Journal of Plant Protection Research ISSN 1427-4345

ORIGINAL ARTICLE

Characterization of Turkish isolates of *Pseudocercospora griseola* the causal agent of angular leaf spot of common beans

Sirel Canpolat[1]*, Salih Maden[2]

[1] Department of Phytopathology, Ankara Plant Protection Central Research Institute, Ankara, Turkey
[2] Department of Plant Protection, Faculty of Agriculture, Ankara University, Ankara, Turkey

Vol. 61, No. 1: 95–102, 2021

DOI: 10.24425/jppr.2021.136273

Received: July 7, 2020
Accepted: August 24, 2020

*Corresponding address:
sirelozan_18@hotmail.com

Abstract

Characterization of angular leaf spot (ALS) disease of beans caused by *Pseudocercospora griseola* (Sacc.) Crous & Braun along with its occurrence was investigated using 118 isolates obtained from beans grown in greenhouses in the western Black Sea region of Turkey. Incidences of ALS disease ranged between 77–100% and 82–100% for summer and autumn sown bean cultivations while the disease severity was in the ranges of 66–82% and 74–86% for the same periods, respectively. All of the 118 isolates of *P. griseola* yielded 500–560 bp PCR products from ITS1 and ITS4 primers, while 45 isolates yielded 200–250 bp products from actin genes primer and 5 isolates yielded 300–350 bp from calmodulin primer. The form of the Turkish isolates of *P. griseola* was determined as f. *griseola* since ITS sequences of 118 isolates of *P. griseola* showed between 98–100% similarity to the isolates of *P. griseola* f. *griseola* deposited in GenBank and our isolates took place on the same branch on the phylogenetic tree formed by the representative isolates in GenBank. The actin sequences did not give a clear differentiation for the forms of *P. griseola*. The phylogenetic trees generated by ITS1, ITS2 and actin genes formed similar branches. Each had two main clade and similar sub clades.

Keywords: angular leaf spot, bean, disease, fungus

Introduction

Common bean (*Phaseolus vulgaris* L.) when used together with cereals is a valuable crop to maintain a balanced diet in order to prevent some serious diseases such as coronary diseases and diabetes (Viguiliouk *et al*. 2017). Angular leaf spot (ALS) disease of common bean occurs in many tropical and subtropical countries on all the continents of the world, especially in areas having humid and warm climates. Although the disease takes its name from the leaf spot symptoms, it affects all the plant parts causing brown circular spots on the pods and seeds. ALS can cause severe crop damage, and up to 70% loss.

ALS on common bean is caused by a fungus which has recently been named as *Pseudocercospora griseola* (Sacc.) Crous & Braun based on the sequence analysis of the small subunit (SSU) region of nuclear ribosomal DNA (nrDNA), since it showed indistinguishable characteristics from other hyphomycete anamorph genera associated with *Mycosphaerella*, namely *Pseudocercospora* and *Stigmina* (Crous *et al*. 2006). These researchers also identified two gene pools in the population of *P. griseola* and named them Andean and Middle-American. They also delineated two groups within *P. griseola*, which are recognised as two formae, namely f. *griseola* and f. *mesoamericana* by further analysis of morphology, cultural characteristics, and DNA sequences of the internal transcribed spacer (ITS), calmodulin, and actin gene regions. The causal agent is also known as *Isariopsis griseola* (Sacc.) and *Phaeoisariopsis griseola* (Sacc.) Ferraris.

Common bean cultivation in greenhouses especially in two provinces in the western Black Sea region

of Turkey, Zonguldak and Bartın, has increased. Recently, a new disease, causing gray to brown, angular leaf spots on the leaves has been determined. When the intensity of the disease was high, leaf shed following the yellowing of the leaves occurred. The causal agent of the disease was initially identified as *P. griseola*. This study was aimed to determine dissemination and severity of the disease, which is probably one of the greatest problems of greenhouse bean cultivation in the region. This paper also deals with further identification and molecular characterization of ALS disease occurring in Turkey.

