agronomy

MDPI

*Article*

# Development of Quantitative Real-Time PCR Assays to Quantify *Erysiphe pisi* and *Erysiphe trifolii* and Its Implementation for Monitoring Their Relative Prevalence in Pea Crops in Spain and Tunisia

**Sara Fondevilla [1,*], Mª José González-Bernal [1], Noura Omri Ben Youssef [2] and Diego Rubiales [1]**

[1] Institute for Sustainable Agriculture, CSIC, Avda. Menéndez Pidal s/n, 14004 Córdoba, Spain; mjgb1988@gmail.com (M.J.G.-B.); diego.rubiales@ias.csic.es (D.R.)
[2] Field Crop Laboratory, National Agronomic Research Institute of Tunisia, Carthage University, Hedi Karray Street, Ariana 2049, Tunisia; noura.mori@gmail.com
* Correspondence: sfondevilla@ias.csic.es

**Abstract:** *E. pisi* was thought to be the only causal agent of powdery mildew in peas, with three genes, *er1*, *er2* and *Er3*, conferring resistance to this pathogen. Recently, *E. trifolii* has also been found to cause this disease in peas in different countries, but its relevance in pea powdery mildew disease worldwide is unknown. The objective of this study was to develop a method to identify and quantify *E. pisi* and *E. trifolii* and use it to analyze the relative prevalence of *E. pisi* and *E. trifolii* in pea fields in Spain and Tunisia. We also wanted to discern the effect of the *er1*, *er2* and *Er3* resistance genes on the relative amount of *E. pisi*/*E. trifolii*. Using the polymorphic sites present between *E. pisi* and *E. trifolii* ITS sequences, we developed a qPCR method capable of identifying and quantifying these pathogens. Our results revealed, for the first time, the occurrence of *E. trifolii* in Tunisia and that the presence of *er1*, *er2* and *Er3* genes have a clear effect on the ratio *E. pisi*/*E. trifolii* in both countries.

**Keywords:** pea powdery mildew; *Erysiphe pisi*; *Erysiphe trifolii*; qPCR; resistance; field pea



**Citation:** Fondevilla, S.; González-Bernal, M.J.; Omri Ben Youssef, N.; Rubiales, D. Development of Quantitative Real-Time PCR Assays to Quantify *Erysiphe pisi* and *Erysiphe trifolii* and Its Implementation for Monitoring Their Relative Prevalence in Pea Crops in Spain and Tunisia. *Agronomy* **2022**, *12*, 334. https://doi.org/10.3390/agronomy12020334

Academic Editor: Gavin Ash

Received: 21 December 2021
Accepted: 24 January 2022
Published: 28 January 2022



## 1. Introduction

Pea powdery mildew is an airborne disease of worldwide distribution, being particularly important in late sowings, reducing yield and quality [1]. Until recently, *Erysiphe pisi* DC was thought to be the only causal agent of powdery mildew in peas (*Pisum sativum* L.) and three genes for resistance to *E. pisi*, named *er1*, *er2* (recessive genes) and *Er3* (dominant gene) [2–4] have been described so far. However, *E. trifolii* Grev. has also been found to infect peas in different countries, including the USA, India and Spain [5,6]. Most pea breeding programs in past decades relied solely on the use of *er1*, whose resistance was considered durable [7]. However, *E. trifolii* is able to break down the resistance conferred by *er1* [5,6], reinforcing the need to monitor its distribution and to consider resistance to *E. trifolii* as well in pea breeding programs.

Unfortunately, *E. pisi* and *E. trifolii* cannot be visually distinguished by the symptoms they cause. The two species can be differentiated by nrRNA internal transcribed spacer sequence (ITS) and teleomorphid features, mainly morphology of chasmothecial appendages [5,8]. However, measurement of teleomorphid features is not easy and is highly time consuming, being also not applicable to quantify the presence of both species when co-infecting the same leaf. Therefore, other methods need to be developed to study the relevance of these pathogens in pea powdery mildew disease worldwide.

The objectives of this study were: (1) To develop a quantitative real-time PCR (qPCR) assay to identify and quantify *E. pisi* and *E. trifolii* in pea infected samples, (2) to analyze the relative prevalence of *E. pisi* and *E. trifolii* in pea fields in Spain and Tunisia, (3) to discern the effect of *er1*, *er2* and *Er3* resistance genes on the relative amount of *E. pisi*/*E. trifolii*

infecting a leaf, and (4) to know whether the relative amount of *E. pisi*/*E. trifolii* changes along the crop cycle, and between leaves and stems in the case of *er2* carrying lines.

