important for the infection of *Plasmodiophora* have been studied by exploring the responses to both natural and induced (mutagenic) variation in host genes affecting infection (Siemens *et al*., 2002; Alix *et al*., 2007). *Arabidopsis* has been used to visualise infection biology of *P. brassicae* (Mithen & Magrath, 1992). The availability of synteny maps between *Arabidopsis* and *Brassica* species has allowed identification of resistance loci in *Brassica* species first identified in *Arabidopsis* (Suwabe *et al*., 2006). Global analysis of host gene expression at different time points post infection has been possible using *Arabidopsis* genome arrays and this has allowed identification of host genes that may be important for infection by *Plasmodiophora* (Siemens *et al*., 2006). Genes of interest can then be studied further by transforming into *Arabidopsis* or by utilising the bank of insertion lines available in *Arabidopsis* (Puzio *et al*., 2000; Siemens *et al*., 2006)

Many of the host plants that *Polymyxa* species infect are not well characterised genetically, have fewer genetic tools available and they have long generation times. Also, the roots of cereals can be difficult to visualize by microscopy as they are

thicker in diameter than those of *Arabidopsis*. This can sometimes make visual detection of *Polymyxa* in roots difficult. Therefore, if infection of *Arabidopsis* by *Polymyxa* species can be demonstrated, this could be a valuable tool in increasing our understanding of plant-*Polymyxa* interactions. This study aimed to look at the potential for infection of *Arabidopsis* by *Polymyxa* spp. under controlled environment conditions using *Polymyxa* infested soils.

Materials and Methods

Arabidopsis thaliana ecotypes Landsberg *erecta* (L*er*-0) and Columbia (Col-0) were used for this study (supplied by A. Cuzick, Rothamsted Research, UK). These ecotypes were chosen because they are genetically distinct and mapping populations are available. Seeds were sown into sterile Levingtons No. 2 compost containing sand, and stratified for four days in the dark at 4°C. Pots were then removed and placed in a greenhouse under short day length conditions (8 hr day at 20°C, 16°C night, light levels 200 - 300 $\mu mol.m^{-2}.sec^{-1}$). Once the seedlings had produced their first true leaves, they were transferred to 10 cm pots containing infectious soils diluted 1:2, soil to sterile sand. Two UK soils were used, one from Wiltshire which was infested with SBCMV (Lyons *et al*., 2008) and one from Woburn where *Polymyxa* was present but no associated virus had ever been identified (Ward *et al*., 2005, R. Lyons, (Rothamsted Research) pers. com). For each soil, 5 seedlings of each ecotype were planted. Plants were then allowed to grow for two months. Flowering bolts were removed upon development to prolong vegetative growth.

Once roots had been removed from pots and undergone vigorous washing in sterile, distilled water, three sets of 3 cm bunches of root, one from the base of the plant, one from the middle of the root mass and one from the root tip, were examined using an Axiophot (Zeiss) light microscope and bright field illumination. Portions of root were mounted in sterile water under a coverslip.

DNA was extracted from root material as described by Ward *et al*. (2005). *Polymyxa*-specific rDNA primers Pxfwd1 (5'CTG CGG AAG GAT CAT TAG CGT T 3') and Pxrev7 (5' GAG GCA TGC TTC CGA GGG CTC T 3') were used in PCR (Ward *et al*., 1994). For sequencing studies, the *Polymyxa*-specific forward primer Pxfwd1 and the generic fungal ITS4 reverse primer (5' TCC TCC GCT TAT TGA TAT GC 3') (White & Bruns *et al*., 1990), were used to amplify rDNA. Each reaction mix (50 µL) contained: 0.2 µM primers, 1U *Taq* DNA polymerase (MBI), 0.2 mM deoxyribonucleoside triphosphates (Sigma), 1x PCR buffer NH_4 (MBI), 0.02 mg/µL BSA. Cycling conditions were 2 min at 95°C, then 30 cycles of: 94°C for 30s, 50°C for 1 min, 72°C for 2 min, followed by 72°C for 10 min. Products were analysed in 1% agarose gels.

PCR products were cloned into the pGEM®-T Easy vector (Promega Corporation, Madison, WI, US). Plasmid DNA was prepared using the QIAprep spin miniprep kit (Qiagen, Crawley, UK) and sequenced using the ABI PRISM™ Big-Dye version 1.1 kit using primers M13SeqF (5' GCC AGG GTT TTC CCA GTC ACG A 3') and M13SeqR (5' GAG CGG ATA ACA ATT TCA CAC AG 3') and run at the Geneservice sequencing facility (http://www.geneservice.co.uk).

