



## Occurrence of angular leaf spot caused by *Pseudocercospora griseola* in *Phaseolus vulgaris* in Asturias, Spain

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### Abstract

Angular leaf spot (ALS) symptoms were observed in 2015 in common bean fields at four locations in Asturias, NW Spain. This disease is frequent in tropical areas and we have no record of its presence in our region, at least in the last 30 years. However, since its detection its presence in the crops has been increasing. Symptoms were necrotic spots on leaves and reddish-brown to black circular spots on pods, stems, branches and petioles. The damage observed in the mentioned crops was highly variable (between 60% and 100% affected leaves), being most severe in crops where no agrochemical treatment were applied. Three strains were selected and identified based in morphological features as *Pseudocercospora griseola*. The ITS region was amplified by PCR obtaining a sequence that was identical for the three isolates (Acc. No. LT222499). This sequence showed 99-100% similarity with those deposited in databases corresponding to *P. griseola*. To fulfill Koch's postulates, a pathogenicity test was carried out in two common bean cultivars ('Andecha' and 'Maruxina'). *P. griseola* was re-isolated from inoculated plants and not from control plants. In cv. 'Andecha', chlorosis was observed in all the inoculated plants, before the appearance of spots. Consequently this is the first confirmed report of this pathogen in our region.

**Additional key words:** bean; *Phaeoisariopsis griseola*; ALS; ITS; molecular identification.

**Authors' contributions:** Conceived, designed and performed the experiments: EL, AJG. Analyzed the data, and wrote the paper: ET and AJG. Contributed reagents/materials/analysis tools: EL, MB and AJG. All authors read and approved the final manuscript.

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### Introduction

The angular leaf spot of bean (ALS) is caused by a pathogen first described by Saccardo in 1878 in bean crops in Italy, which was called *Isariopsis griseola*. Later, Ferraris (1909) concluded that the genus *Isariopsis* was identical to the genus *Phaeoisariopsis* and proposed the new combination of *Phaeoisariopsis griseola* (Sacc.) Ferr. To further complicate the denomination, Ellis (1881) had also described the same fungus and had called it *Graphium laxum* Ell. Finally, in the last review of the genus (Crous *et al.*, 2006 and 2013), it was named *Pseudocercospora griseola* (Sacc.) Crous & Braun, although many references still use the old nomenclature.

*P. griseola* presents a high variability. Two major groups have been described: (i) Andean, corresponding to *P. griseola* f. *griseola*, and (ii) Middle-American,

corresponding to *P. griseola* f. *mesoamericana*, that cannot be differentiated by the symptoms produced or its morphology (Guzmán *et al.*, 1995). Furthermore, pathogenic races have been described according to its pathogenicity in the different bean cultivars (Pereira *et al.*, 2011).

ALS is a disease described in about 80 countries that can cause severe yield losses in tropical and subtropical countries. Losses of 40% to 80% were described under favourable conditions (Guzmán *et al.*, 1999). In Brazil and Colombia losses of 45% and 80%, respectively, were described in absence of disease control measures (Bergamin *et al.*, 1997). In Africa, losses of 50-60% were reported (Golato & Meossi, 1972). In the USA and Europe, the disease produces damage occasionally.

In Spain, this fungus was described in 1927 in Barcelona, in 1937 in Vizcaya and in 1944 in Malaga (Benlloch, 1944), although no pathogenicity tests were

carried out. Until now, these are the last references of this disease in the scientific literature from Spain.

In Asturias (NW Spain), with a mild and humid climate, the bean type "granja asturiana" is grown. This type reaches a high value in the market and is the basis of an emblematic dish of the local gastronomy, the "fabada". In September 2015, symptoms of ALS were observed in a bean field located in the council of Valdés, where these symptoms had already been observed in the previous year although no samples were taken. Moreover, similar symptoms were also observed in another bean field in the council of Coaña and in two others belonging to the council of Tineo, all located within the western zone of the Principality of Asturias. The objective of this work was to identify the causal agent of this disease.

## Material and methods

**Identification:** Morphological features were observed and compared with those described by Crous *et al.* (2006, 2013).

**DNA isolation:** DNA of fungal isolates was obtained by "Fungal DNA Mini kit" from Omega Bio-Tek (USA) following the manufacturer's instructions.

