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Molecular identification and pathogenicity assessment of a rust fungus infecting common ragweed (Ambrosia artemisiifolia) in its native North American range

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Molecular identification and pathogenicity assessment of a rust fungus infecting common ragweed (*Ambrosia artemisiifolia*) in its native North American range

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Abstract

A rust fungus collected from common ragweed (*Ambrosia artemisiifolia*) in Texas, USA, was identified as belonging to the *Puccinia xanthii* morphospecies based on its nrDNA ITS sequence. Pathogenicity studies carried out with this rust accession under quarantine conditions in the UK showed that the fungus was highly virulent on *A. artemisiifolia* plants from Australia. Recently, *P. xanthii* has been proposed as a potential classical biological control agent (CBCA) for common ragweed in its invasive range, focusing on Europe, despite previous doubts about its biocontrol potential. The results of the pathogenicity tests reported here support the suitability of this pathogen as a CBCA for common ragweed.

Keywords: allergenic weed, classical biological control, fungal species concept, *Pucciniaceae*, *Pucciniomycetes*, invasive alien species

Introduction

Common ragweed (*Ambrosia artemisiifolia*) is a North American native that was introduced repeatedly and inadvertently to Europe in the eighteenth century (Chauvel et al. 2006; Gaudeul et al. 2011; Gladieux et al. 2011) and has since become invasive and problematic in a number of countries. Besides its economic impact on crop yield, this plant presents a major health and social problem because of its highly allergenic pollen. As a consequence, common ragweed has become the best-known alien weed in the affected European regions - namely Central and Eastern Europe, southern France, and northern Italy - due to campaigns launched to bring attention to this noxious weed (Kiss 2007a). Thereby, *A. artemisiifolia* has, like no other plant, raised the awareness of invasive plants in Europe (Gerber et al. 2011).

In addition to more traditional herbicide and mechanical control methods, biological control has also been considered as a strategy to deal with the ragweed invasion in Europe (Kiss 2007a; Gerber et al. 2011). Research into the suitability of fungal plant pathogens as classical biological control agents (CBCA) represents a special field of applied mycology and, in particular, some species of the rust fungi (*Pucciniomycetes*) have already successfully been used against invasive alien weeds (Evans 2013). Well known examples include: the Madagascan rust *Maravalia cryptostegiae* against *Cryptostegia grandiflora* (rubber-vine) and the South African rust *Puccinia myrsiphylli* against *Asparagus asparagoides* (bridal creeper), both exotic and invasive plant species in Australia; the rust *Uromycladium tepperianum* controlling its invasive Australian host *Acacia saligna* (Port Jackson willow) in South Africa; and, the Neotropical rust *Puccinia spegazzinii* employed successfully against invasive *Mikania micrantha* (mile-a-minute weed) in a number of Asian and South Pacific countries. Based on these successes, Gerber et al. (2011) suggested exploring the potential of natural

55 enemies, including the microcyclic autoecious rust *Puccinia xanthii*, for classical biological
56 control of common ragweed in its introduced range. Gerber et al. (2011) proposed to study the
57 *P. xanthii* lineage infecting *A. artemisiifolia* in its native range because it has been posited that
58 *P. xanthii* represents a morphospecies comprising distinct accessions each of which is
59 specialized to one or a few hosts within the Asteraceae (Seier et al. 2009). This has been
60 exemplified for the specific hosts *Xanthium occidentale*, *X. italicum*, *Parthenium*
61 *hysterophorus* and *A. trifida* (Batra 1981; Morin et al. 1993; Lu et al. 2004; Kiss 2007b, Seier
62 et al. 2009; Zhang et al. 2011). Two other little known rust species, the autoecious *P. conoclinii*
63 and the heteroecious *P. canaliculata*, have also been listed as pathogens of *A. artemisiifolia* in
64 the USA (Farr et al. 2015), but were not identified here as potential CBCAs of common
65 ragweed.

66 During the study of the narrow host specialization of selected *P. xanthii* lineages, Seier
67 et al. (2009) introduced the variety, *P. xanthii* var. *parthenii-hysterophorae*, for the rust
68 accession infecting *Pa. hysterophorus*, which has been released as a CBCA against this weed in
69 Australia (Tomley et al. 2004; Seier 2005). Seier et al. (2009) further concluded that other *P.*
70 *xanthii* lineages specialized on different asteraceous hosts should also be assigned varietal
71 status; however, to date, this has not been done. For example, the rust accession infecting giant
72 ragweed (*A. trifida*), but not *A. artemisiifolia* or other asteraceous species (Batra 1981; Lu et al.
73 2004; Zhang et al. 2011), should formally be described as a variety, although it was named by
74 Batra (1981) as a *forma specialis*, *P. xanthii* f. sp. *ambrosia-trifidae*. This specific accession
75 had already been proposed as a CBCA of invasive giant ragweed in China, even before it had
76 become widespread on *A. trifida* in that region (Lu et al. 2004; Zhang et al. 2011).