Results and Discussion

Examination by microscopy showed the presence of *Polymyxa*-like spores in numerous root hairs (but not the main root) of *Arabidopsis* ecotype L*er*-0 plants grown in the Woburn soil (Figure 1). Two of the Col-0 plants grown in the Woburn soil contained structures that resembled *Polymyxa* zoosporangia (Figure 2). Three of these structures were seen in total and they were all located in the main root system rather than the root hairs. No spore clusters were observed. In the root sections examined from *Arabidopsis* plants grown in the Wiltshire soil, no *Polymyxa*-like spores or zoosporangia were identified.

PCR with the *Polymyxa*-specific primers Pxfwd1/Pxrev7 demonstrated the presence of *Polymyxa* species in the roots of all four combinations of *Arabidopsis* ecotypes and soils (Figure 3). A total of 28 clones were sequenced following amplification of rDNA products from *Arabidopsis* roots using primers Pxfwd1/ ITS4. Eleven of these sequences showed significant identity to *P. graminis* F1 ITS ribosomal DNA (Table 1) and one to *P. betae* F67 ITS rDNA. Of the remaining sequences, nine showed 98-100% nucleotide identity to *Arabidopsis* rDNA, two to uncultured *Basidiomycetes*, one to an uncultured *Helotiale*, one to *Urostyla grandis*, one had very partial identity to *Anguina agrapyri*, another had partial identity to an Ectomycorrhizal fungus and one had no known homology to any sequence in Genbank. The identification of *Arabidopsis* and other non-*Polymyxa* sequences in the roots is not unexpected, as only one of the primers used (Pxfwd1) is *Polymyxa* -specific whereas the ITS4 primer is a generic, ‘fungal’ rDNA primer.

158 Sequences from these experiments were aligned with existing *Polymyxa*
159 rDNA sequences and phylogenetic analyses were performed in MEGA4 (Figure 4).
160 With the exception of LeWil clone 34, which grouped with *P. betae*, all of the other
161 *Polymyxa* sequences obtained from *Arabidopsis* root samples formed a clade with the
162 *P. graminis* F1 (ribotype I) isolate (AY12824, 99% support from bootstrapping).
163 There was strong bootstrap support (99%) separating the Col-0 Woburn clone 3
164 sequence from the other sequences in this clade.
165 Collectively, our results indicate that *Arabidopsis* is susceptible to infection by
166 *Polymyxa* spp. *Polymyxa*-like spore clusters were identified in root hairs of
167 *Arabidopsis* L*er*-0 plants and structures resembling young *Polymyxa*-like
168 zoosporangia in the roots of Col-0 plants. The putative zoosporangium is not like that
169 of any of the other plasmodiophorid genera. Although these structures were not
170 observed in all plants, it is possible that they were present in parts of the root system
171 other than those examined by microscopy. The spores, although similar in appearance
172 to *Plasmodiophora*, were aggregated together in clusters whereas *Plasmodiophora*
173 spores do not form aggregates. In addition no galls were observed in the roots of these
174 plants, as would occur in *Plasmodiophora* infections.
175 Using *Polymyxa*-specific PCR assays, *Polymyxa* was detected in all four
176 combinations of *Arabidopsis* ecotypes and soils, and this was confirmed by rDNA
177 sequencing; sequences either had high nucleotide identity to the rDNA sequence from
178 ribotype I *P. graminis* or to *P. betae*. None showed close identity to *P. graminis* type
179 II despite this ribotype being present in both soils (Ward *et al.*, 2005; Lyons *et al.*,
180 2008). Although temperate ribotypes of *P. graminis* have been shown mainly to infect
181 monocotyledonous plants, *P. betae* and tropical isolates of *P. graminis* have been

shown to infect dicotyledonous plants (Barr, 1979; Ratna *et al*., 1991; Barr & Asher, 1992; Legrève *et al*., 2000).

The observation of spores in the root hairs of the *Arabidopsis* ecotype L*er*-0 plants is interesting as *Polymyxa* species are not routinely reported infecting root hairs, although this has been observed infrequently (M. Smith, M. J. Adams, unpublished). However, it is not unreasonable to expect changes in morphology and tissue colonisation in this alternative host.