Materials and Methods

Sampling and isolations

ALS symptoms were observed in many greenhouses growing beans in Bartın and Zonguldak provinces, Turkey. *Pseudocercospora griseola* isolates were obtained from samples collected from naturally infected leaves and pods of common bean. Greenhouses growing beans were visited once a week and samples were collected from the plants showing ALS symptoms. The samples were taken at seedling-flowering and pod-harvest stages from the greenhouses representing the provinces by the random sampling method described by Bora and Karaca (1970). Data regarding cultivars, their seed size and color, growth habits, and location were recorded during the collection of diseased samples. For fungal sporulation, tissues from infected bean leaves were placed on moistened filter papers in Petri dishes, and were incubated at room temperature for 3 to 4 days. Conidia from sporulated ALS lesions were picked up with a tiny piece of agar placed on the tip of a sterile dissecting needle, streaked onto V-8 juice agar, and then incubated for 24 h at 24°C. Individual germinated conidia were then transferred to V-8 juice agar to obtain monosporic cultures for each *P. griseola* isolate.

Disease incidence and severity

Surveys were conducted in Karabük, Bartın and Zonguldak provinces of the north-western part of Turkey. Observations were made between May–December 2011. Locations were selected based on their bean production intensity comprising 39% of the total production area. Sampling was carried out randomly by selecting 42 greenhouses per location 2 km away from each other. In each greenhouse plot, incidence and severity were estimated visually at several systematically selected sampling sites. Disease incidence was expressed as the percentage of infected plants of the 50 plants picked. Disease severity was assessed by evaluating 50 bean leaves from different parts of the greenhouse taking a transact walk along the rows and across the selected greenhouse. Disease severity was evaluated by using the 1 to 9 scale of Schoonhoven and Pastor-Corrales (1987) as follows: 1 – no visible symptoms of the disease, 3 – presence of a few, small non-sporulating lesions covering approximately 2% of the leaf area, 5 – the presence of several, generally small lesions with limited sporulation covering approximately 5% of the leaf area, 7 – abundant and generally large sporulating lesions covering approximately 10% of the leaf area, 9 – 25% or more of the leaf area covered by large sporulating and often coalescing lesions. The coordinates of each sampled site in the greenhouse was recorded by GPS. Disease severity was calculated by Townsend and Heuberger's (1943) formula as below:

$$\text{Disease severity (\%)} = \frac{\sum(\text{No. of lives at each scale} \times \text{Scale value})}{\text{Highest scale value (9)} \times \text{Total no. of leaves (50)}} \times 100.$$

DNA extraction and PCR amplification

For DNA extraction, 100 mg *P. griseola* mycelium grown on malt extract agar (MEA) was scraped, weighed and treated with liquid nitrogen. After freezing and crushing, DNA extraction was performed according to the protocol of DNeasy plant kit (QIAGEN Inc. Valencia, CA). The obtained DNAs were stored at –20°C for use of PCR. The amount and purity of the obtained DNAs were determined as ng · μl^{-1} by using the NANODROP spectrophotometer (Thermo Scientific 2000) by reading at absorbance values of 260, 280, 260/280, and 260/230 nm, using ultra-pure water as a blind. Spectrophotometric readings were performed with three replications.

PCR amplification of the DNAs was performed by using general primers of three different conserved regions of the causal agent which were, respectively, ACT-512F ATGTGCAAGGCCGGTTTCGC, ACT-783R TACGAGTCCTTCTGGCCCAT (200–300 bp), CAL-228F AGTTCAAGGAGGCCTTCTCCC, CAL-737R CATCTTTCTGGCCAT (300–350 bp), ITS1: TCCG TAGGTGAACCTGCGG, ITS4: TCCTCCGCTTATT GATATGC (500–560 bp). The implemented PCR cycle program was followed (Crous *et al.* 2006). The PCR products were carried out and monitored by electrophoresis at 100 volts in 1× TBE (40 mM Tris-borate, 1 mM EDTA, pH 8.0) buffer loaded onto 1.5% agarose gel in the following order. DNA sequences were performed by GENOKS (Gazi Mah. Silahtar Cd. No: 67, 06560, Yenimahalle/Ankara). In addition, DNA sequences of PCR products using general primers were extracted and compared with respective sequences in the NCBI (National Centre for Biotechnology Information).