## 2. Materials and Methods

### *2.1. Development of a qPCR Assay to Identify and Quantify E. pisi and E. trifolii*

*E. pisi* isolate CO-10A and *E. trifolii* isolate CO-11B were used to obtain pure DNA from these fungal species for the qPCR probes. These isolates were derived from single colony isolates and their identity was confirmed by sequencing their ITS sequences [6]. The DNA was extracted from fresh spores of these isolates using the CTAB method described by [9], including RNase treatment with RNase A (Panreac Química SLU). The DNA concentration in each of the samples was quantified by qubit fluorometric quantitation. Integrity of DNA was checked on agarose gels and its purity was determined using a NanoDropND1000 (NanoDropTechnologies, Inc., Wilmington, NC, USA).

ITS sequences from *E. pisi* and *E. trifolii* were retrieved from the Gene Bank database and aligned using "Multiple Alignment" tool included in the Geneious Prime version 2020.1.2 (Biomatters Ltd., Auckland, New Zealand) software, with default parameters. Based on this alignment, consistent polymorphic sites between these species were identified. Then, primer pairs suitable for qPCR amplifications were designed including these polymorphic sites to specifically amplify *E. pisi* or *E. trifolii*. Primers were designed using Probe Finder version 2.53 (Roche, Basel, Switzerland) and its specificity was checked using the Primer-Blast NCBI tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/ accessed on 24 July 2017). Two primer pairs per species (EPFw1/EPRv; EPFw2/EPRv; ETFw1/ETRv1; ETFw2/ETFw2) were tested. The sequence of these primers is shown in Table 1. Each set of primers was used to amplify DNA from *E. pisi*, from *E. trifolii* and from peas. Polymerase chain reactions were performed in a StepOne Real Time PCR System (Applied Biosystems, Foster City, CA, USA), using SYBR Green to monitor dsDNA synthesis. Reactions contained 5 µL Fast Start Universal SYBR Green Master (ROX), 1 µL (1 ng) DNA, and 0.3 µM of each gene-specific primer in a final volume of 10 µL. The following standard thermal profile was used for all PCR reactions: Polymerase activation (95 °C for 10 min), amplification and quantification (40 cycles; 95 °C for 15 s, annealing temperature for 1 min) and dissociation curve generation (95 °C for 1 min, 60 °C for 15 s, 95 °C for 15 s). In order to optimize the qPCR assay, three different annealing temperatures (60 °C, 62 °C and 64 °C) were tested. The PCR efficiency of each primer pair was calculated using LingRegPCR version 7.5 software [10].

**Table 1.** Primer sequences for the amplification of *Erysiphe pisi* or *Erysiphe trifolii* by qPCR.

| Primer | Species | Sequence |
|---|---|---|
| EPFw1 [1] | *Erysiphe pisi* | GCTCAGTCG**T**GGC**A**TCTGC**T** [2] |
| EPFw2 | *Erysiphe pisi* | AGGCTCAGTCG**T**GGC**A**TCTGC**T** |
| EPRv | *Erysiphe pisi* | GGCCCGCCAAAGCAACAAGA |
| ETFw1 | *Erysiphe trifolii* | GTCGCTGT**T**CGCA**A**GGA |
| ETRv1 | *Erysiphe trifolii* | AGCTGAGACGACACAAACAA |
| ETFw2 | *Erysiphe trifolii* | TACAGAGTGCGAGGCTCA |
| ETRv2 | *Erysiphe trifolii* | GCA**G**GTCC**T**TGCG**A**ACA |

[1] Fw indicates forward primer and Rv reverse primer. [2] Polymorphic sites between *E. pisi* and *E. trifolii* ITS sequences are shown in bold.

### *2.2. Analysis of the Relative Prevalence of E. pisi and E. trifolii in Pea Fields in Spain and Tunisia*

The pea accessions JI2302 (carrying *er1* resistance gene), JI2480 (carrying *er2* gene), cv. Eritreo (carrying *Er3* gene) and cv. Messire (susceptible) were sown in different locations of Spain (Córdoba and Almodóvar del Río) and Tunisia (Mornag and Béja) during different seasons (Figures 1–4). Plants were sown in a randomized complete block design with three blocks, each block having 1 m row with 20 seeds of each accession. Naturally infected leave samples were taken from each genotype and replicated in these experiments. In the

case of the line carrying the *er2* gene, as in Spain and in the experiments carried out by other authors, this gene only confers leaf resistance [3,11]; leave and stem samples were taken separately in the experiment performed in Córdoba during 2017. We also wanted to know whether the relative prevalence of *E. pisi* and *E. trifolii* changes along the crop cycle. Therefore, in the experiments carried out in Spain, leave samples were taken at different stages of the crop cycle. Additionally, leaf samples were collected in commercial pea fields in Spain (pea fields at Espiel and Santaella) and Tunisia (pea fields at Bizerte, Kairouan and El Kef).

