Characterisation of Angular Leaf Spot Pathogen and Identification of Source(s) of Resistance in Common Bean

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"PARENTS"

Someone who holds us in arms as we enter this world

Someone who encourages all our efforts and appreciate us wholeheartedly

Someone who listens patiently to our never ending doubts

Some who's eyes shine with pride and happiness at our every little achievement

Someone who helps us to smile instead shedding a tear

Someone who is our life-long friend

DEDICATE MY THESIS

"To serve whom was my dream and

Dream of serving them remain forever" …

My Beloved Parents…

And

...My brothers...

Mudasir Gani and Arif Gani...

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Sopore 193 201

Certificate – I

This is to certify that the thesis entitled, **"Characterisation of Angular Leaf Spot Pathogen and Identification of Source(s) of Resistance in Common Bean"** submitted in partial fulfilment of the requirements for the award of the degree of **Master of Science in Agriculture (Plant Pathology),** to the **Faculty of Agriculture, Sher-e-Kashmir University of Agricultural Sciences and Technology of Kashmir** is a record of bonafide research work carried out by **Ms. Saima Gani (Regd. No. MSA/2019/1297)** under my supervision and guidance. No part of the thesis has been submitted for any other degree or diploma.

It is further certified that any help or information received during the course of investigation has duly been acknowledged.

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We, the members of the Advisory Committee of **Ms. Saima Gani (Regd. No. MSA/2019/1297)** a candidate for the degree of **Master of Science in Agriculture (Plant Pathology)** have gone through the manuscript of the thesis entitled, **"Characterisation of Angular Leaf Spot Pathogen and Identification of Source(s) of Resistance in Common Bean"** and recommend that it may be submitted by the student in partial fulfilment of the requirements for the award of the degree.

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Certificate – III

This is to certify that the thesis entitled, **"Characterisation of Angular Leaf Spot Pathogen and Identification of Source(s) of Resistance in Common Bean"** submitted by **Ms. Saima Gani (Regd. No. MSA/2019/1297)** to the **Faculty of Agriculture, Sher-e-Kashmir University of Agricultural Sciences and Technology of Kashmir** in partial fulfilment of the requirements for the award of the degree of **Master of Science in Agriculture (Plant Pathology)** was examined and approved by the Advisory Committee and External Examiner on ……

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Title of the Thesis	:	**"Characterisation of Angular Leaf Spot Pathogen and Identification of Source(s) of Resistance in Common Bean"**

ABSTRACT

The present study entitled **"Characterization of Angular Leaf Spot Pathogen and Identification of Source(s) of Resistance in Common Bean"** was conducted to characterize common bean angular leaf spot pathogen based on morphology and Internal Transcribed Spacer (ITS) region, and to identify the sources of resistance. Two isolates Pg01 and Pg02 obtained in this study were identified as *Pseudocercospora griseola* on the basis of morphological characteristics. The isolates Pg01 and Pg02 were slow growing with 2.0 mm and 2.5 mm colony diameter, respectively on V-8 agar juice medium after incubation of 5 days at 25±1^{0}C. Colony colour of isolate Pg01 was initially greyish which later became black, and colony colour of isolate Pg02 was initially greyish white which later became dark olivaceous. The mycelium of both the isolates was septate, irregularly branched and hyaline with a diameter of 3.47-4.01 µm (Av.3.7 µm). Both the isolates were characterized by having synnematous and pigmented conidiophores. The size of conidiophores was 90-158 × 3.26-4.6 µm with an average of 138×3.6 µm in isolate Pg01 and 91.5-156 × 3.22-4.5 µm with an