Determination of the form of *Pseudocercospora griseola* and phylogenetic analysis of the isolates

PCR products were separated in 1.5% agarose gels, stained with ethidium bromide and visualized under UV light. Sequence analysis of the products was done. For determination of the forms, nucleotide sequences were blasted to NCBI BLAST search, and the form of the isolates was identified according to the homology with sequences already present in GenBank. The nucleotide sequences of the bean isolates were stored in Gen -Bank with accession numbers MK483911-MK483944, MK483661-MK483699, MT445197-MT445217. Maximum parsimony (MP) analysis was used in phylogenetic analysis of DNA sequences to specify genetic differences among isolates. For phylogenetic analyses, sequence information was transferred to MEGA 6.0 program and genetic similarity between isolates was evaluated by creating a dendrogram (Tamura *et al.* 2013). A phylogenetic tree was also constructed by using 21 isolates of ours and some isolates of f. *griseola* and f. *mesoamericana* deposited in GenBank. Phylogenetic trees were also constructed by using both 73 isolates selected from 118 ITS gene sequences.

Results

Characteristics of angular leaf spot disease

Although the disease takes its name from the leaf spots seen on leaves (Fig. 1A), it also infects the pods and produces round, slightly depressed lesions (Fig. 1B). The fungus *P. griseola* profusely sporulated on leaf lesions when humidity was high and produced fasciculate conidiophores (Fig. 1C) forming multi-celled, dark colored, elongate conidia (Fig. 1D). *Pseudocercospora griseola* grew very slowly on culture media, producing dark colored colonies on MEA (Fig. 1D) and V8 juice agar (Fig. 1E).

Disease incidence and severity in the western Black Sea region of Turkey

About 73, 44 and 30% of the total greenhouse areas of Karabük, Bartın and Zonguldak provinces were monitored, respectively. All isolates were obtained from common beans showing angular leaf spot symptoms. ALS disease incidence ranged between 77–100% and 82–100% for summer and autumn sown bean cultivations while disease severity was in the ranges of 66–82% and 74–86% for the same periods, respectively. Autumn sowing periods were slightly higher (Table 1). GPS points of sampling greenhouses are given in Figure 2.

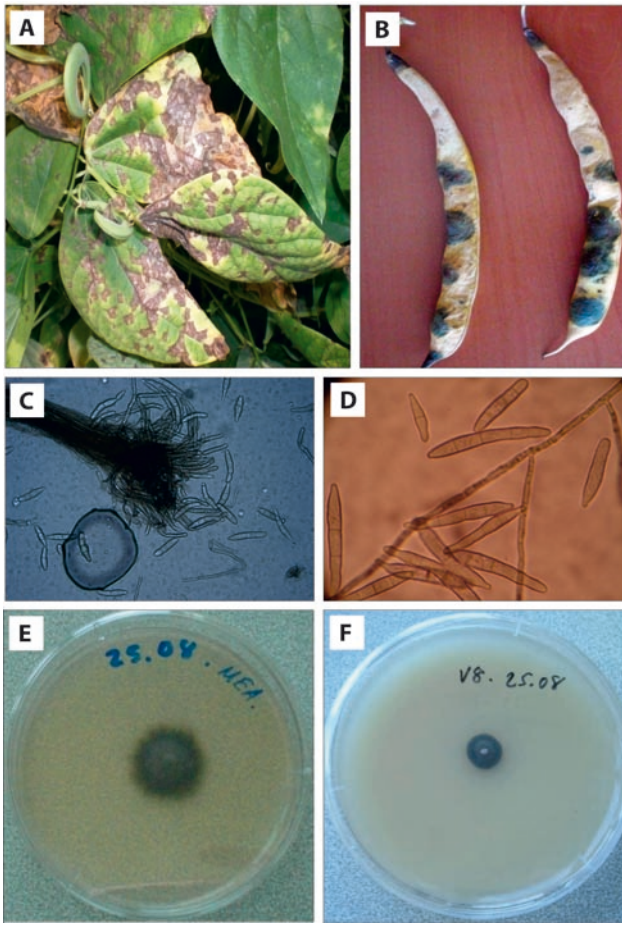

Fig. 1. Some characteristics of angular leaf spot (ALS) disease caused by *Pseudocercospora griseola*: A – leaf spots, B – pod spots, C – conidiophores, D – conidia, E – fourteen-day-old colony on MEA, F – on V8 juice agar

Molecular characterization of Turkish isolates of *Pseudocercospora griseola*

All of the 118 isolates of *P. griseola* yielded 500–560 bp PCR products from ITS1and ITS4 primers (Fig. 3A), while 45 isolates yielded 200–250 bp products from actin genes (Fig. 3B) and five isolates yielded 300–350 bp from calmodulin primer (Fig. 3C).