77 Amongst the *P. xanthii* lineages, the one infecting *A. artemisiifolia* is one of the lesser
78 researched varieties within this morphospecies and, to date, no detailed studies have been

undertaken with this lineage; however, pathogenicity studies were performed under quarantine conditions at CABI in the UK in the 1980s. Interestingly, while herbarium specimens document the presence of the rust on *A. artemisiifolia* in the USA between 1855 and 1963, attempts to re-collect this accession in the field in North America in 2002-2003 were unsuccessful (Kiss 2007b). This recent failure to find *P. xanthii* on *A. artemisiifolia* in the USA may be explained by the fact that the surveys were not conducted in most of the places where herbarium material had been collected previously (Kiss 2007b). Nevertheless, there is a lack of any data concerning *P. xanthii* on *A. artemisiifolia* in Canada, where other *P. xanthii* lineages commonly occur on *A. trifida* and *Xanthium* spp. (Parmelee 1977; Ginns 1986). This, in addition to the unsuccessful attempts to collect the rust on *A. artemisiifolia* in the USA, suggests that *P. xanthii* occurs only infrequently on *A. artemisiifolia*, possibly causing little damage and no noticeable epidemics. Thus, doubt has been cast on the suitability of this pathogen as a CBCA of common ragweed in its exotic range (Kiss 2007b).

However, the results presented here seem to contradict this scenario. We report as yet unpublished pathogenicity studies carried out in 1989 with a rust accession collected from *A. artemisiifolia* in Texas, USA (W. A. Palmer, pers. comm. 1989) and deposited as a voucher specimen in the CABI Herbarium (Herb IMI), now hosted by RBG Kew, under the accession number IMI 503827. These results were not published earlier because the identity of this rust has only recently been confirmed, based on a re-examination of the original herbarium specimen. The rust fungus had been tentatively identified as *P. xanthii* in 1989, based on morphology, but molecular support to confirm its identity was considered to be essential. Therefore, the main goals of this work were to (i) determine the internal transcribed spacer (ITS) sequence of the nuclear ribosomal DNA (nrDNA) in the rust specimen IMI 503827, and

compare this ITS sequence with that of other *P. xanthii* lineages, and (ii) report the pathogenicity tests carried out with this rust accession.

Materials and methods

Fungal and plant material

An accession of *P. xanthii* ex *A. artemisifolia* collected in Austin, Texas, USA, on 5 October 1989 was used for pathogenicity tests and molecular studies. Infected leaf material bearing telia free of hyperparasites was dried in a plant press, to prevent teliospore germination due to excess humidity during transport, and shipped to the quarantine facilities of the International Institute of Biological Control (IIBC) of CABI, Silwood Park, Ascot, UK. Upon arrival, teliospore material was used immediately for pathogenicity studies under quarantine greenhouse conditions. *Ambrosia artemisiifolia* plants were grown from seeds obtained from Australia (Queensland). Seeds were sown in seed trays filled with sterilized John Innes Seed Compost (2 parts sterilized loam : 1 part peat : 1 part sand; 0.6 kg ground limestone and 1.2 kg superphosphate added per m^3 of mix) and maintained at a temperature regime of 25/13°C day/night under natural light conditions. Established young plants were transplanted into 10 cm diameter plastic pots filled with a 1:1 mixture of John Bowers Multi Purpose Compost (containing peat, composted wood, green compost, fertiliser and non-ionic surfactant) and John Innes No. 2 soil-based compost (7 parts loam : 3 parts peat : 2 parts sand; 0.6 kg ground limestone, 2.4 kg hoof and horn meal, 2.4 kg superphosphate and 1.2 kg potassium sulphate added per m^3 of mix). Prior to experimental use, plants were maintained in a quarantine greenhouse fitted with negative pressure and HEPA filtration at an average temperature of 25°C day/20°C night, and an average relative humidity of 60% day / 80% night. Supplementary

lighting was provided by metal halide and sodium lamps (full spectrum, light intensity ranging from 8,000 to 13,000 lux) for 16 hours daily.