Pea samples from Spain were taken from the fields and stored at −80 °C until DNA extraction. Samples taken from Tunisian fields were lyophilized. The DNA was extracted using the CTAB method described by [9] including RNase treatment with RNase A (Panreac Química SLU). DNA was quantified using qubit fluorometric quantitation. The DNA integrity was checked on agarose gels and its purity was determined using a NanoDropND1000 (NanoDropTechnologies, Inc., Wilmington, NC, USA).

To quantify *E. pisi* and *E. trifolii* in these samples, 1 ng of their DNA was subjected to qPCR using the specific primers developed for *E. pisi* and *E. trifolii* and the protocol described above. A dilution series of pure DNA from *E. pisi* and *E. trifolii* (10, 1, 0.1, 0.01, 0.001, 0.0001, 0.00001 and 0.000001 ng/uL) were used in each qPCR assay, in duplicate, as a template, for constructing standard curves. Low inter-assay variation was confirmed by checking the reproducibility of standards indicating no inhibition of the target amplification. Calibration curves were generated and their correlation coefficient, efficiencies and slopes were calculated using the software StepOne v 2.1(Applied Biosystems, Foster City, CA, USA).

## 3. Results and Discussions

### *3.1. Development of a qPCR Assay to Identify and Quantify E. pisi and E. trifolii*

*E. pisi* was thought to be the only causal agent of powdery mildew in pea till Attanayake et al. [5] described *E. trifolii* causing powdery mildew symptoms on peas in the USA. Since then, *E. trifolii* has also been identified infecting *er1* pea lines in Spain and India [6], suggesting that *E. trifolii* might be very widely distributed, even when not studied in many countries. In fact, *E. trifolii* has also been reported infecting other legume crops close to peas, such as lentils, grass peas and red peas [12,13], that can serve as a green bridge for spore dispersion, reinforcing the need to monitor *E. trifolii* distribution. The fact that resistance against *E. pisi* and *E. trifolii* might have different genetic controls not only on peas [6] but also on related species [13] reinforces the need to run specific *E. trifolii* resistance breeding in peas. This is complicated by the difficulty to distinguish between *E. pisi* and *E. trifolii* symptoms. Both species can be distinguished using morphological characters, but this method is highly time consuming, if even possible, as cleistotecia are not always formed, and is unsuitable for a fast analysis of the amount of each species infecting a plant. Therefore, there is a general lack of knowledge about the relative relevance of *E. trifolii* as a pea pathogen in pea producing areas. Our study provided a useful tool to fill this gap. We took advantage of the polymorphism present between *E. pisi* and *E. trifolii* ITS sequences to develop a quantitative real-time PCR method that enables the fast identification and quantification of *E. pisi* and *E. trifolii*.

Two primer pairs per species and three annealing temperatures were tested for the qPCR amplifications. According to the specificity and efficiency obtained, the primer pairs EPFw1/EPRv (amplicon 78 pb) for *E. pisi* and ETFw2/ETRv2 (amplicon 123 pb) for *E. trifolii* and an annealing temperature of 62 °C was selected for further qPCR assays. For the primers selected, efficiency at this temperature was 2 for the primers specific for *E. pisi* and 1.927 for the primers specific for *E. trifolii*, and the melting curves showed that only one target sequence was amplified. Assays confirmed that the selected primers were specific for *E. pisi* or *E. trifolii*. Thus, these primers correctly amplified the ITS sequence when using DNA from their specific species but did not produce amplification when using DNA from the other *Erysiphe* species or from *Pisum sativum* (only a residual presence of unspecific

(off-target) signals yielding Cq values >31 in the non-target species, compared to the Cq values around 16 obtained for the target species, was observed; see Table 2). Therefore, these primers are suitable for the identification and quantification of *E. pisi* and *E. trifolii* in any pea sample.