The form of our isolates of *P. griseola* was determined as f. griseola since ITS sequences of our 118 isolates of *P. griseola* showed between 98–100% similarity to the isolates of *P. griseola* f. *griseola* deposited in GenBank. The similarity of the ITS sequences of these isolates to *P. griseola* f. *mesoamericana* was 90–92%. In the tree generated by our 21 selected isolates and four isolates from each formae of *P. griseola* deposited in GenBank, all of our isolates took place in the same clade with f. *griseola* (Fig. 4). Based on morphological characteristics (Fig. 1) and sequencing the ITS (Fig. 4), our isolates were identified as *P. griseola* f. *griseola*. The genetic relatedness between ITS and actin sequences obtained from the present study, selected as

Table 1. Percent disease incidence and severity of angular leaf spot disease of common bean in the greenhouses in districts of three provinces of western Anatolia region of Turkey

Provinces/district		Percent of disease incidence		Percent of disease severity	
		spring sown	autumn sown	spring sown	autumn sown
Bartın	Central	70.5–100	82.7–100	66.3–82.7	74.4–86.3
Zonguldak	Devrek	77.1–100	85.1–100	71.4–100	86.7–100
	Gökçebey	81.8–100	87.1–100	78.5–100	85.7–100
	Çaycuma	78.7–100	89.1–100	81.7–100	84.9–100
Karabük	Yenice	no ALS was found in commercial bean growing areas. Only one plant had disease in a home garden with a transient plastic cover			

Fig. 2. Distribution of the greenhouses sampled in three provinces

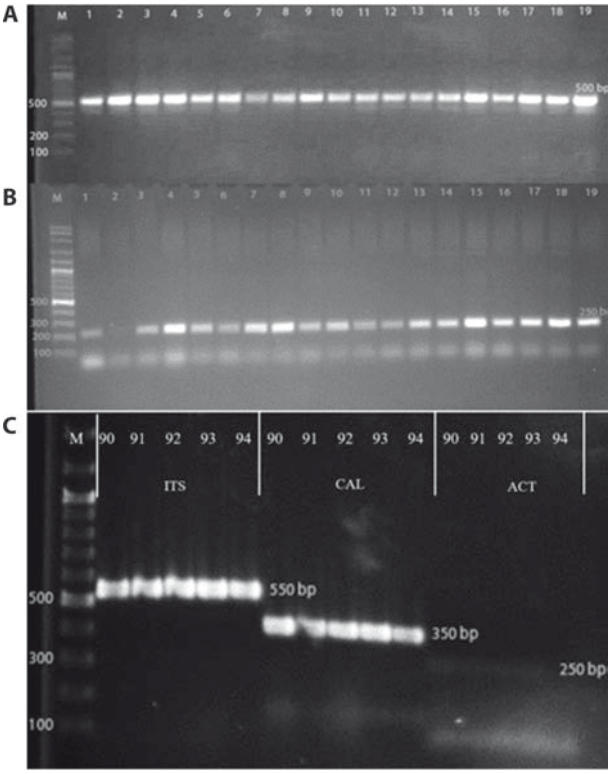

Fig. 3. PCR products of 500–560, 200–250, and 300–350 bp generated by: A – ITS1 and ITS4, B – actin, C – calmodulin primers of *Pseudocercospora griseola* isolates, respectively

representative of *Pseudocercospora griseola* f. *griseola*, is shown in dendrograms of Figures 5 and 6. As shown in both of the dendrograms, the genetic diversity of almost all the isolates was clearly settled and showed a similar pattern for ITS and actin gene sequences.