Pathogenicity tests

Pathogenicity studies were undertaken using vigorously growing *A. artemisiifolia* plants past the six leaf stage. Plants to be inoculated were placed in a dew chamber (Mercia Scientific, Birmingham, UK) underneath a fine mesh which was suspended at a distance of *ca* 5 cm above the foliage. Five to eight leaves per plant were inoculated by positioning pieces of rust-infected leaf material bearing up to three telia onto the mesh, telia facing down, directly above individual leaves. Plants were kept in the dew chamber running at 18 °C for 48 h and then removed and maintained in a designated greenhouse compartment under controlled temperature, relative humidity and light conditions, as outlined above. Inoculated plants were assessed at three-day intervals over a four-week period for the appearance of disease symptoms, in the form of leaf chlorosis and telia formation. Three replicate plants were used and the experiment was repeated once. All plant and fungal material used in the quarantine facility was incinerated after the study.

DNA extraction and PCR amplification of the nrDNA ITS region

To extract the total genomic DNA from the IMI 503827 specimen, teliospores were picked up with sterile glass needles under a dissecting microscope, or small pieces of infected host materials were excised from the dried leaves, placed in Eppendorf tubes, and processed using a DNeasy Plant Mini Kit (Qiagen). The nrDNA ITS region was PCR-amplified separately in five DNA samples obtained as described above using the rust specific primers ITS5-u and ITS4-u

148 (Pfunder et al. 2001). PCRs were done in 20 µl total volume containing 10 µl Dream Taq Green
149 Master mix (Fermentas), 0.75 µl DMSO, 50 pmol of each primers (SIGMA), 6.25 µl mQ water,
150 and 2 µl isolated genomic DNA template. PCR conditions were as follows: 5 min at 94ºC
151 followed by 35 cycles of 45s at 94ºC , 45s at 50 ºC and 1 min at 72 ºC, followed by 10 min at
152 72 ºC.

153

154 Cloning and sequencing of the ITS region

155 PCR products were purified with a PCR Clean up-M kit (Viogene, Hong-Kong, China) and
156 cloned into a pGEMT Easy Vector system (Promega, Madison, WI, USA). The purified
157 amplicons were A-tailed using a normal Taq polymerase and dATP (MBI Fermentas, Vilnius,
158 Lithuania) before cloning, and purified again using the PCR Clean up-M kit. Subsequent steps
159 of the cloning procedure were performed as described by Kovács et al. (2007). At least three
160 positive clones from each amplicon were sent for sequencing to LGC Genomics (Berlin,
161 Germany) using universal primers. Altogether, the ITS region was successfully sequenced in 12
162 clones.

163

164 Data analysis

165 Sequences were compiled from electrophoregrams using using Pregap4 and Gap4 (Staden et al
166 2000), aligned with Multalin (Corpet 1988) and subsequently checked and adjusted manually
167 with ProSeq 2.9 (Filatov 2002). The newly obtained sequences were aligned together with
168 those reported by Morin et al. (2009) and Seier et al. (2009) for *Puccinia* spp. Further, more than
169 90% similar, ITS sequences were also sourced from GenBank using BLAST searches.

Results and Discussion

The ITS sequence for the herbarium specimen IMI 503827 was deposited in GenBank under the accession number KM114871. The sequence is 553 bp long and was identical in all the 12 clones sequenced. This is important to note because in some rust specimens, ITS sequences can exhibit considerable intra-sample variability, occasionally up to a few dozen variable nucleotide positions (e.g., Alaei et al. 2009; Feau et al. 2011; Tanner et al. 2015). The ITS sequence in the *P. xanthii* specimen studied here is identical to that of *P. xanthii* var. *parthenii-hysterophorae* collected from *Pa. hysterophorus* in Australia (EU659697), and approximately 98% similar to four other accessions of *P. xanthii* which, in turn, were identical to each other despite their diverse geographic origins (Fig. 1). During BLAST searches, no other ITS sequences showed more than 90% similarity with the sequence determined in the rust accession used in greenhouse tests. This strongly suggests that the rust isolate tested in quarantine in the UK in 1989, is indeed a *P. xanthii* accession. Also, it has become clear that this group of rusts, forming the *P. xanthii* morphospecies, and its closest relatives, are still poorly known from a molecular point of view, currently being represented by only six ITS, and a very few other DNA sequences in GenBank. Although the ITS sequence of the *P. xanthii* accession used in the pathogenicity tests is identical to that of *P. xanthii* var. *parthenii-hysterophorae*, preliminary cross-inoculation studies showed that *Pa. hysterophorus* was not susceptible towards the rust accession ex *A. artemisiifolia*. Conversely, the rust lineage ex *Pa. hysterophorus*, introduced as a CBCA for this invasive plant in Australia, proved not to be infective to *A. artemisiifolia* (unpublished data).