One way to absolutely determine the organism producing the structures observed in the roots of the *Arabidopsis* plants would be to use a technique such as laser capture micro-dissection although this would be technically challenging (Emmert-Buck *et al*., 1996; Kerk *et al*., 2003; Day *et al*., 2005).

Further experiments would be required to optimise the system, to establish the range of *Polymyxa* isolates capable of infecting *Arabidopsis* and to determine whether there were any links between the type of infection seen (location and developmental stage) and the *Arabidopsis* ecotype used.

This is the first report to demonstrate that infection of *Arabidopsis* by *Polymyxa* spp. is possible. Both *P. graminis* and *P. betae* sequences were found in infected *Arabidopsis* roots and extends the range of known hosts for both species. This important finding opens up the exciting possibility of using a model system for studying *Polymyxa* infections with a wide range of available tools, and that is much more amenable to study than using sugar beet or cereal hosts.

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333 Table 1. Results of BLAST hits for sequenced clones with significant nucleotide
334 identity to *Polymyxa* rDNA.
335

336

Arabidopsis ecotype	Soil	Clone No.	BLAST Hit[a]	Genbank Accession No.
Landsberg	Woburn	LeWob34	98% *P. graminis* F1[b]	FN393973
Landsberg	Wiltshire	LeWil3	99% *P. graminis* F1	FN393974
Landsberg	Wiltshire	LeWil7	99% *P. graminis* F1	FN393967
Landsberg	Wiltshire	LeWil34	99% *P. betae* [c]	FN393976
Landsberg	Woburn	LeWob8	97% *P. graminis* F1	FN393968
Columbia	Woburn	CoWob3	93% *P. graminis* F1	FN393971
Columbia	Woburn	CoWob10	99% *P. graminis* F1	FN393972
Columbia	Woburn	CoWob11	99% *P. graminis* F1	FN393966
Columbia	Woburn	CoWob29	99% *P. graminis* F1	FN393975
Columbia	Wiltshire	CoWil1	99% *P. graminis* F1	FN393969
Columbia	Wiltshire	CoWil7	99% *P. graminis* F1	FN393970

337

338 [a]Closest match showing percentage nucleotide identity between the query and database sequences, [b]
339 Accession Number : AY12824, [c] Accession Number:Y12827