Discussion

Angular leaf spot disease of common beans caused by *P. griseola* was previously found harmful and widespread in greenhouse bean production in Bartın and Zonguldak provinces of the north western part of Turkey. Inoculum sources, survival of the disease and disease outbreak were also determined in this area (Canpolat and Maden 2017). Studies carried out in other countries show that up to 50–80% disease severity (Ddamulira *et al*. 2014) and our study also showed that disease incidence and severity up to 92% occurred in greenhouse bean production areas in the country, the autumn sowing having higher rates than the spring sowings in the two provinces, Bartın and Zonguldak. In Karabük province, where commercial greenhouse bean production is not economically significant, the disease was only found in a house garden. The reason for the non-existence of the disease in the fields could be the lower relative humidity, less than 90%, which is required for disease outbreak. The disease incidence and severity had been very high on the local bean variety grown in this area for a long time. However, while some commercial varieties did not show higher disease severity, we think that they have no resistance (Canpolat and Maden 2020). Occurrence of higher intensities of the disease could be due to the use of a susceptible local cultivar in autumn sowings, the use of infected seeds harvested from spring sowings and the higher relative humidity in the autumn. Commercial cultivars

Fig. 4. Dendrogram showing genetic relatedness between 21 Turkish isolates of *Pseudocercospora griseola* f. *griseola* (marked with red spots) with the forms of *griseola* and *mesoamericana* deposited in GenBank

sown in some greenhouses showed lower disease incidence, which might have some tolerance to the disease. This situation was also shown by our previous study (Canpolat and Maden 2020). The high incidence of disease and severity in the region may also be due to unsuitable greenhouse structure, frequent sowing, the use of more seed per bed, establishment of greenhouses in the wrong direction for example not being perpendicular to the prevailing wind and improper cultural practices.

Molecular characterization of 118 isolates of *P. griseola*, specifically the subtype and genotypic differentiation was done for the first time in this study in Turkey. From the three primer sets, only ITS primers

