

DECIPHERING THE BIOLOGY OF *PHYLLACHORA AMBROSIAE*, AN ENIGMATIC FUNGAL PATHOGEN OF *AMBROSIA ARTEMISIIFOLIA*, AND A HIDDEN FRIEND OF ALLERGIC PEOPLE

SCIENTIFIC REPORT

**on an STSM carried out in the framework of the COST ACTION FA1203 entitled
'Sustainable management of *Ambrosia artemisiifolia* in Europe (SMARTER)'**

Start date: 1 July 2015 **End date:** 31 July 2015

STSM grantee:

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Host Details:

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Main objectives of the STSM:

1. A detailed study of the *P. ambrosiae* specimens collected in Florida, USA, in 2014 using molecular tools.
2. The study of as many other *P. ambrosiae* specimens as possible available at CABI and borrowed from other herbaria (e.g. BPI, USDA).
3. Analysis of the TEM work done by the applicant's group in Hungary.
4. Understanding the presence of phylogenetically diverse *P. ambrosiae* strains in ragweed populations in different parts of the world.
5. Deciphering the complete, apparently mostly intracellular, life cycle of *P. ambrosiae* in *A. artemisiifolia* plants and explaining why/how is this pathogen able to seriously damage its plant host in some years, and cause a huge reduction in its allergenic pollen production.

Background:

Phyllachora ambrosiae is a little known, enigmatic fungal pathogen of common ragweed (*Ambrosia artemisiifolia*), capable, infrequently, maybe once in a decade, to naturally kill the pollen-producing inflorescences of this noxious plant in large areas (Bohár, Vajna & Kiss, 2000), but being almost undetectable in ragweed populations most of the time (Kiss 2007). The last comprehensive paper about this apparently obligate biotrophic fungus was published 60 years ago in *American Journal of Botany* (Miller 1954). Since then, there are only sporadic reports on its first detection in Europe (Bohár, Vajna & Kiss, 2000) and its occurrence in Ukraine (Hayova 2006). Gerber et al. (2011), when reviewing all the potential arthropod and fungal biocontrol agents

(BCAs) of *A. artemisiifolia*, rightly pointed out that its little known biology makes *P. ambrosiae* currently unsuitable to be considered as a potential BCA of common ragweed.

During the past years L. Kiss' research group made some progress in understanding how *P. ambrosiae* infects and damages ragweed plants (unpublished results). However, some aspects of this host-pathogen interaction should still be clarified. Also, more *P. ambrosiae* materials, including samples collected by Prof. Heinz Müller-Schärer in 2014 in Florida, USA, in the framework of SMARTER, should be included in this work. In fact, there are only a very few authentic specimens of *P. ambrosiae* worldwide. CABI's UK Centre offers both expertise in weed pathology and access to more *P. ambrosiae* specimens. The main objectives of the STSM were to (i) collaborate with CABI experts in the study of *P. ambrosiae*, and (ii) publish jointly all the results on this enigmatic pathogen.

Main results of the one-month STSM:

1. Molecular analysis of *P. ambrosiae* samples collected in the USA in 2014

During a SMARTER field trip in summer 2014, Professor Heinz Müller-Schärer and his colleagues collected two samples of *P. ambrosiae* in Florida, USA, designated as FL3 and FL4. We determined the nrDNA ITS and 28S sequences in these samples which were identical to those determined by L. Kiss' group earlier in another North American *P. ambrosiae* specimen collected by Dr. Claudia Nischwitz in Tifton, GA, in 2005, and deposited by L. Kiss at U.S. National Fungus Collections (standard abbreviation: BPI, <http://nt.ars-grin.gov/fungaldatabases/specimens/specimens.cfm>) in Beltsville, MD, under the accession number BPI 880509.