340

341

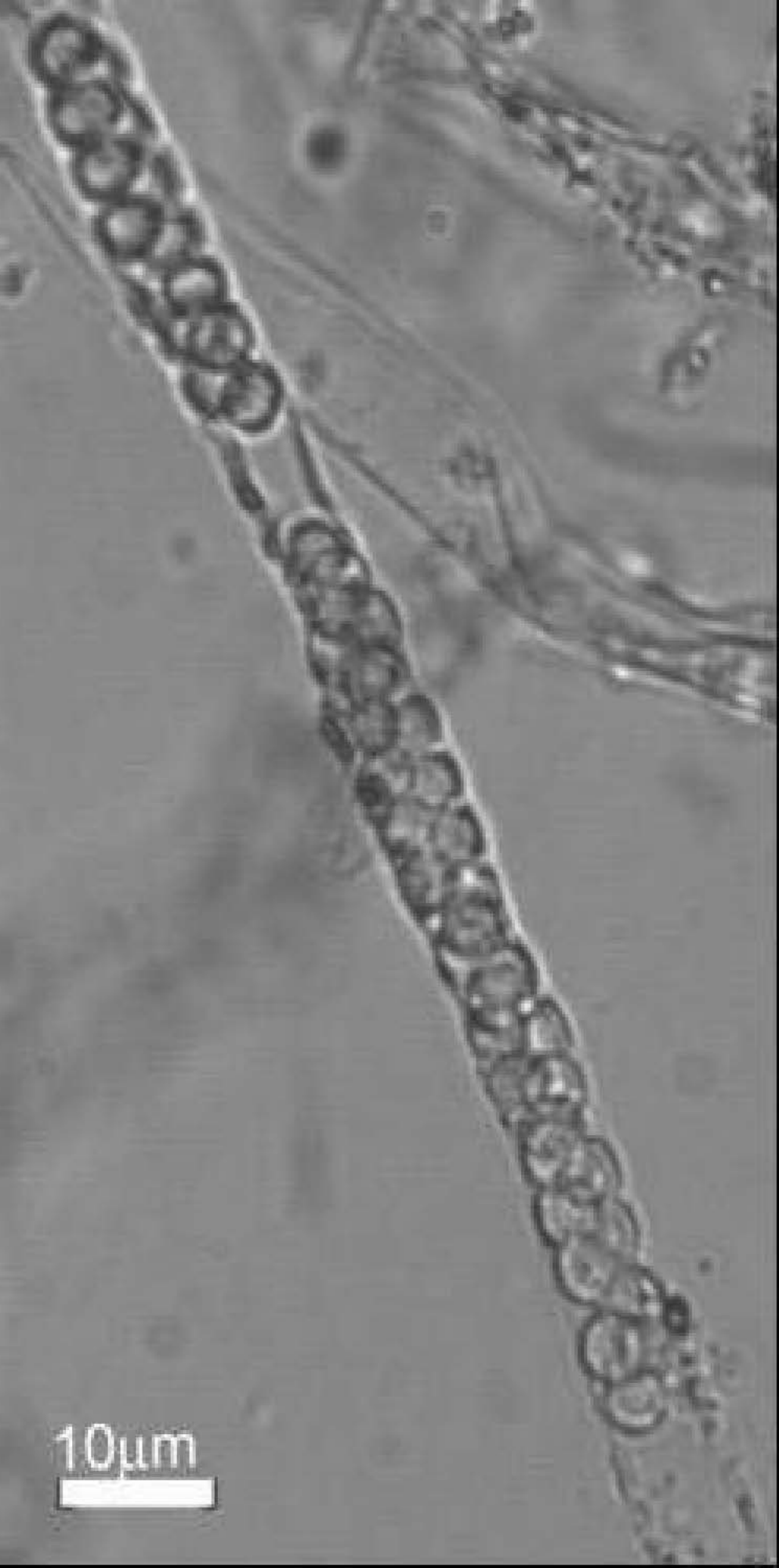
Figure legends

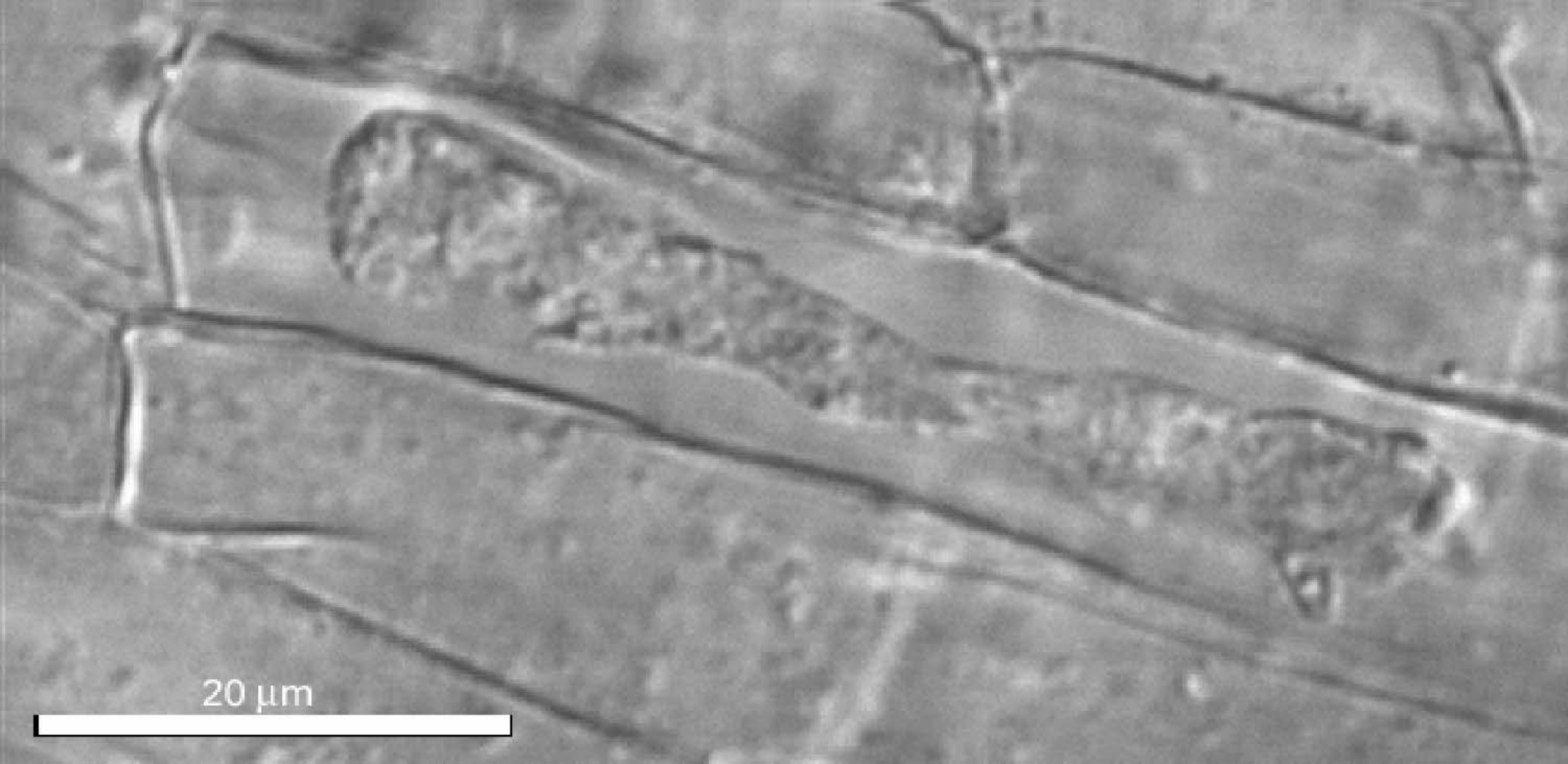
Figure 1. Root hair from *Arabidopsis* L*er*-0 plant grown in Woburn soil containing plasmodiophorid-like spore clusters