average of 139 × 3.2 µm in isolate Pg02. The conidia of both the isolates were cylindrical in shape, olivaceous grey to olive brown in colour having 3 to 8 septa. Conidial dimensions varied from 38.10-49.04 × 3.1-6.29 µm in isolate Pg01 and 38.16-49.0 × 3.2-6.26 µm in Pg02. Based on morphological characterization, the pathogen isolates were identified as *Pseudocercospora griseola*. In order to decipher the formae to which these isolates belong, Internal transcribed spacer (ITS) regions of rDNA of two isolates were amplified using ITS1 and ITS4 primers and amplified products were custom sequenced. The sequences were retrieved from chromatograms and the sequence information was analyzed through BLASTn programme which indicated that the sequences showed maximum similarity with *Pseudocercospora griseola* f.sp. *griseola* sequences available at NCBI, and subsequently two sequences were submitted to NCBI with Accession Numbers MZ452242 and MZ413342. In phylogenetic analysis, isolates Pg01 and Pg02 were clustered with *P. griseola* f.sp. *griseola* sequences retrieved from GenBank, whereas the sequences of *P. griseola* f.sp. *mesoamericana* were clustered separately. For germplasm evaluation under field conditions, out of eighty-eight different genotypes twenty-two genotypes were resistant, forty-nine genotypes showed intermediate reaction and seventeen genotypes were susceptible. Among fifty-four genotypes screened in greenhouse under artificial inoculation with Andean race Pg02, eleven genotypes were resistant, thirteen genotypes showed intermediate reaction while as rest of the genotypes were susceptible.

Key words: Angular leaf spot, Common Bean, Germplasm evaluation, Molecular characterization, *Pseudocercospora griseola*

Signature of Student
Dated:_____

Signature of Major Advisor
Dated:_____

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Place: Wadura, Sopore

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Chapter-1

INTRODUCTION

Common bean (*Phaseolus vulgaris* L., 2n=2x=22) is world's most important legume crop (Nay *et al*., 2019) and most widely consumed grain legume in the world (Singh, 2001; Romero-Arena *et al*., 2013). It is popularly known as rajmash or hembs in North-Western Himalayan states and it is a non-centric crop with Andean and Mesoamerican as two different major gene pools (Singh *et al*., 1991). The Mesoamerican gene pool consists of small seeded varieties (< 25g/100 seed) domesticated from small seeded wild forms whereas the Andean gene pool has much larger seeded varieties (medium size of 25- 40 g/100 seed and large seed > 40 g/100 seed) domesticated from large seeded wild forms (Gepts *et al*., 1986). In India, it grows in hilly areas of Himachal Pradesh, Jammu and Kashmir, Uttrakhand and North-Eastern states during Kharif, while it is grown as Rabi crop in parts of Uttar Pradesh, Maharashtra, Karnataka and Andhra Pradesh.

Globally, common bean is grown on an area of about 29.39 million ha with an annual production of about 18 million metric tonnes. In India it is grown on an area of 0.23 million ha with a production and productivity of 0.66 million tonnes and 2.81 t ha^{-1}, respectively (FAO, 2017). As vegetable crop, common bean is grown in Kashmir over an area of 2000 ha with an annual production of 400 metric tonnes (Bhat *et al.,* 2017).

It is considered as nutritional powerhouse crop because it is important source of protein, fiber, calories and vital micronutrients (Singh, 1999; Broughton *et al*., 2003). Dry beans are considered as important functional food with high levels of chemically diverse components (phenols, starch, vitamins especially vitamin C and pro-vitamin A and fructo-oligosaccharides) (Hazra and Som, 2005) providing protection against conditions like oxidative stress, cardiovascular

diseases, diabetes, metabolic syndrome and different types of cancers (Camara *et al*., 2013).

The occurrence of biotic and abiotic stress hampers the bean yield to varying extent and the main contributing factors of yield reduction are diseases like ascochyta blight (*Ascochyta phaseolorum*), white mould *(Sclerotinia sclerotiorum*), anthracnose (*Colletotrichum lindemuthianum*), rust (*Uromyces appendiculatus*), leaf spot (*Cercospora cruenta*), powdery mildew (*Erysiphe polygoni*), charcoal rot (*Macrophomina phaseolina*), dry root rot and wilt (*Fusarium solani),* bacterial blight (*Xanthomonas phaseoli)*, bean common mosaic virus (BCMV), bean yellow mosaic virus (BYMV) and angular leaf spot (*Phaeoisariopsis griseola*) (Schwartz and Harveson, 2015).

Angular leaf spot (ALS) is one of the most serious diseases of common bean worldwide (Correa-Victoria *et al*., 1989). This disease is a major constraint in common bean production and yield losses up to 80 per cent have been reported (Schwartz *et al*., 1981; Saettler, 1991; Liebenberg & Pretorius, 1997; Junior *et al*., 2001). Stenglein *et al*. (2003) reported that every 10 per cent increase in disease severity results in 7.9 per cent yield loss under favourable environment conditions. Seed quality is deteriorated when pods are affected; thereby affecting the marketability of bean seeds (Pastor-Corrales *et al*., 1998). The disease produces symptoms on leaves, stems and pods of common bean throughout the growing season and is very devastative in areas where warm and moist conditions are accompanied by abundant inoculum from infected plant residues and contaminated seed (Allorent and Savary, 2005).