**PCR amplification:** The internal transcribed spacers (ITS) region was amplified by PCR using the primers described by White *et al.* (1990) in a PTC 100 thermocycler (MJ Research, CA, USA). Conventional amplifications were performed in a final volume of 50  $\mu$ L each containing 1  $\mu$ L of DNA, 5  $\mu$ L of 10x PCR buffer (supplied by the manufacturer), 200  $\mu$ M of each deoxyribonucleotide, 20 pmol of each primer, and 2.0 U of DyNAzyme II DNA polymerase (Finnzymes Oy, Espoo, Finland). Volume was made up to 50  $\mu$ L with sterile bi-distilled water. After a 4 min denaturation step at 94°C, the reaction mixture was run through 35 cycles of denaturation at 95°C for 30 sec, annealing at 55°C for 45 sec, and extension at 72°C for 1 min, followed by a final step at 72°C during 10 min. The PCR products were purified with the Ultraclean™PCR clean up DNA purification kit (MO-BIO, Inc., USA), as recommended by the manufacturer, and used directly for sequencing analysis (Secugen, Madrid).

The sequences obtained were compared to those deposited in the databases by BLAST (Altschul *et al.*, 1997).

**Phylogenetic tree:** The sequences of isolates and those deposited in databases corresponding to *P. griseola* f. *griseola* and *P. griseola* f. *mesoamericana*, were aligned by Clustal W (Thompson *et al.*, 1994). Two methods were used, maximum likelihood based on the Tamura-Nei model (Tamura & Nei, 1993)

and neighbor-joining (Saitou & Nei, 1987) with the Kimura-2 model (Kimura, 1980), both with a bootstrap of 1000 replicates, by Mega 6 software (Tamura *et al.*, 2013).

**Pathogenicity tests:** The pathogenicity tests were conducted on two different cultivars of "granja asturiana" bean, 'Andecha' and 'Maruxina'. Three strains were selected, one for each affected field, which were grown on V8 agar for 22 days (Tello *et al.*, 1991). The inoculum was prepared in a mixer using two plates with the fungus and one litre of sterilized and distilled water (Castellanos *et al.*, 2011). Four seedlings were irrigated per inoculated strain. The same number of seedlings processed and irrigated in the same way, but fungus-free medium were used as control. Both control and inoculated seedlings were covered with plastic bags for 48 h to saturate moisture and were kept in a climatic chamber, with 80% relative humidity, photoperiod of 16 h light and 23 ° C. The assay was repeated twice and held for 45 days.

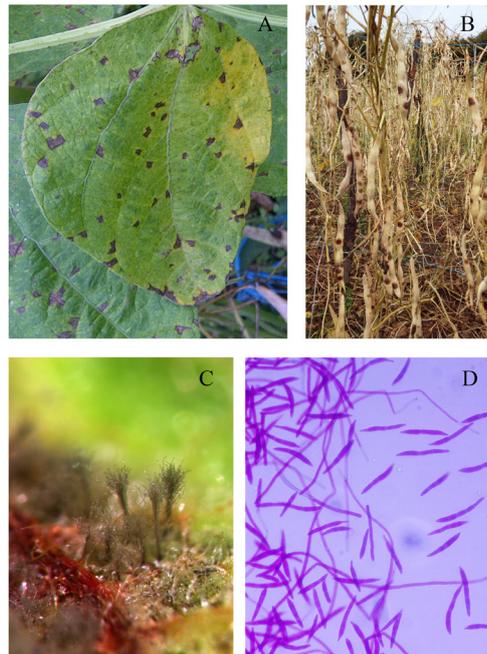
## Results and discussion

The symptoms observed in the field consisted of angular spots on leaves. Initially, some grey dots appear on the undersides of the leaves. On some varieties, a yellow halo is also present. Spots may coalesce and later, defoliation can occur. On pods, circular spots can range from reddish brown to black in colour, which can stain the seed. Brown and elongated lesions appeared on stems, branches and petioles (Fig. 1A and 1B). The three strains selected were identified as *P. griseola* by observing the morphological characteristics of the fungus (hyphae/conidiophores grouped in dense fascicles, forming synnematosous conidiomata, cylindrical conidia with several septa, etc.) and comparing them with those described by Crous *et al.* (2006, 2013).