All pathogenicity tests undertaken with the rust accession ex *A. artemisiifolia* from Austin, Texas, resulted in heavily infected *A. artemisiifolia* plants grown from seeds collected

in Australia. Disease symptoms first became visible as chlorotic leaf spots which appeared, on average, nine days after inoculation, with telia formation commencing after a further 2-3 days. Telia developed predominantly on the lower leaf surface, spreading outwards from the centre of the initial chlorotic lesion. Over time, the disease progressed and on some inoculated plants sporulation covered most of the lower leaf surface, frequently including the petiole (Figure 2a and b).

The virulence of the rust accession observed during our greenhouse studies contradicts to some extent the reported "elusiveness" of the pathogen encountered during recent field surveys in the USA (Kiss 2007b). It could be assumed that such a virulent pathogen should be more widespread, unless the host is able to occupy a wider ecological niche than the fungus. Such a scenario has been documented for two rust species infecting *Pa. hysterophorus* in its native range in Mexico: *Puccinia abrupta* var. *partheniicola*, the winter rust, being restricted to the dry cool highlands (>700 m); whilst *P. melampodii*, the summer rust (= *P. xanthii* var. *parthenii-hysterophorae*), occurs only in the humid sub-tropical regions, below 600 m (Evans 1997, 1998; Evans and Ellison 1990). Theoretically, therefore, it is possible that *A. artemisiifolia* is able to persist in regions where critical abiotic factors, such as temperature are suboptimal for severe rust infection. However, even if this assumption was correct, it would be expected that *P. xanthii* should be more abundant on its host in some areas of its North American range. The *P. xanthii* accessions infecting *Xanthium* spp. and *A. trifida*, respectively, are widespread in North America wherever their host plants are found (Parmelee 1977; Ginns 1986; Farr et al. 2015), and it is unlikely that their climatic requirements are very different from those of the accessions infecting common ragweed. Another possible explanation for the scarceness of *P. xanthii* on *A. artemisiifolia* in the USA could be that the native *A. artemisiifolia* biotypes have developed an increased resistance towards the rust, which would

either enable the plant to tolerate the pathogen without exhibiting symptoms of disease, or prevent fungal infection altogether. In contrast, however, the Australian biotype of *A. artemisiifolia* used during our pathogenicity tests proved to be highly susceptible to the rust accession from Texas, under the prevailing optimum conditions for spore germination and infection.

Clearly, more comprehensive cross-inoculation studies are needed to ascertain the host specificity and varietal status of the rust lineage from *A. artemisiifolia.* A more detailed molecular characterization of this lineage - based, for example, on sequences of the translation elongation factor (TEF) gene available for some *P. xanthii* accessions (Seier et al. 2009) - would also facilitate its taxonomic classification. Our attempts to amplify the TEF gene in the herbarium specimen IMI 503827 failed, thus the ITS sequence reported here is the only molecular marker currently available for this fungus.

Marigold (*Calendula officinalis*) is of particular interest in host range studies, since this non-host species has previously been shown to be susceptible to *P. xanthii* lineages in host-range screening studies (Alcorn 1976; Seier et al. 1997). However, to our knowledge, no viable *P. xanthii* accessions infecting *A. artemisiifolia* are currently available worldwide; therefore, at present, it is not possible to carry out pathogenicity tests with this rust. More extensive surveys focusing on sites in North America where the *P. xanthii* on *A. artemisiifolia* has been previously collected, and more detailed studies with newly collected isolates are needed to investigate the suitability of this rust as a CBCA of *A. artemisiifolia* outside the native range of its host plant, especially in Europe.