Angular leaf spot (ALS) pathogen exhibits high genetic diversity (Guzman *et al*., 1995; Mahuku *et al*., 2002; Wagara *et al*., 2004) and it has undergone parallel evolution with the host resulting in the formation of two main groups i.e., Andean and Mesoamerican (Mahuku *et al*., 2002; Crous *et al*., 2006). Several scientists have differentiated these two pathogen groups with the use of molecular markers (Guzman *et al*., 1999; Mahuku *et al*., 2002; Crous *et al*., 2006). In order

to develop effective management strategies, characterisation of the pathogen has a pivotal importance and the molecular tools in this respect prove to be more effective and authentic.

Although fungicides are an option for the management of angular leaf spot, they are often expensive and are not environment friendly or not readily available to marginal farmers, the predominant producers of dry bean. Cultivars with resistance to *Phaeoisariopsis griseola* offer a cost-effective, easy-to-use, readily available and environmentally friendly management strategy (Pastor- Corrales *et al*., 1998). Several sources of ALS resistance have been identified among primary and secondary gene pools of *Phaseolus vulgaris* (Pastor-Corrales *et al*., 1998; Busogoro *et al*., 1999; Mahuku *et al*., 2003, 2009, 2011). However, development of common bean cultivars with durable ALS resistance is difficult to maintain due to the broad and changing virulence diversity of the ALS pathogen that renders varieties that are resistant in one year or location susceptible in another (Pastor-Corrales *et al*., 1998; Mahuku *et al*., 2002; Nay *et al*., 2019).

Therefore, there is a need to identify new sources of resistance that can be regularly used to introgress desired genes into commercial cultivars. In order to identify new source(s) of resistance for sustainable management of ALS, the present investigation was undertaken with the following objectives:

- To characterise common bean angular leaf spot pathogen with sequence information of ribosomal DNA using Internal Transcribed Spacer (ITS) markers.
- To identify source(s) of resistance to angular leaf spot among diverse common bean genotypes.

Chapter-2

REVIEW OF LITERATURE

Angular leaf spot disease caused by *Pseudocercospora griseola* is one of the most destructive diseases of common bean. It is a facultative hemibiotrophic and/or necrotrophic fungal pathogen that deteriorates plant health, grain yield and seed quality in common bean. The disease is favoured by intermittent dry-wet and warm-cool weather (Correa-Victoria *et al*., 1989). Under favourable temperature conditions of 18 to 24^{o}C, high humidity (>70%) and in the presence of a susceptible host, the pathogen has the ability to colonize different parts of plants including leaves, pods and seeds (Stenglein *et al*., 2003). Leaf and stem infections by *P. griseola* result in premature defoliation, shrivelled pods and shrunken seeds, thus reducing the yield potential of beans (Stenglein *et al*., 2003). The pathogen has great variability, which explains the large number of existing races and the complexity of genetic resistance (Damasceno-Silva *et al*., 2008). The literature pertaining to present investigation entitled: "Characterisation of angular leaf spot pathogen and identification of source(s) of resistance in common bean and has been reviewed as follows:

2.1 Morphological characterization of *Pseudocercospora griseola*

2.2 Molecular characterization of *Pseudocercospora griseola*

2.3 Identification of sources of resistance to angular leaf spot

2.1 Morphological characterization of *Pseudocercospora griseola*

The pathogen of common bean angular leaf spot was first described as *Isariopsis griseola* by Saccardo (1878) and then successively as *Graphium laxum* Ell. (Ellis, 1881), *Isariopsis laxa* (Ell.) Sacc. (Saccardo, 1886), *Cercospora stuhlmanni* Henn and *Cercospora columnare* (Ellis and Everbart, 1893). Ferraris (1909) recognized the pathogen as *Phaeoisariopsis griseola*. Harter and Zaumeyer (1944) concluded that these were all synonyms of *Isariopsis griseola* based on