This was very important because before 2014 the specimen collected in Tifton, GA, was the only North American sample of *P. ambrosiae* for which molecular identification was possible. (All our PCR amplification trials failed in all the other, much older *P. ambrosiae* specimens available at BPI, probably because the DNA in those old specimens was degraded.) Therefore, as a result of this step, we have now molecular data for three North American *P. ambrosiae* specimens, collected in two states of the USA, Georgia and Florida, in 2005 and 2014. This is an important achievement in the case of such a hard-to-find fungal pathogen where only a very few authentic samples exist.

The ITS and 28S sequences determined in FL3 and FL4 were analyzed together with the DNA sequences determined previously in our laboratory in Hungarian, Korean, Ukrainian and USA samples (see Result #4 below). The newly determined sequences will be made publicly available in NCBI GenBank (<http://www.ncbi.nlm.nih.gov/genbank/>) as soon as the paper using these DNA data is accepted for publication in an international journal. Currently, not a single DNA sequence is available in GenBank for *P. ambrosiae* or any closely related *Phyllachora* spp., therefore our results are crucial for the precise, reliable identification of this pathogen of common ragweed.

2. Study of *P. ambrosiae* specimens available at CABI and other herbaria

As the whole plant pathological and mycological herbarium collection of CABI (standard abbreviation: IMI) was moved to the Fungarium of the Royal Botanic Gardens (RBG) in Kew (<http://www.herbimi.info/herbimi/home.htm>), I visited Prof. Paul Cannon, the internationally recognized expert of the Phyllachoraceae, at Jodrell Laboratory, RBG Kew, to examine the *P. ambrosiae* specimens available in the Fungarium, and to discuss the details of our *P. ambrosiae* project. Unfortunately, only the following two IMI specimens were found in the catalogue of the Fungarium, none of which were collected from *Ambrosia artemisiifolia* (Table 1).

Table 1. *Phyllachora ambrosiae* specimens at RBG Kew

Accession number	Name, associated organism	Place of collection	Year of collection
IMI 15116	<i>Phyllachora ambrosiae</i> associated with <i>Iva xanthifolia</i>	Montana, USA	1913
IMI 154443	<i>Phyllachora ambrosiae</i> associated with <i>Ambrosia psilostachya</i> var. <i>californica</i>	California, USA	1938

However, none of these two specimens were found *de facto* in the collection during my visit at RBG Kew. Fortunately, I was previously able to study the following *P. ambrosiae* specimens available at U.S. National Fungus Collections (BPI); however, as specified below (Table 2), only two BPI specimens, nos. 636220 and 636225, contained perithecia of the pathogen:

Table 2. *Phyllachora ambrosiae* specimens available at BPI (Beltsville, MD, USA) and studied by L. Kiss