**Table 2.** Quantification cycle (Cq) values of EPFw1/EPRv and ETFw2/ETFw2 primer pairs analyzed in DNA from *Erysiphe pisi*, *Erysiphe trifolii* and *Pisum sativum* using 62 °C as the annealing temperature.

| Primer Pair | Species | | |
|---|---|---|---|
| | ***E. pisi*** | ***E. trifolii*** | ***Pisum sativum*** |
| EPFw1/EPRv | 16.45 | 31.02 | 31.93 |
| ETFw2/ETFw2 | 34.44 | 16.46 | 36.99 |

Our quantitative real-time PCR method was successfully used by us to study the relative prevalence of these two species in Spanish and Tunisian pea fields, and could be used to carry out similar studies in other countries. Molecular techniques have been previously widely used as a fast and reliable method to identify and quantify pathogens in samples. In particular, the nrRNA internal transcribed spacer sequence (ITS) is highly conserved within individuals from the same species and has been used in many studies as a reliable tool to identify and quantify different organisms [14–16].

### *3.2. Analysis of the Relative Prevalence of E. pisi and E. trifolii in Pea Fields in Spain and Tunisia*

A linear response was observed over the serial dilutions included. Standard curves obtained in the different plates were suitable for the quantification of both pathogens, showing good correlation coefficient ($R^2$) values (ranging from 0.842 to 0.998, being generally >0.98), and efficiencies between 84.199 and 125.629. Linear regression slope values ranged from −3.962 to −2.267. According to the results obtained in the qPCRs assays, the amount of *E. pisi* and *E. trifolii* was estimated in the different pea samples and used to calculate the percentage of *E. pisi* and *E. trifolii*. The percentages of each pathogen in the different lines and experiments are shown in Figures 1–4.

Our study shows, for the first time, the presence of *E. trifolii* infecting peas in Tunisia, being even more important than in Spain, as, in most of the lines studied, the percentage of *E. trifolii* compared to *E. pisi* was higher than in Spain. It is also shown that *E. trifolii* is not only able to infect *E. pisi*-resistant pea lines carrying the *er1* gene, but also any pea line carrying either *er2* or *Er3*, or none of them. Still, the presence of *er1*, *er2* and *Er3* genes have a clear effect on the relative prevalence of *E. pisi* and *E. trifolii*.

JI2302 (*er1*) showed incomplete resistance in Spain and Tunisia, with powdery mildew symptoms starting later and progressing slower than on the susceptible accessions. For instance, at Córdoba 2017, disease severity (DS) on JI2302 was only 10% by the first scoring date (3 May), when it was already 100% on cv. Messire. DS on JI2303 increased to a maximum of 30% by 16 May. Similarly, in the following season (Córdoba 2018) DS on JI2302 was 28% by the first scoring date, 11 May, reaching a maximum of 32% by 22 May, when it was already 100% on Messire by 11 May. DS scores in Tunisia also show a reduced powdery mildew on JI2302, with DS 43% at Mornag 2017 compared to 90% on Messire, and 5% on Béja 2019, compared to 92% infection of Messire. Analysis of the infected samples showed that the pathogen infecting *er1* plants was mostly *E. trifolii* (>98% in all cases) (Figure 1). These results confirm previous studies reporting the stable resistance that the *er1* gene confers to *E. pisi* and the capability of *E. trifolii* to break this resistance down [6,17].

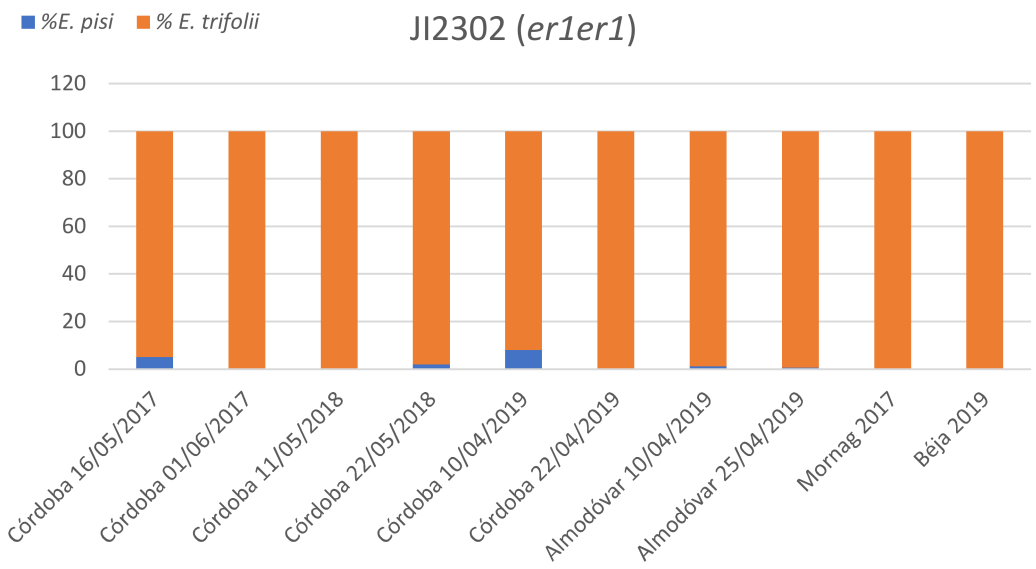


**Figure 1.** Percentage of *E. pisi* and *E. trifolii* in the different samples collected in accessions JI2302. Percentages shown are the average of three biological replicates. Each sample was named by the location where it was taken, followed by the date when it was sampled.