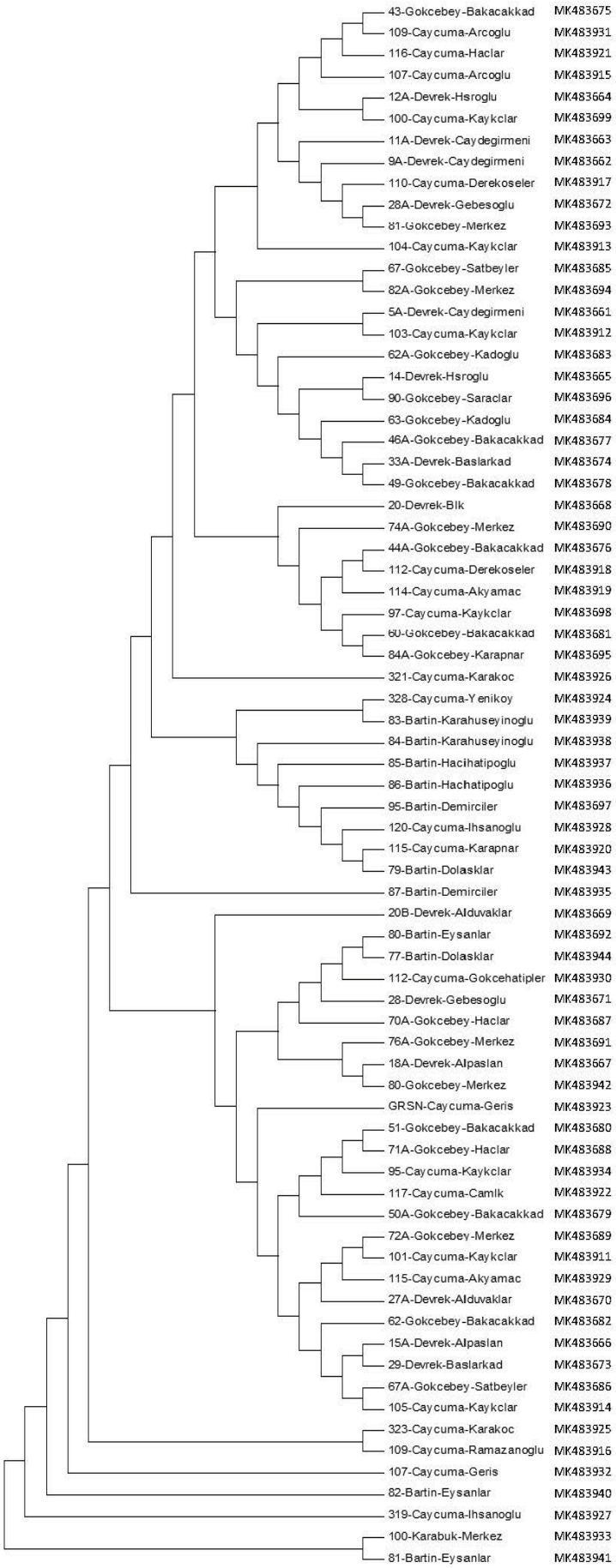

Fig. 5. Dendrogram showing genetic relatedness of 73 Turkish isolates of *Pseudocercospora griseola* f. *griseola* formed by DNA sequences generated by ITS1 and ITS4 primers

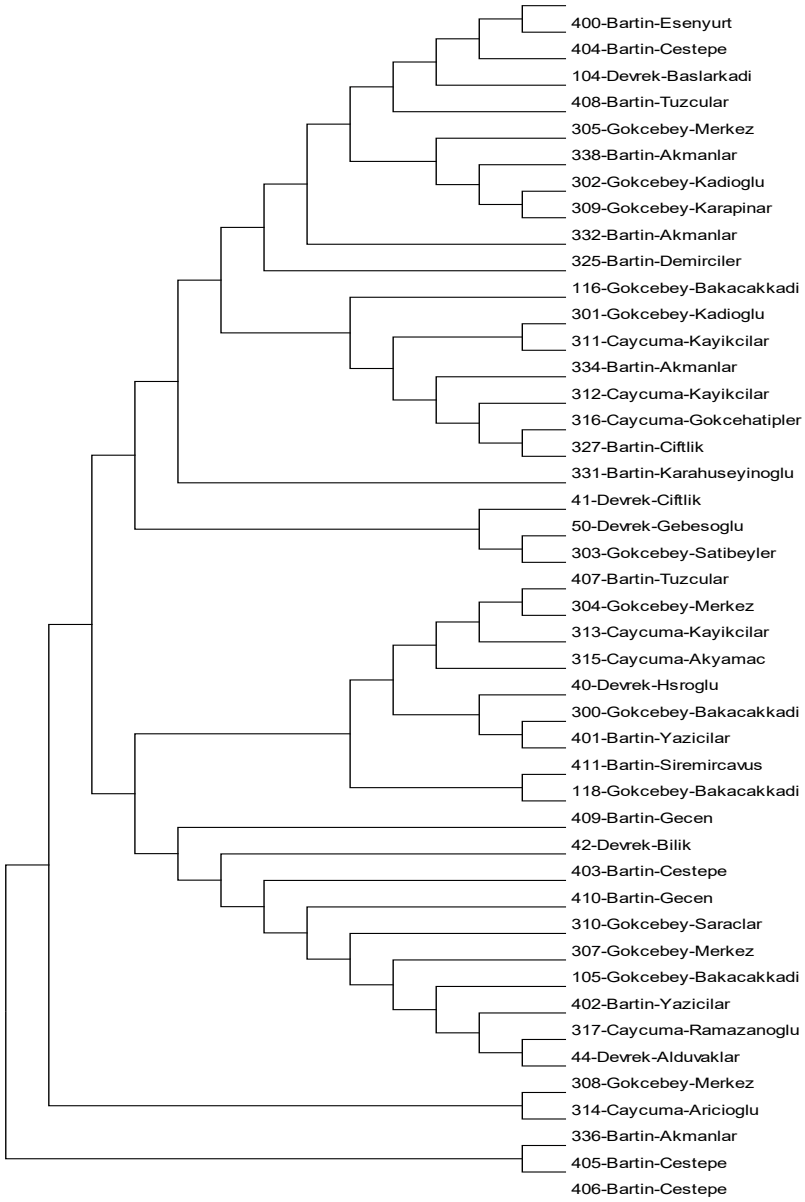

Fig. 6. Dendrogram showing genetic relatedness of 45 Turkish isolates of *Pseudocercospora griseola* f. *griseola* formed by DNA sequences generated by actin primers