Accession number	Name, associated organism	Place of collection	Year of collection	Notes
BPI 636251	not specified	Mississippi, USA	1893	no <i>P. ambrosiae</i> structures found
BPI 636213	<i>Phyllachora ambrosiae</i> associated with <i>Ambrosia artemisiifolia</i> (?)	Colombia	1940	no <i>P. ambrosiae</i> structures found
BPI 636214	<i>Phyllachora ambrosiae</i> associated with <i>Ambrosia artemisiifolia</i> (?)	Colombia	1910	no <i>P. ambrosiae</i> structures found
BPI 636215	<i>Phyllachora ambrosiae</i> associated with <i>Ambrosia artemisiifolia</i>	Mississippi, USA	1922	no <i>P. ambrosiae</i> structures found
BPI 636216	<i>Phyllachora ambrosiae</i> associated with <i>Ambrosia artemisiifolia</i>	Mississippi, USA	1921	no <i>P. ambrosiae</i> structures found
BPI 636217	<i>Phyllachora ambrosiae</i> associated with <i>Ambrosia artemisiifolia</i>	Mississippi, USA	1921	no <i>P. ambrosiae</i> structures found
BPI 636218	<i>Phyllachora ambrosiae</i> associated with <i>Ambrosia artemisiifolia</i>	Colombia	1930	no <i>P. ambrosiae</i> structures found
BPI 636220	<i>Phyllachora ambrosiae</i> associated with <i>Ambrosia artemisiifolia</i>	Tuskegee, Alabama, USA	1935	Perithecia, asci and ascospores of <i>P. ambrosiae</i> present in the <i>A. artemisiifolia</i> leaves
BPI 636221	<i>Phyllachora ambrosiae</i> associated with <i>Ambrosia artemisiifolia</i>	Virginia, USA	1938	no <i>P. ambrosiae</i> structures found
BPI 636223	<i>Phyllachora ambrosiae</i> associated with <i>Ambrosia artemisiifolia</i>	Amery, WI, USA	1959	no <i>P. ambrosiae</i> structures found
BPI 636224	<i>Phyllachora ambrosiae</i> associated with <i>Ambrosia artemisiifolia</i>	Pine Bluff, WI, USA	1962	no <i>P. ambrosiae</i> structures found
BPI 636225	<i>Phyllachora ambrosiae</i> associated with <i>Ambrosia artemisiifolia</i>	Gainesville, FL, USA	1912	Perithecia, asci and ascospores of <i>P. ambrosiae</i> present in the <i>A. artemisiifolia</i> leaves

It is noteworthy that the collector of the specimen BPI 636220, G. W. Carver, mentioned that *P. ambrosiae* was 'very destructive' when found in Tuskegee, AL, USA, in 1935 (Fig. 1):

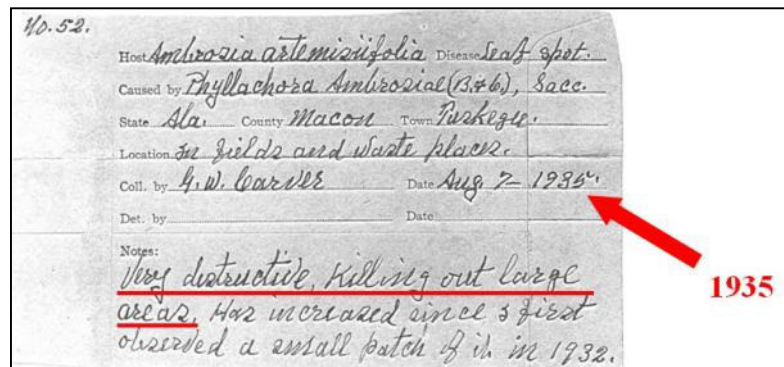


Fig. 1. The label of the herbarium specimen BPI 636220.

3. Analysis of the TEM work done by L. Kiss' group in Hungary

A comprehensive (and as yet unpublished) transmission electron microscopy (TEM) work done by Dr. Károly Bóka (Eötvös Univ., Dept. Plant Anatomy, Budapest, Hungary) and L. Kiss resulted in a large number of images of *P. ambrosiae* hyphae localized inside the epidermal and mesophyll cells of common ragweed leaves (e.g., Figs. 2-4). These images revealed unknown characteristics of the infection process, such as the way of spread of *P. ambrosiae* hyphae from one plant cell to another (Figs. 2 and 3) and the thickening of the fungal cell wall when the host plant cells are finally killed (Fig. 4). During the STSM this TEM pictures were further analyzed in the context of the biology and infection process of the pathogen..