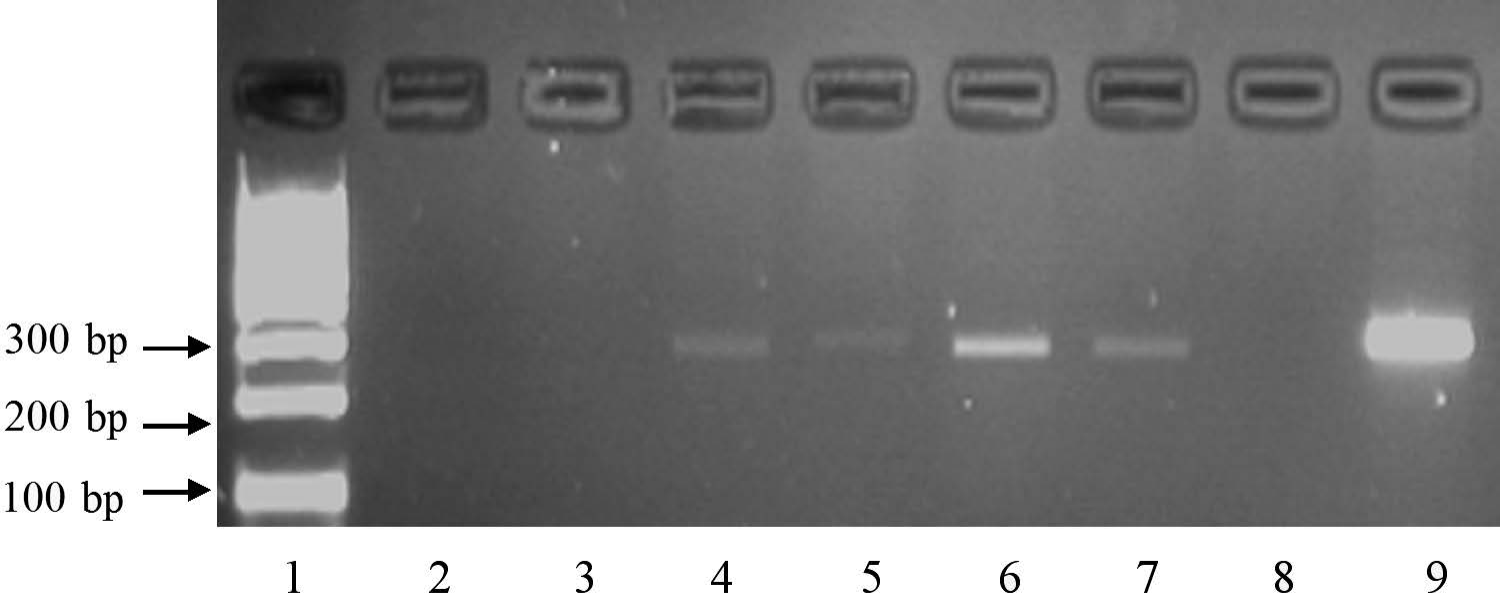
Figure 2. *Polymyxa*-like zoosporangial structure in a main root cell of an *Arabidopsis* Col-0 plant grown in Woburn soil.