amplified 500–560 bp products from all of the isolates, while actin primes amplified 200–250 bp from 45 isolates and calmoludin primers 300–350 bp from only five isolates. The ITS gene sequences of all the Turkish isolates showed similarity between 98–100% to *P. griseola* f. *griseola*, the form which is present in many parts of the world (Crous *et al*. 2006; Chilagane *et al*. 2016), while the similarity of the isolates to *P. griseola* f. *mesoamaricana* ranged from 90–92%. ITS sequences of our 21 selected isolates took place in the same clade in the phylogenetic tree generated with the isolates of f. *griseola* deposited in GenBank. This clearly shows that all the Turkish isolates of *P. griseola* belong to *P. griseola* f. *griseola*.

In this study, genetic diversity levels between angular leaf spot isolates collected from the western Black Sea region of Turkey were determined by using different primers. When the obtained results were evaluated, all isolates were separated from each other and there was a variation between them. The genetic separation of the isolates in the study supports the existence of different pathotypes of the causal agent. This study is the first study in this field in Turkey and in the future, larger and detailed studies will reveal important information about the diversity and distribution of the pathotypes of angular leaf spot.

Angular leaf spot disease has been reported to be one of the most important disease factors seen everywhere with bean cultivation, and it has been reported that there are many strains and pathotypes in the world. This number may increase with the emergence of new strains due to pathogenic changes between

among populations. We intended to determine the pathotypes of *P. griseola* in Turkey and we obtained a small number of 12 international pathotype differentiation seed set from the breeders from South Africa. In order to reproduce the seeds, the 12-seed set was planted in both Antalya and Ankara, but four varieties of the differential set did not bloom and produce pods although they grew profusely. Therefore, pathotypes of *P. griseola* could not be determined either by using international differential set or molecular differentiation which has not been characterized yet.

Various molecular techniques have been used to identify genetic variations of *P. griseola*, including random amplified polymorphic DNA (RAPD) and inter simple sequence repeats (ISSR), ISSR-PCR (Abadio *et al*. 2012) and recently random amplified microsatellite (RAMS), BOX AIR, ERIC and REM primers (Ddamulira *et al*. 2014). All of the above-mentioned studies found a great variation among the isolates of *P. griseola* f. *griseola*. Our findings with the use of ITS and actin gene primers also showed variation among the Turkish isolates of P.g.

Pseudocercospora griseola has a great pathogenic variability and this situation is described as pathotypes and Quantitative Trait Loci (QTL). Nay *et al.* (2019) reviewed the current resistance mechanism and reported that five QTL's, three of them (Phg-1, Phg-4, and Phg-5) are from common bean cultivars of the Andean gene pool and two of them (Phg-2 and Phg-3) are from beans of the Mesoamerican gene pool. They also reported other resistance loci on seven chromosomes (Pv1, Pv3, Pv5, Pv6, Pv8, Pv9 and Pv11) and drew a map showing all the resistance linkages including QTL's and pathotypes. The wide pathogenic variability, which has not been settled yet, was also reflected in our phylogenetic tree generated by ITS and actin gene regions. The presence of various pathotypes is expected in Turkey too and the determination of the pathotypes the QTL's would be more beneficial for breeding resistance in Turkey.

In our previous work, we tested 17 commercial bean cultivar reactions against 10 different aggressive isolates of *P. griseola* and three Turkish bred and two foreign cultivars were found to be resistant to all tested isolates (Canpolat and Maden 2020). Resistance of many bean cultivars grown in Turkey should be determined by checking whether they have some of the existing resistance loci or not. This procedure might facilitate breeding resistance since chemical control of the disease is neither effective nor safe.