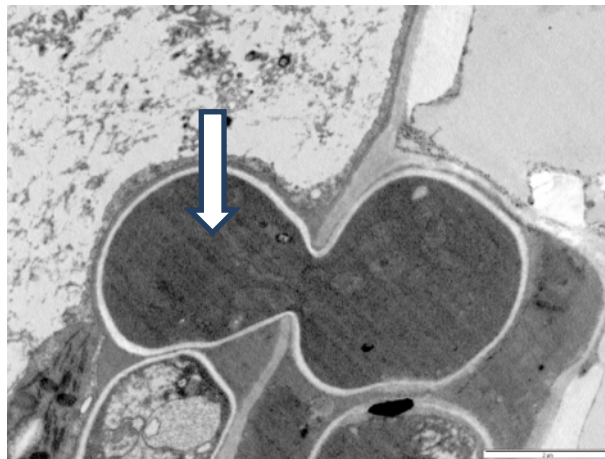


Fig. 2. *P. ambrosiae* hyphae (white arrow) do not immediately destroy the plant cell membrane in the penetrated plant cell when growing from one cell of the common ragweed tissue to another; the penetrating hyphae are first surrounded by a matrix deposited between the external part of the fungal cell wall and the cell membrane of the penetrated plant cell.

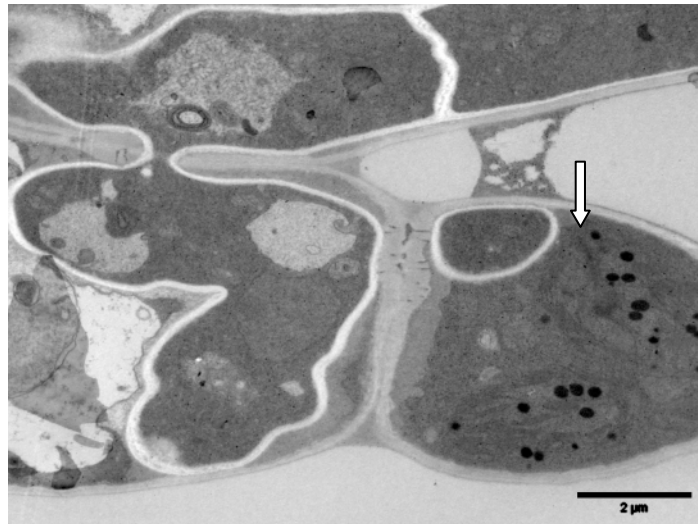


Fig. 3. *P. ambrosiae* hyphae in the cells of common ragweed; note the almost intact cytoplasm in the ragweed cell indicated with a white arrow.

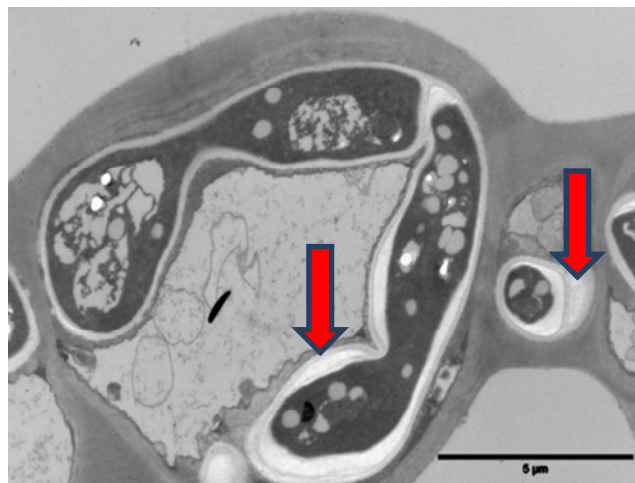


Fig. 4. *P. ambrosiae* hyphae in epidermal cells of a common ragweed leaf; note the thickened fungal cell walls in these cells (red arrows) with almost completely destroyed cytoplasm.