The *er2* resistance gene remained highly effective protecting against powdery mildew infection in Spain, with accession JI2480 displaying only very few symptoms by the end of the crop cycle. Our study showed that these few symptoms are caused mainly by *E. pisi* (Figure 2). These results are in agreement with previous studies showing that *er2* resistance to *E. pisi* is temperature- and leaf age-dependent, with complete resistance expressed only at high temperatures (25 °C) or in mature leaves [17]. In Tunisia, this line could be more infected, but still showing incomplete resistance (DS lower than 27%). The species infecting JI2480 in Tunisia was also mainly *E. pisi*, but the percentage of *E. trifolii* increased compared to Spain. Expression of *er2* against *E. trifolii* was not affected by growing temperatures in the range of 20–25 °C in a previous study [6], but this outcome suggests that resistance to *E. trifolii* conferred by the *er2* gene can also be influenced by other environmental conditions or other variables not known yet. Therefore, more studies are needed to clarify environmental effects on the expression of *er2* resistance against *E. trifolii*.

The few powdery mildew symptoms observed in Spain on JI2480 (*er2*) were mainly on stems rather than on leaves (in leaves only a thin mycelium was observed in the lower side of the leaves). Tiwari et al. [11] also reported a higher infection in stems compared to leaves, and Heringa et al. [3] reported that lines carrying the *er2* gene had only leaf resistance. Our study showed that these few symptoms are caused mainly by *E. pisi* both in leaves and stems, not supporting the hypothesis that *E. pisi* and *E. trifolii* might differ in their ability to infect leaves or stems.

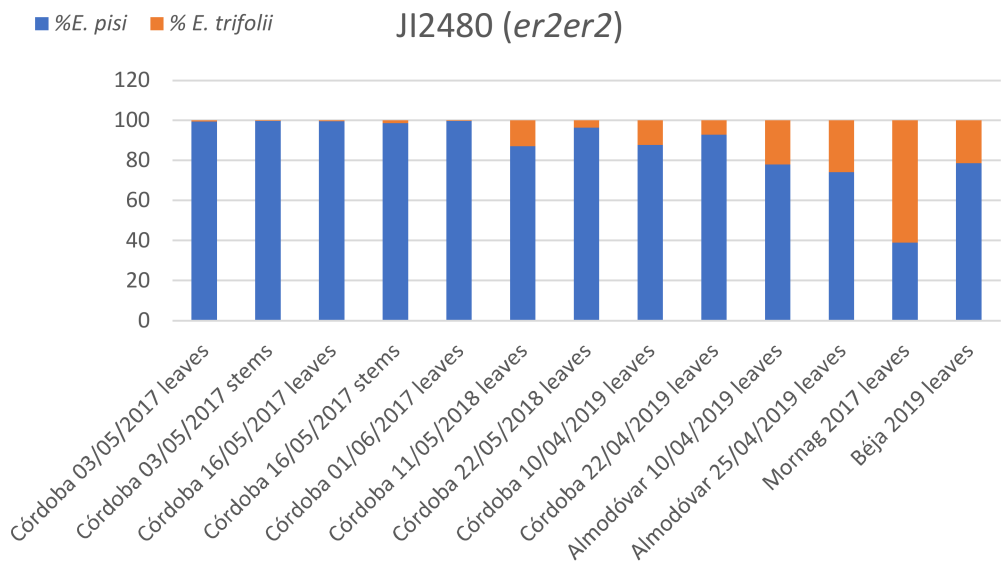


**Figure 2.** Percentage of *E. pisi* and *E. trifolii* in the different samples collected in accessions JI2480. Percentages shown are the average of three biological replicates. Each sample was named after the location where it was taken, followed by the date when it was sampled. It is also specified after the date whether samples were taken from leaves or stems.