mycelial characteristics, conidial septation, pigmentation, conidiophore and stroma characteristics. *Phaeoisariopsis griseola* (Sacc.) Ferr is a deuteromycetous fungus belonging to class-Hyphomycetes, order-Stilbellales and family-stilbellaceae (Sutton, 1980). Conidiophores are produced in groups (synnemata) of 8 to 40 which are loosely fused together along most of their length to form a strand, splaying out only at the apex (Miles, 1917). Conidiophores are 30-80 µm in length and 3.0-8.8 µm in width. Conidia are septate usually 3-6, but sometimes may range from zero to seven (Hocking, 1967; Ellis, 1971). Buruchara (1983) reported considerable variation in conidial size and septation between and within isolates. Sharma and Sohi (1978) reported that the morphological characters are slightly different from those reported by Saccardo (1886), Miles (1917) and Hocking (1967) for Indian isolate of *Phaeoisariopsis griseola* with respect to length and width of synnemata, conidiophores, conidia and also the conidial septation. Bose and Sindhan, (1972) reported that synnemata arise from pear shaped stromata within the mesophyll and are 48 µm in diameter and 65 µm in height. The conidia of pathogen are pale grey, cylindrical to spindle shaped and tapering and rounded at one or both ends (Bose and Sindhan, 1972; Karanja *et al*., 1994). Deighton (1990) re-evaluated the genus and considered the synnematous arrangement of conidiophores to be unsuitable as main character for generic differentiation and subsequently he placed *Phaeoisariopsis* to a few species similar to *P. griseola*, having non-geniculate conidiogenous cells with flattened, but conspicuous scars. Von Arx (1983) and Braun (1992, 1995a) preferred to support *Phaeoisariopsis*, because of synnematous conidiomata and conspicuous (slightly thickened, not darkened) conidiogenous loci. Crous *et al*. (2006) found that the pathogen produced moderate aerial mycelium on PDA, pale olivaceous-grey to olivaceous-grey surface in the central part with iron-grey margin.

2.2 Molecular characterisation of *Pseudocercospora griseola*

Common bean angular leaf spot disease has been described to be one of the most serious disease with many strains and pathotypes in the world. Several

molecular techniques have been used to identify genetic changes in *P. griseola,* including ITS sequence analysis which is reviewed as under:

Guzman *et al*. (1999) differentiated *P. griseola* (Andean and Mesoamerican) forms as two major groups from infected common bean (*Phaseolus vulgaris*) leaves based on PCR amplification of different-sized fragments of DNA using group-specific primers.

Mahuku *et al*. (2002) elucidated the relationships among the Afro-Andean, Andean and Meso-American groups of *Phaeoisariopsis griseola* using different markers viz., Random amplified microsatellites, RAPD and restriction digestion of amplified ribosomal intergenic spacer region. Molecular data analysis revealed that Andean and Meso-American isolates formed separate groups and the Afro-Andean isolates clustered with Andean isolates. It was therefore, concluded that Afro-Andean isolates do not constitute a new *Phaeoisariopsis griseola* group and do not represent long-term evolution of the pathogen genome, but is likely the consequents of point mutations in genes for virulence.

Crous *et al.* (2006) re-evaluated the taxonomic status of *Phaeoisariopsis griseola* and the sequence analysis of the smaller subunit region of nuclear DNA revealed that the genus *Phaeoisariopsis* is not differentiated from other hyphomycete anamorph genera associated with *Mycosphaerella*, namely *Pseudocercospora* and *Stigmina*. A new combination was therefore, proposed in the genus *Pseudocercospora* over *Phaeoisariopsis* and *Stigmina*. Further, comparisons by means of morpho-cultural characteristics, and ITS sequence analysis of DNA, calmodulin and actin gene regions delineated two groups within *Phaeoisariopsis griseola*, recognised as two formae, namely f. *griseola* and f. *Mesoamericana*. *Phaeoisariopsis griseola* (Sacc.).

Abadio *et al*. (2012) found that ISSR-PCR technique was suitable for assessing intraspecific variability of *Phaeoisariopsis griseola.*

Landeras *et al*. (2015) identified Angular leaf spot (ALS) as *Pseudocercospora griseola* based on morphological features and the sequence analysis of ITS (Internal Transcribed Spacer) region.