4. Understanding the presence of two phylogenetically different *P. ambrosiae* lineages in ragweed populations in different parts of the world

As mentioned in Result #1, the nrDNA ITS and 28S sequences newly determined in specimens FL3 and FL4 were identical to those determined by L. Kiss' group earlier in another North American *P. ambrosiae* specimen, BPI 880509. On the other hand, all the ITS and 28S sequences determined in a number of *P. ambrosiae* samples collected in Hungary, Ukraine and Korea by L. Kiss and his co-workers from 1999 to 2014, were identical to each other, and different from the North American ITS and 28S sequences, respectively. (The ITS sequences of the Eurasian and North American specimens differed in 45 nucleotide positions from each other.) This is a surprising result and clearly indicates that the Eurasian *P. ambrosiae* specimens are different from those examined from North America, representing two distinct, but closely related lineages (species?). Also, BLAST searches and, consequently, the phylogenetic analyses showed that the two *P. ambrosiae* lineages are only distantly related to any known lineages within the Phylum Ascomycota. This in itself makes these fungi very interesting from a fungal phylogenetic point of view.

Common ragweed was introduced from North America to Europe in the 19th century (Chauvel et al. 2006), and even later, probably in the 20th century, to some parts of Asia, such as Korea (Gaudeul et al. 2011). This noxious plant is an annual, reproducing exclusively by seeds, and *P. ambrosiae* is not known to be seed-transmitted. If we assume that *P. ambrosiae* has no host plant other than *A. artemisiifolia* in Eurasia, how can it be explained that its Eurasian ITS-haplotype spread so efficiently in the introduced Eurasian ragweed populations in less than 150 to 200 years and that the same haplotype is being found in distant regions, from Hungary to Korea? Also, it is clear that this Eurasian ITS-haplotype was not introduced from North America during the past one or two centuries, together with common ragweed, because the consistent 45 nucleotide difference in the ITS sequences of the North American and Eurasian haplotypes simply refute this possibility. Thus, our current working hypothesis, developed during this STSM, explains the results obtained with molecular markers in as described below.

5. Deciphering the complete life cycle of *P. ambrosiae* in *A. artemisiifolia* plants and explaining why/how is able to seriously damage its plant host in some years, and cause a huge reduction in its allergenic pollen production?

As described in Result #4, the idea of the spread of *P. ambrosiae* in the newly introduced Eurasian ragweed populations without any alternative hosts cannot be explained based on molecular data, and it is also hard to explain in the light of the life cycle of this pathogen as revealed by L. Kiss' group. The production of asexual spores, i.e. conidia, is unknown in this fungus. Apparently, ascospores are the only propagation structure of *P. ambrosiae*, and ascospores, in general, are not considered to contribute much to the long-distance dispersal of ascomycetes. Moreover, L. Kiss' group showed that the life cycle of this pathogen is completed on *A. artemisiifolia* with ascosporic infections, because, in greenhouse experiments, potted ragweed plants infected with *P. ambrosiae* ascospores collected from the field developed perithecia with mature ascospores in 20 to 25 days. These artificial infections were repeated successfully 4 times with new sets of potted ragweed plants, using ascospores produced in the previous inoculation experiments. These experiments demonstrated that ascospores support the polycyclic, within-season spread of *P. ambrosiae* in common ragweed populations; their role as overwintering inocula cannot be excluded, but attempts to demonstrate this failed.

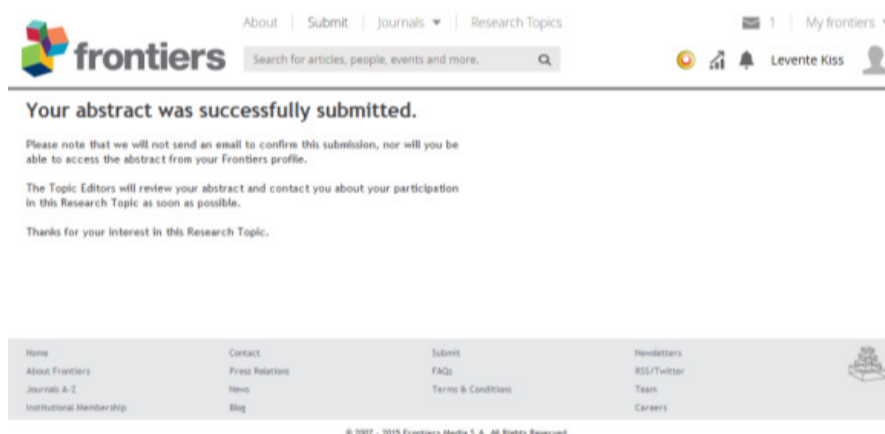
As mentioned above, *P. ambrosiae* is not known to be seed-transmitted neither is it known to be soil-borne. Alternative host plants of *P. ambrosiae* are unknown in either North America or Eurasia, although its presence in other plant species cannot be excluded. Common ragweed cannot be considered as an 'accidental host' of the Eurasian and/or North American lineages of *P. ambrosiae* because both herbarium specimens collected in 1912 and 1935 in the USA and deposited at BPI (Table 2), and our samples collected in Eurasia from 1999 to 2014 and in the USA in 2005 and 2014 clearly show that *P. ambrosiae* regularly infects *A. artemisiifolia*, although it was neither found every year nor consistently in high numbers, in most of the sampled areas.