The cultivar Eritreo (*Er3*) was highly resistant in both Spain and Tunisia (showing up to DS 10%). Therefore, the *Er3* gene provided resistance to both pathogens. It is worth mentioning that two recent studies in southern Spain showed high powdery mildew resistance in *Er3* carrying gene breeding lines, whereas all other lines and cultivars, some of which might have *er1*, were heavily infected [18,19]. Our results show that the few symptoms observed were caused by both *E. pisi* and *E. trifolii*. In Spanish samples, the percentage of *E. trifolii* ranged from 37 to 70% depending on the region and time point. In Tunisian samples, *E. trifolii* was more abundant (88 and 93% in 2017 and 2019, respectively) (Figure 3). *E. trifolii* was more abundant in most samples, which is in agreement with the reported capability of *E. trifolii* to overtake *Er3* resistance at higher temperatures [6]. Still, some proportion of *E. pisi* was also found in these lines. Our previous study [20] indicates that, in *Er3* lines, *E. pisi* is able to develop some hyphae. Its growth is stopped soon after due to hypersensitive response in most of the colonies (95.5%) but not all. The small amount of *E. pisi* found in *Er3* lines may correspond to this small amount of mycelium produced by *E. pisi* or, alternatively, there may exist an environmental factor not identified yet that could affect *Er3* expression and allow a small amount of growth of *E. pisi* in *Er3* lines.

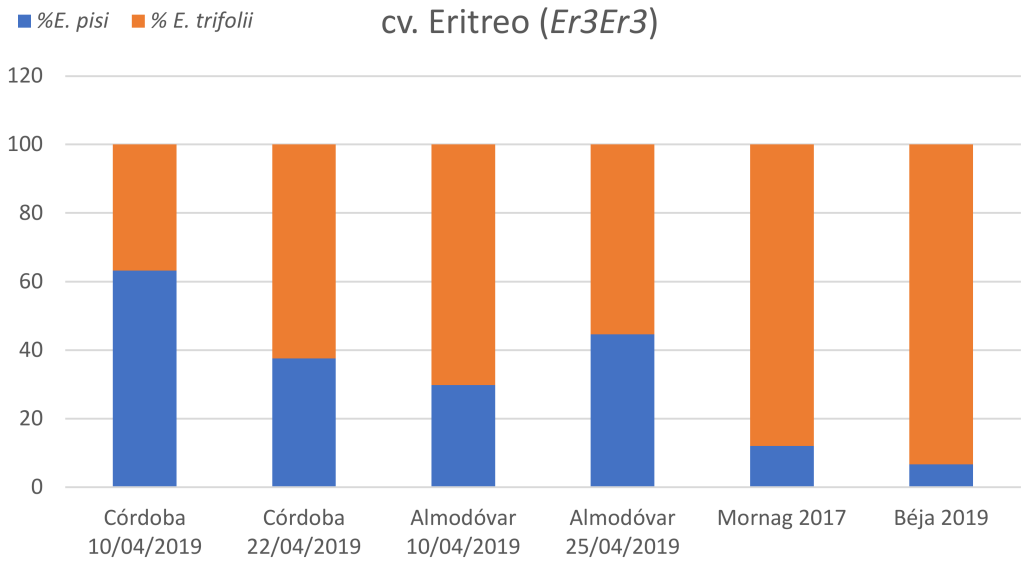


**Figure 3.** Percentage of *E. pisi* and *E. trifolii* in the different samples collected in cv. Eritreo. Percentages shown are the average of three biological replicates. Each sample was named after the location where it was taken, followed by the date when it was sampled.

In cv. Messire (lacking *er1*, *er2* and *Er3* mediated resistance) and in the other varieties collected in different commercial pea fields in Spain and Tunisia, the relative prevalence of *E. pisi* with respect to *E. trifolii* varied between varieties, locations and time points, but *E. pisi* was frequently more abundant than *E. trifolii*. In general, *E. trifolii* was more relevant in Tunisia than in Spain, even exceeding *E. pisi* in some samples (Figure 4).