Chilagane *et al*. (2016) characterized *Phaeoisariopsis griseola* and its host in Tanzania, by sampling for both pathogen as well as host. For characterization of the host from gene pool origin, Phaseolin DNA markers were used, whereas, for pathogen characterization the Internal Transcribed Spacer region (ITS) was used. Phylogenetic analysis revealed the presence of 69.7% Andean and 30.3% Mesoamerican strains of *P. griseola* in Tanzania. Population of common bean host consisted of 84.2% Andean and 15.8% Mesoamerican types. Therefore, it was concluded that Andean races of *Phaeoisariopsis griseola* and Andean common bean genotypes were predominant in Tanzania**.**

Serrato-Diaz *et al*. (2020) demonstrated that *Phaeoisariopsis griseola* and *Phaseolus vulgaris* have co-evolved and evaluated the diversity of *Phaeoisariopsis griseola* isolates from four countries. Total of 171 *P. griseola* isolates were evaluated from Puerto Rico, Honduras, Guatemala and Tanzania. Sequences obtained from four nuclear genes (β-tubulin, actin, the internal transcribed spacer (ITS) region of the ribosomal DNA and the SSU ribosomal RNA gene) were used in phylogenetic analysis by Bayesian inference. Phylogenetic trees grouped all isolates from Puerto Co (43), Honduras (25) and Guatemala (14) in the Middle American clade of the 89 Tanzanian isolates, 37 were Middle American and 40 Andean and a third population with 12 isolates was identified as Afro-Andean.

Canpolat and Maden (2020) characterized 118 isolates of *Pseudocercospora griseola* (Sacc.) isolated from common beans grown in controlled conditions of greenhouses in the western Black Sea region of Turkey. All 118 *P. griseola* isolates yielded 500-560 base pair PCR amplified products from ITS1 and ITS4 primers. The form of the Turkish isolates of *Phaeoisariopsis griseola* was determined as f. *griseola* since ITS sequences of 118

Phaeoisariopsis griseola isolates showed 98-100% similarity to the *Pseudocercospora griseola* f. *griseola* isolates deposited in GenBank.

2.3 Identification of sources of resistance to angular leaf spot

Breeding for resistance is the most economical approach for the management of Angular leaf spot, but high genetic diversity in the pathogen makes it difficult to achieve durable resistance (Mahuku *et al*., 2003). Therefore, it is important to characterise the pathogen and continually identify the new sources of resistance against the disease.

On the basis of morphological and molecular markers, two gene pools (groups) related to common bean origin have been defined namely; Andean, and the Middle American. The isolates of Andean origin are pathogenic to large seeded (Andean), while the Middle American isolates are pathogenic to both large and small seeded common beans (Mesoamerican) (Pastor-Corrales *et al*., 1998). Varieties of Andean origin such as AND-277, G5-686 and Mexico 54 have been identified as good sources of resistance (Pastor-Corrales *et al*., 1998; Nietsche *et al*., 2001; Aggarwal *et al*., 2004). However, several researchers across different countries from time to time have identified different sources of resistance which is reviewed as under:

Schwartz (1982) evaluated 13000 CIAT common bean accessions for their reactions to the angular leaf spot disease for three years and found that thirty accessions showed resistant reaction to numerous races obtained from other parts of the world, including Europe.

Pastor-Corrales *et al*. (1998) screened 22,832 common bean accessions against Angular leaf spot disease under natural conditions at CIAT's Experiment Station, Quilichao, Colombia, between 1985 and 1992. The resulting 123 accessions showed intermediate reaction (scores of 4 to 6). The resistant accessions were then tested in the controlled conditions of greenhouse against selected 14 *Phaeoisariopsis griseola* isolates of diverse origins. Nineteen common

bean accessions showed intermediate or resistant reaction to at least 13 of 14 pathogen isolates tested. Further, they evaluated 13,219 bred lines in the field of which 89 lines showed intermediate or resistant reaction and 33 bred lines proved intermediate or resistant to at least eight of nine isolates in the controlled conditions of greenhouse. Therefore, it was suggested that populations of common bean should be developed from crosses between Andean and Middle American gene pools to breed for durable resistance to common bean angular leaf spot disease.

Sartorato (2002) collected fifty one isolates of *Phaeoisariopsis griseola* in five Brazilian States and tested these isolates on a set of 12 international differential cultivars in the greenhouse of which seven different pathotypes were identified.