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350 FIGURE LEGENDS

351 **Fig. 1.** Nucleotide positions with variable characters detected when the nrDNA ITS sequences
352 were compared in the following six *Puccinia xanthii* specimens: (1) the isolate used in this
353 work (IMI 503827 / KM114871*); (2) *P. xanthii* var. *parthenii-hysterophorae* collected from
354 *Parthenium hysterophorus* in Australia (BRIP 51793 / EU659697); (3) *P. xanthii* collected
355 from *Xanthium italicum* in Hungary (BRIP 48819 / EU659694); (4) *P. xanthii* collected from *X.*
356 *strumarium* sensu lato in Brazil (BRIP 48822 / EU659695); (5) *P. xanthii* collected from *X.*
357 *strumarium* sensu lato (BRIP 48821 / EU659696); and (6) *P. xanthii* collected from *X.*
358 *occidentale* in Australia (BRIP 49131a / EF635903).

359

360 *voucher / GenBank accession number of the ITS sequence; BRIP = Plant Pathology
361 Herbarium, Queensland Department of Primary Industries and Fisheries, Australia; IMI =
362 CABI Herbarium (Herb IMI), Kew Gardens, London, UK.

363

364 **Fig. 2.** *Ambrosia artemisiifolia* severely infected with *Puccinia xanthii* in a quarantine
365 greenhouse at CABI: **a.** leaf showing telia on the lower surface and the petiole; **b.** extensive
366 telial sporulation causing leaf necrosis and die-back.

367

line figure Click here to download line figure Fig 1 Puccinia xanthii on Ambrosia.pdf

ITS sequence accession number	Nucleotide positions with variable characters*						
	63–65	111	133–138	144	154	175	502
KM114871	ttt	c	------	c	–	t	a
EU659697	ttt	c	------	c	–	t	a
EU659696	a--	t	tttttt	a	t	–	g
EU659695	a--	t	tttttt	a	t	–	g
EU659694	a--	t	tttttt	a	t	–	g
EF635903	a--	t	tttttt	a	t	–	g

**Nucleotide positions were numbered starting with the first position in the KM114871 sequence.*

colour figure

Click here to download colour figure P xanthii - Fig 2a HCE.jpg

colour figure

Click here to download colour figure P xanthii - Fig 2b HCE.jpg

REPLY TO THE EDITOR'S AND THE REVIEWER'S COMMENTS

MANUSCRIPT NO.: EJPP-D-15-00300
TITLE: "Molecular identification and pathogenicity assessment of a *Puccinia xanthii* accession infecting common ragweed (*Ambrosia artemisiifolia*) in its native North American range"
AUTHORS: Kassai-Jáger et al.

REPLY TO THE EDITOR'S COMMENTS

Dear Professor Jeger,

We are very grateful for your comments on our work and also for judging it as suitable for publication in EJPP after a major revision. Apologies for the very long delay in submitting the revised version of this work.

Please find below our point-by-point replies and reactions to the Reviewer's comments. The manuscript was fully revised in line with these comments.

REPLY TO THE REVIEWER'S COMMENTS

We are grateful to the Reviewer for his/her comments, shown in red colour below, and also for the time spent on reviewing our work. We do hope our reply to the comments and the changes made during revision are appropriate and the revised version is suitable for publication in EJPP.

Our point-by-point answers to the specific comments are as follows:

I would change 'Puccinia xanthii' to 'rust fungus' in the title since it doesn't make sense to have 'Molecular identification.... of a Puccinia xanthii accession'... How can you identify something that you have already given a name to?

Title changed as suggested.

The abstract will most likely have to be rewritten after the paper has been revised.

Done.

I must say I struggled while reading the introduction. It just didn't flow well. Starting with a paragraph on rust fungi used for weed biological control is fine, but it should be followed with a paragraph on common ragweed, providing details on its importance in Europe, some info on its biology and why biological control is being considered.

The Introduction was fully re-structured based on these comments. The revised Introduction starts with a paragraph on common ragweed and continues with the first paragraph of the original Introduction, etc.

Then it would be logical to focus on the rust fungi known to occur on common ragweed in the USA (P. conoclinii, P. canaliculata and P. xanthii) and why the most promising is P. xanthii (i.e. known to represent a morphospecies that comprises different lineages each specialized on different hosts and thus highly specific; doesn't have an alternate host (as P. canaliculata does); successfully used for biological control of other weeds).

It is beyond the goals of this work to evaluate the biocontrol potential of *P. xanthii*, *P. canaliculata* and *P. conoclinii* against *A. artemisiifolia*. Therefore, we did not address this question in the manuscript, neither in the original nor in the revised version, but mentioned these two other rust species right after introducing the paper by Gerber et al. (2011), as suggested by the Reviewer. Gerber et al. (2011) identified *P. xanthii* only as a promising CBCA of *A. artemisiifolia*, and, as explained in our manuscript, it was their paper which triggered our work.