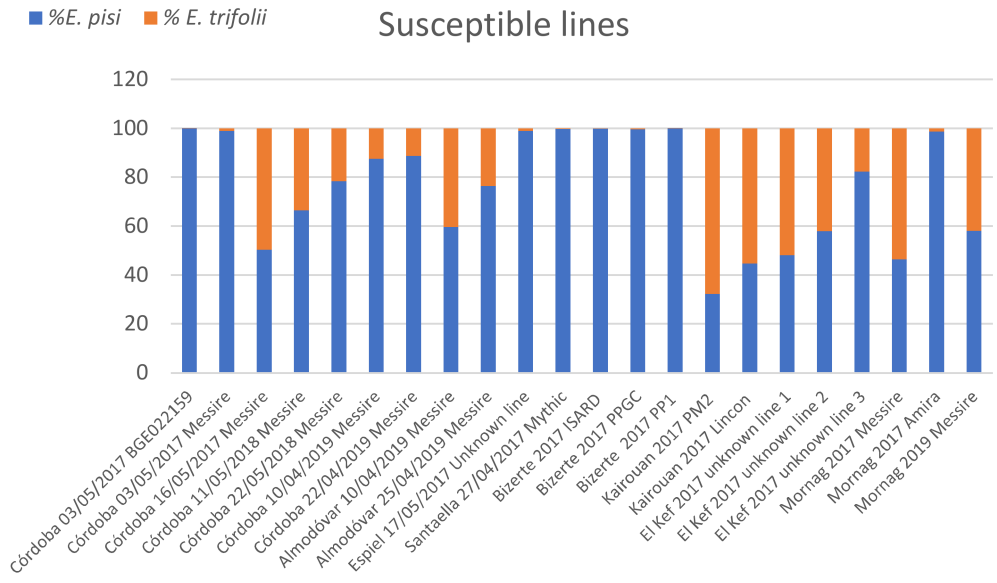


**Figure 4.** Percentage of *E. pisi* and *E. trifolii* in the different samples collected in susceptible lines. For Messire, percentages shown are the average of three biological replicates. Each sample was named after the location where it was taken, followed by the date when it was sampled. The cultivar from which the sample obtained is also specified after the date.

The evolution on the percentage of both pathogens along the crop cycle did not show a clear trend, with *E. trifolii* increasing a bit in the later sampling dates during 2017 and 2019, but showing the opposite trend during 2018.

## 4. Conclusions

In conclusion, our study provides, for the first time, a qPCR method that allows the identification and quantification of *E. pisi* and *E. trifolii*. Its implementation to analyze the amount of these pathogens in pea samples allowed us to analyze the relative prevalence of these pathogens in Spanish and Tunisian fields, and open the door to carry out similar analyses in other countries. Our study showed, for the first time, the presence of *E. trifolii* infecting peas in Tunisia, being even more important than in Spain, as, in most of the lines studied, the percentage of *E. trifolii* compared to *E. pisi* was higher than in Spain. Our study also reported, for first time, the effect of *er1*, *er2* and *Er3* genes on the ratio between *E. pisi* and *E. trifolii* infecting a plant. Our results reveal that *E. trifolii* is more important than previously thought, showing that this pathogen is able to defeat the *er1* resistance gene, but also to infect any pea line. This, joined to the fact that *E. trifolii* has a broad host range and was reported to infect other legumes close to peas reinforces the need to monitor its distribution and to consider resistance to *E. trifolii* as well in pea breeding programs.

**Author Contributions:** Conceptualization S.F. and D.R.; Investigation S.F., M.J.G.-B. and N.O.B.Y.; Writing—Review and Editing, S.F., D.R. and N.O.B.Y.; Funding Acquisition, S.F. and D.R. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research was funded by the AGL2014-52871-R and PID2020-11468RB-100 projects (MCIN/AEI/10.13039/501100011033).