Currently, as a result of the analysis of all the data available, we assume that the main host species of the Eurasian and North American lineages of *P. ambrosiae* should be perennial species, in which the *P. ambrosiae* infection has not been discovered yet, and which have been widespread in both Eurasia and North America long before common ragweed appeared in their environments. An annual plant, such as *A. artemisiifolia*, cannot support in itself the establishment, and evolution, of a mostly intracellular, obligate biotrophic ascomycetous pathogen which reproduces solely by ascospores, without the most typical within-season way of spread of this fungal group, i.e. production of asexual spores (conidia), and apparently lacks efficient overwintering structures, as well. Thus, it is unlikely that the existence of *P. ambrosiae* populations is limited to ragweed populations, especially because of the apparent lack of efficient mechanisms for season-to-season spread in this annual plant.

We suppose that *P. ambrosiae* may have, at least in part, an endophytic way of life in its main, and currently unknown, perennial host plants, and its 'jump' on *A. artemisiifolia*, which sometimes leads to devastating epidemics on this host, could be triggered by as yet unknown factors. This working hypothesis does not answer all the open questions concerning the enigmatic nature of the *P. ambrosiae* epidemics, but could explain why extensive sequencing of all kinds of endophyte-like fungi in a large number of plant species, done by different laboratories worldwide, have already revealed a few ITS sequences which are closely related to those determined in several *P. ambrosiae* specimens from common ragweed. Clearly, many more samples of *P. ambrosiae* from *A. artemisiifolia* would be needed for a more extensive study of this plant-pathogen interaction; however, it should be noted that this work included all the currently available herbarium and fresh *P. ambrosiae* samples worldwide (!) and analyzed molecular markers in all the samples where PCR amplification of fungal DNA markers was at all possible.

Concluding remarks

A reduction in the production of highly allergenic ragweed pollen using different weed management strategies, including biological control, is one of the main outcomes expected from SMARTER. *Phyllachora ambrosiae* is one of the fungal pathogens of common ragweed known to seriously reduce, although infrequently, its pollen production in the field (Vajna et al. 2000, Kiss 2007). To date, almost nothing is known about the background of the *P. ambrosiae* epidemics in common ragweed populations. During this STSM, we obtained new results, and analyzed these results in the light of all the data available in this respect (most of which are still unpublished), and developed a working hypothesis to explain the largely enigmatic nature of *P. ambrosiae* epidemics in ragweed populations. It is expected that all these achievements will be published in *Frontiers in Plant Science* (impact factor: 3.9/2014) based on an invitation received by L. Kiss. The pre-submission query, based on an extended abstract, has already been submitted to *Frontiers in Plant Science*:

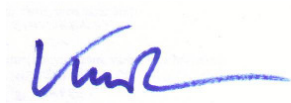


Contribution of the planned STSM to SMARTER

This STSM is in agreement with the objectives of WG1 and WG4 of SMARTER.

References

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