Acknowledgements

This study is the Ph.D. thesis of Ankara University Graduate School of Natural and Applied Sciences Department of Plant Protection named "Determination of the Groups of *Pseudocercospora griseola* Causing Angular Leaf Spot in Protected Beans in West Black Sea Region, Investigations on Prevalence, Searches of Inoculum and Reactions of Some Bean Cultivation Against This Agent" and this project was supported by the Turkish Ministry of Agriculture and Forestry with Project number TAGEM-BS-/10/10-01/02-05 under National Integrated Greenhouse Disease Management Program.

References

Abadio A.K.R., Lima S.S., Santana M.F., Salamao T.M.F., Sartorato A., Mizubuti E.S.G., Araujo E.F., Queiroz de M.V. 2012. Genetic diversity analysis of isolates of the fungal bean pathogen *Pseudocercospora griseola* from central and southern Brazil. Genetics and Molecular Research 11 (2): 1272–1279. DOI: 10.4238/2012.May.14.1

Bora T., Karaca İ. 1970. Kültür Bitkilerinde Hastalığın ve Zararın Olçülmesi. [Measurement of Disease and Damage in Cultivated Plants]. Ege University, Faculty of Agriculture Auxiliary Textbook, No. 167. (in Turkish)

Canpolat S., Maden S. 2017. Determination of the inoculum sources of angular leaf spot disease caused by *Pseudocercospora griseola*, on common beans. Plant Protection Bulletin 57 (1): 39–47 (in Turkish, with English abstract). DOI: 10.16955/bitkorb.299016, ISSN 0406-3597

Canpolat S., Maden S. 2020. Reactions of some common bean cultivars grown in Turkey against some isolates of angular leaf spot disease, caused by *Pseudocercospora griseola*. Plant Protection Bulletin 60 (2): 45–54. (in Turkish, with English abstract). DOI: 10.16955/bitkorb.630968

Chilagane L.A., Nchimbi-Msolla S., Kusolwa P.M., Porch T.G., Diaz L.M.S., Tryphone G.M. 2016. Characterization of the common bean host and *Pseudocercospora griseola*, the causative agent of angular leaf spot disease in Tanzania. African Journal of Plant Science 10 (11): 238–245. DOI: https://doi.org/10.5897/AJPS2016.1427

Crous P.W., Lienbenberg M.M., Braun U., Groenewald J.Z. 2006. Re-evaluating the taxonomic status of *Phaeoisariopsis griseola*, the causal agent of angular leaf spot of bean. Studies in Mycology 55 (1): 163–173. DOI: 10.3114/sim.55.1.163

Ddamulira G., Mukankusi C.M., Ochwo-Ssemakula M., Edema R., Sseruwagi P., Gepts P.L. 2014. Distribution and variability of *Pseudocercospora griseola* in Uganda. Journal of Agricultural Science 6 (6): 16–29. DOI: 10.5539/jas.v6n6p16

Nay M.M., Souza T.L.P.O., Gonçalves-Vidigal M.C., Raatz B., Mukankusi C.M., Gonçalves-Vidigal M.C., Abreu A.F.B., Melo L.C., Pastor-Corrales M.A. 2019. A review of angular leaf spot resistance in common bean. Crop Science 59: 1376–1391. DOI: 10.2135/cropsci2018.09.0596

Sartorato A. 2004. Pathogenic variability and genetic diversity of *Phaeoisariopsis griseola* isolates from two counties in the State of Goias, Brazil. Journal of Phytopathology 152: 385–390.

Schoonhoven A., Pastor-Corrales M.A. 1987. Standard system for the evaluation of bean germplasm. Centro Internacional de Agricultura Tropical, CIAT Apartado Areo 6713 Cali, Colombia, p. 56.

Tamura K., Stecher G., Peterson D., Filipski A., Kumar S. 2013. MEGA 6: Molecular evolutionary genetics analysis version 6.0. Molecular Biology and Evolution 30 (12): 2725.

Townsend G.K., Heuberger J.W. 1943. Methods for estimating losses caused by diseases in fungicide experiments. Plant Disease Report 27: 340–343.

Viguiliouk E., Mejia S.B., Kendall C.W., Sievenpiper J.L. 2017. Can pulses play a role in improving cardiometabolic health. Evidence from systematic reviews and meta-analyses. Annuals of the New York Academy of Sciences 1392 (1): 43.