**Data Availability Statement:** Data are contained within the article.

**Conflicts of Interest:** The authors declare no conflict of interest.

## References

1. Fondevilla, S.; Rubiales, D. Powdery mildew control in pea. A review. *Agronon. Sustain. Dev.* **2012**, *32*, 401–409. [CrossRef]
2. Harland, S.C. Inheritance of immunity to mildew in Peruvian forms of *Pisum sativum*. *Heredity* **1948**, *2*, 263–269. [CrossRef] [PubMed]
3. Heringa, R.J.; Van Norel, A.; Tazelaar, M.F. Resistance to powdery mildew (*Erysiphe polygoni* D.C.) in peas (*Pisum sativum* L.). *Euphytica* **1969**, *18*, 163–169.
4. Fondevilla, S.; Cubero, J.I.; Rubiales, D. Confirmation that the *Er3* gene, conferring resistance to *Erysiphe pisi* in pea, is a different gene from *er1* and *er2* genes. *Plant Breed.* **2010**, *130*, 281–282. [CrossRef]
5. Attanayake, R.N.; Glawe, D.A.; McPhee, K.E.; Dugan, F.M.; Chen, W. *Erysiphe trifolii*—A newly recognized powdery mildew pathogen of pea. *Plant Pathol.* **2010**, *59*, 712–720. [CrossRef]
6. Fondevilla, S.; Chattopadhyay, C.; Khare, N.; Rubiales, D. *Erysiphe trifolii* is able to overcome *er1* and *Er3* resistance genes but not *er2*. *Eur. J. Plant Pathol.* **2013**, *136*, 557–563. [CrossRef]
7. Rubiales, D.; González-Bernal, M.J.; Warkentin, T.; Bueckert, R.; Vaz Patto, M.C.; McPhee, K.; McGee, R.; Smýkal, P. Advances in breeding of peas. In *Achieving Sustainable Cultivation of Vegetables*; Hochmuth, G., Ed.; BurleigDodds Science Publishing Limited: Cambridge, UK, 2019; pp. 1–33. [CrossRef]
8. Baiswar, P.; Ngachan, S.; Verma, V.; Kumar, R.; Jha, A.; Chandra, S. Molecular evidence of *Erysiphe pisi* on pea and *E. trifoliorum* on white clover in northeast India. *Australas. Plant Dis. Notes* **2015**, *10*, 1–3. [CrossRef]
9. Torres, A.M.; Weeden, N.F.; Martín, A. Linkage among isozyme, RFLP and RAPD markers in *Viciafaba*. *Theor. Appl. Genet.* **1993**, *85*, 937–945. [CrossRef] [PubMed]
10. Ramakers, C.; Ruijter, J.M.; Lekanne Deprez, R.H.; Moorman, A.F.M. Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. *Neurosci. Lett.* **2003**, *339*, 62–66. [CrossRef]
11. Tiwari, K.R.; Penner, G.A.; Warkentin, T.D. Inheritance of powdery mildew resistance in pea. *Can. J. Plant Sci.* **1997**, *77*, 307–310. [CrossRef]
12. Attanayake, R.N.; Glawe, D.A.; Dugan, F.M.; Chen, W. *Erysiphe trifolii* causing powdery mildew of lentil (*Lens culinaris*). *Plant Dis.* **2009**, *93*, 797–803. [CrossRef] [PubMed]
13. Santos, C.; Martins, D.; Rubiales, D.; Vaz Patto, M.C. Partial resistance against *Erysiphe pisi* and *E. trifolii* under different genetic control in *Lathyrus cicera*: Outcomes from a linkage mapping approach. *Plant Dis.* **2020**, *104*, 2875–2884. [CrossRef] [PubMed]
14. Norini, M.P.; Secher, C.; Lollier, M.; Jézéquel, K.; Cornu, J.Y.; Lebeau, T. Quantification of the 16S–23S rRNA internal transcribed spacers of *Burkholderia xenovorans* strain LB400 using real-time PCR in soil samples. *Lett. Applied Microbiol.* **2013**, *56*, 366–372. [CrossRef] [PubMed]
15. Bokulich, N.A.; Mills, D.A. Improved Selection of Internal Transcribed Spacer-Specific Primers Enables Quantitative, Ultra-High-Throughput Profiling of Fungal Communities. *AEM* **2013**, *79*, 2519–2526. [CrossRef] [PubMed]

16. Ieda, S.; Moriyama, M.; Takashita, T.; Maehara, T.; Imabayashi, Y.; Shinozaki, S.; Tanaka, A.; Hayashida, J.N.; Furukawa, S.; Ohta, M.; et al. Molecular Analysis of Fungal Populations in Patients with Oral Candidiasis Using Internal Transcribed Spacer Region. *PLoS ONE* **2014**, *9*, e101156. [CrossRef]
17. Fondevilla, S.; Carver, T.L.W.; Moreno, M.T.; Rubiales, D. Macroscopic and histological characterisation of genes *er1* and *er2* for powdery mildew resistance in pea. *Eur. J. Plant Pathol.* **2006**, *115*, 309–321. [CrossRef]
18. Rubiales, D.; Osuna-Caballero, S.; González-Bernal, M.J.; Cobos, M.J.; Flores, F. Pea Breeding Lines Adapted to Autumn Sowings in Broomrape Prone Mediterranean Environments. *Agronomy* **2021**, *11*, 769. [CrossRef]
19. RAEA. *Resultados de Ensayos de Variedades de Guisantes Proteaginosos. Campaña 2020/21*; Junta de Andalucia Ed.: Alcalá del Río, Spain; pp. 1–21.
20. Fondevilla, S.; Carver, T.L.W.; Moreno, M.T.; Rubiales, D. Identification and characterisation of sources of resistance to *Erysiphe pisi* Syd. in *Pisum* spp. *Plant Breed.* **2007**, *126*, 113–119. [CrossRef]