Following this I would include details of where it has been recorded over the years and the failed attempts to collect the specific common ragweed lineage of P. xanthii in 2002-03).

We think it is important to expand the narrow host specialization issue in *P. xanthii* first, as done in the original submission. We did this together with the presentation of the taxonomic aspects of the host range issue which is another side of the same problem, and has to be addressed here because it mirrors the results of host range tests. During revision, we deleted the term 'taxonomy' because the taxonomic issue is not the main message from this part and we think it was misleading to mention this term here.

By the way, this part (the next two paragraphs) contains all the information requested by the Reviewer: we mentioned here *P. xanthii* records in different parts of the world (and the papers cited here contain even more information in this respect) and also the absence of this rust in the surveyed areas of the USA in 2002-2003.

The last paragraph of the introduction should then clearly states what this paper is about, i.e. report on results from i. sequencing of the rust accession collected in 1989 from common ragweed in Texas confirming that it belongs to P. xanthii morphospecies and ii. pathogenicity tests of the rust accession on common ragweed plants from Europe.

Done.

You need to first present results confirming identification and then results from pathogenicity tests. Although these activities were done in reverse, it just doesn't work for the 'story' to present them in chronological order.

We had to explain first where does the herbarium specimen examined with molecular tools come from - we think this is unavoidable before listing the goals (i) and (ii) as suggested by the Reviewer and as it was done in the revised version. Following this explanation, we re-wrote all the parts of the manuscript, including the Results and Discussion part, in line with the Reviewer's suggestions.

I don't think you have to justify why results from the pathogenicity tests have not been published before and keep on repeating throughout the paper that this work was done in 1989.

All parts dealing with this issue were re-written during revision to avoid this repetition.

In the materials and methods, you should be consistent and use pathogenicity tests throughout and not interchange with 'inoculation studies' and 'greenhouse studies'.

Done.

It would be good if the composition of the John Innes products used was included in parentheses.

We added the composition of all the compost types used in this work to the manuscript. However, it should be noted that the seeds of *A. artemisiifolia*, which is a pioneering plant, germinate in almost any kind of soil, thus the lack of information on the exact composition of the composts used would not affect the repeatability of the pathogenicity tests.

P6, L36-39: I don't understand why you have this sentence, considering that you give precise conditions above.

This was a mistake, the sentence was deleted during revision.

You need to state in results and discussion that you obtained similar results in each set of pathogenicity tests performed (I assume it was the case - if not then elaborate).

Done.

P9, L1-10: Your argument here is tenuous. The severe symptoms you obtained in your tests do confirm that the rust accession used was pathogenic on common ragweed, but it doesn't mean that you would necessarily see such symptoms in the field. Plants in your tests were placed for 48 h in a dew chamber - this is not typical field conditions. The common ragweed P. xanthii lineage may be rare in the field simply because environmental conditions are sub-optimal for disease development where the host plant occurs.

We carefully considered each part of our arguments listed in this paragraph and we still think we cannot provide a better discussion of our results. The Reviewer's idea, i.e. this rust is rare because environmental conditions are sub-optimal, was mentioned in this paragraph even in the original submission, but was immediately rejected because other *P. xanthii* lineages infecting *Xanthium* spp. and *A. trifida* are widespread in North America, and it is unlikely that their climatic requirements are very different from those of the accessions infecting common ragweed.

P10, L36 & L41: HC Evans and MK Seier are authors on this paper so it should be 'unpublished data' not 'personal communication'.

Corrected.

P11, L1-7: This sentence is totally out of place.

We do not agree with this comment: it is important to highlight in the discussion that previously some genotypes of *Calendula officinalis* were susceptible to different *P. xanthii* lineages; these infected only their host plants of origin AND *C. officinalis*. To separate this part from the previous one, we placed the information concerning *C. officinalis* in a new paragraph.

P11: You need to add at least one additional paragraph at the very end of the paper to wrap up. The first thing that came to my mind is how you plan to source an accession of the rust fungus for further research considering previous failures. Will you keep on surveying and hoping for the best? Will you rely on collaborators in the US? It would also be good to elaborate on what would be the key research activities that would be undertaken once an accession is found. It is always good to finish a paper by opening up.

Done.

Once again, we would like to acknowledge all the comments on our manuscript. We do hope our replies to the comments and the changes made during revision were appropriate and the revised version is suitable for publication in EJPP.

Sincerely,

Levente Kiss
Corresponding author for this submission