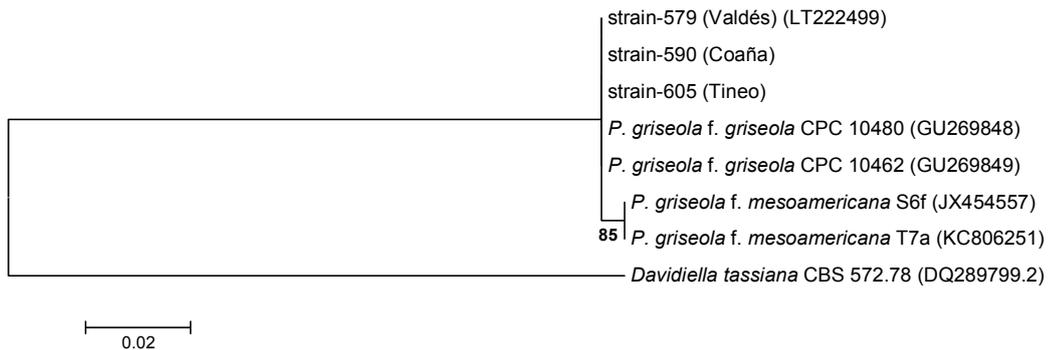
In order to perform a more accurate identification, the ITS of the three strains were sequenced. All three isolates were genetically identical, therefore only one of them corresponding to strain 579, was deposited in the EMBL database (Acc. No. LT222499).

When we compared this sequence with that registered in databases, it presented a similarity of 100% with one corresponding to *P. griseola* (HQ690098) and 99% with others of the same species (JX454556-57, JX454568), which corroborates its identity. Furthermore, our isolates would correspond to *P. griseola* f. *griseola*, because they did not grow at 30° C (Crous *et al.*, 2006).

In the phylogenetic trees (Fig. 2) it can be observed that our isolates clustered with *P. griseola* f. *griseola*, which is consistent with the cultivar origin due to the "granja asturiana" type being of Andean origin. The



**Figure 1.** Symptoms of angular leaf spot of bean: A, leaves. B, pods. C, fasciculate conidiophores. D, conidia.



**Figure 2.** Evolutionary relationships of taxa using the maximum likelihood method with ITS sequences showing the position of the Asturian isolates of *P. griseola* clustered with *P. griseola* f. *griseola*. The evolutionary distances were computed using the Tamura-Nei model. Bootstrap values >50% (based on 1000 replicates) are indicated at branch points. Accession numbers are given in parentheses. *Davidiella tassiana* was used as outgroup.

trees obtained by the two methods showed similar results so only one of them is shown.

To fulfill Koch's postulates, pathogenicity tests were performed by artificial infection of the fungus on bean seedlings. The assay was carried out twice and the pathogen was re-isolated from inoculated plants. Two different bean cultivars, 'Andecha' and 'Maruxina', were used and differences were observed in the symptoms produced. In the cv 'Maruxina' similar spots to the field observations were noted, while in cv 'Andecha' there was a generalized chlorosis of the plants prior to the appearance of the spots, which considerably reduced their vigour. Pereira *et al.* (2015) reported that this

fungus presents a high pathogenic variability, therefore this aspect is highly interesting for later studies in which it would be convenient to characterize the level of susceptibility of the different local bean cultivars to the pathogen.

The damage produced in the affected fields was highly variable, being more severe in two located in Valdés and Coaña. In Valdés, it was estimated that 100% of the leaves and pods were affected, while in Coaña 60% of the leaves and 30% of the pods were symptomatic. In other bean-producing regions, losses above 80% have been described under conditions favorable to the development of the pathogen (Singh & Schwartz, 2010).

We consider that very likely the greater severity of the damage observed in these two locations was due to the fact that these are under organic management, with no chemical treatments. This aspect is relevant, given the increasing importance of organic crops in our region and throughout the country, which may lead to an increase in the incidence of the disease.

Another aspect to be highlighted is the fact that this disease prevails in tropical and subtropical climates, which does not correspond to the climatic conditions in our region. It has been described that the development of the disease is faster at 24° C and with high humidity (León, 2009). Such observations could lead us to hypothesize whether, on the one hand, the globalization of markets, with the consequent increase in international exchange of plant material, as well as global climate change, may be responsible for changes in the known pathosystems. Although, the possible adaptation of the pathogen to this climate cannot be disregarded.

The symptoms observed in the field appear in the final phase of the crop and can be confused with those produced by other pathogens, such as *Colletotrichum lindemuthianum* that cause the anthracnose in beans, or those caused by *P. syringae* pv. *phaseolicola* that produces halo blight. This fact could also explain the limited information that we have regarding this pathogen in Asturias, as either a recently introduced or being unnoticed among other better known pathogens in our region.

Finally, the fungus *Pseudocercospora griseola*, causal agent of the angular spot of bean, has been identified as causal agent of the symptoms observed in cultivated fields located in western Asturias with some differences between the two bean cultivars tested. This fungus has caused greater damage to crops that have not received phytosanitary treatments.

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