Figure 3. Amplification of *Polymyxa*-specific rDNA products from *Arabidopsis* roots. Lane 1, 100 bp size marker (Fermentas); lane 2, healthy uninfected *Arabidopsis* Col-0; lane 3, healthy uninfected *Arabidopsis* L*er*-0; lane 4, *Arabidopsis* Col-0 from Woburn soil; lane 5, *Arabidopsis* L*er*-0 from Woburn soil; lane 6, *Arabidopsis* Col-0 from Wiltshire soil; lane 7, *Arabidopsis* L*er*-0 from Wiltshire soil; lane 8, no DNA control; lane 9, *Polymyxa* DNA positive control.

Figure 4. Phylogenetic relationships between *Polymyxa* ITS rDNA sequences from *Arabidopsis* and other isolates. Sequences PgF1 (*P.graminis* ribotype I, Accession No. Y12824), Pg51 (*P. graminis* ribotype II, Y12826) and PbF67 (P. betae, Y12827) were reported in Ward and Adams, 1998), other sequences were obtained in the current study. The Neighbor-Joining method (Maximum composite likelihood distances) was used in MEGA4 (Tamura *et al*., 2007) with 10000 bootstrap replications.


10µm


20 µm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200 bp
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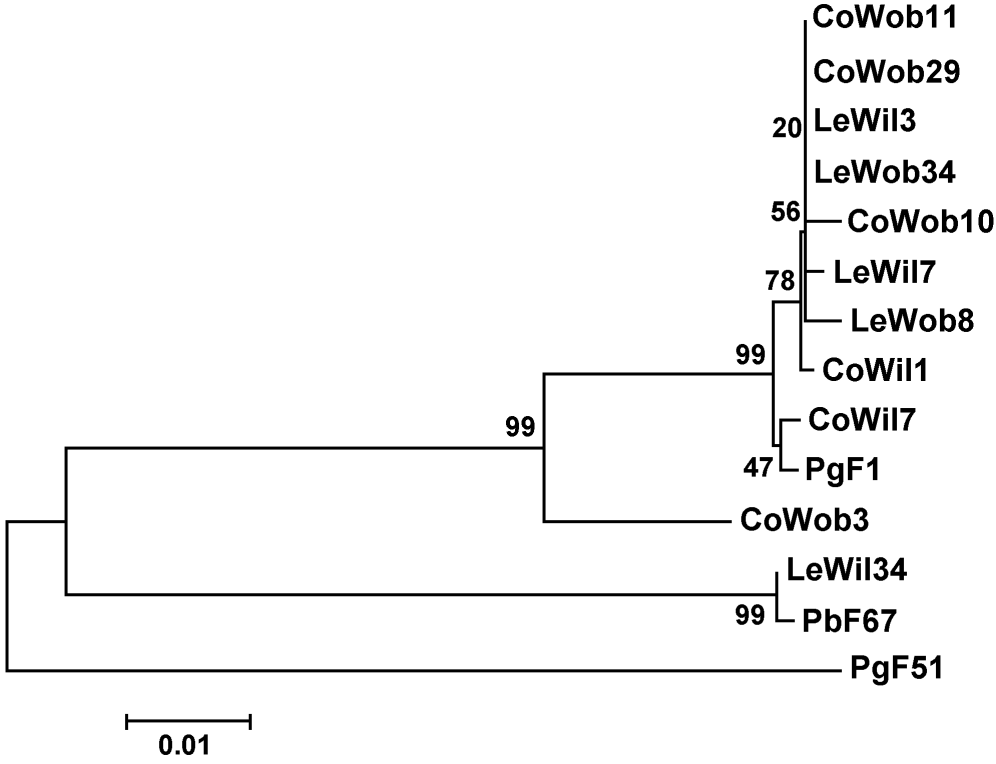
CoWob11
CoWob29
20
LeWil3
LeWob34
56
CoWob10
78
LeWil7
LeWob8
99
CoWil1
99
CoWil7
47
PgF1
CoWob3
LeWil34
99
PbF67
PgF51
0.01