Mahuku *et al*. (2003) evaluated a common bean core collection, primary and secondary gene pools and lines derived from inter-specific crosses of *Phaseolus vulgaris* and *Phaseolus coccineus* or *Phaseolus polyanthus* (secondary gene pool) for resistance to angular leaf spot and found that out of 1441 accessions in the core collection, only 2.2% were resistant to both Andean and Mesoamerican races of *P. griseola*, 28% were resistant only to Andean and 9% to Mesoamerican races. Very few wild *P. vulgaris* accessions (4%), were resistant to ALS. In contrast, high level of resistance (62%) was found in the secondary gene pool. Among the 1010 lines from inter-specific crosses, 109 lines were highly resistant. These genotypes from the primary and secondary common bean gene pools resistant to Andean and Mesoamerican races of *P. griseola* offer a potential for developing broad and durable ALS resistance.

Aggarwal *et al.* (2004) identified CAL 143 as the first Andean bean with resistance to angular leaf spot disease caused by *Phaeoisariopsis griseola*. Further, it was found that CAL 143 was resistant in Zambia, Tanzania, Malawi, South Africa and susceptible in Uganda. They identified two additional Andean

bean lines, AND 277 and AND 279, with resistance to angular leaf spot in Malawi and found that Andean isolates were mostly compatible with Andean cultivars.

Padua *et al*. (2012) evaluated 209 inbred lines to 63-63 race of *P. griseola,* and found that 54 (26%) lines were resistant and 155 (74%) were susceptible.

Sanglard *et al*. (2013) reported the cultivar Ouro Negro possess one ALS resistance gene locus that is different from other resistance sources and this locus confers resistance to two pathogen races 63:23 and 63:39.

Seventy one local landraces in Uganda were screened for ALS resistance and only 14 per cent of the landraces were found resistant under field conditions. However, one land race U00297 was found resistant to all the four races tested under artificial inoculation conditions (Ddamulira *et al*., 2014).

Ddamulira *et al*. (2015) screened four commercial varieties, four landraces and two controls with four races of *P. griseola* (1:6, 17:39, 21:39 and 61:63) against ALS. The experiment was conducted in pots in a screen house to identify resistant genotypes and it was found that landrace (U01597) showed consistent resistance to four *P. griseola* races. But for commercial varieties, NABE 13 was found moderately resistant to four *P. griseola* races while the rest were susceptible.

Adikshita and Sharma (2016) screened 18 common bean germplasm lines for ALS resistance under natural epiphytotic and artificial inoculation conditions and found that none of the lines were resistant.

Kijana *et al*. (2017) screened 37 common bean varieties in Democratic Republic of Congo (DRC) using virulent Andean and Mesoamerican isolates and identified four bean varieties, viz., ARA 4, COD MLV 059, MLV 224/94B, LSA 144 and Mexico 54 resistant to Angular leaf spot.

Mukamuhirwa *et al*. (2017) screened fifty-seven common bean varieties and found that ACC 714 exhibited broad spectrum resistance against both Andean and Mesoamerican isolates of *Phaeoisariopsis griseola*.

Pereira *et al*. (2019) evaluated 144 common bean lines for resistance to *Phaeoisariopsis griseola* in greenhouse experiments (V2 and V3 stages) and in the field. Common bean lines were inoculated with a mixture of spores of pathogen races 63-63 and 63-23. They observed that (31 %), (7 %) and (10 %) of lines were resistant in stages V2, V3 and in the field, respectively. Estimates of coincidence index V2-V3, V2-field and V3-field were (68 %), (69 %) and (88 %) respectively. They found that evaluations at V3 stage and in the field were the phenotyping methods more efficient for recording ALS severity. They further emphasised that greenhouse experiments may be employed in association with the field experiments to increase genetic gain.

Rodriguez *et al*. (2019) studied the reaction of 181 common bean genotypes from USA, Malawi, Tanzania, Angola, Puerto Rico, Honduras, Ecuador and Colombia against two angular leaf spot races (61:11 and 63:5) and reported that 16 lines were identified to have resistance to both the races of *Phaeoisariopsis griseola*. The resistant lines included breeding lines of Andean origin CAL-143, CAL-277 and the Mesoamerican cultivar 'Ouro Negro' that possess the resistance genes Phg-5, Phg-1, Phg-3, respectively.

Rezene and Mekonin (2019) screened 300 common bean lines for resistance against Angular leaf spot disease under natural field conditions and found that only 14 lines showed resistant reaction. Hence, it was speculated that the lines showing resistance under natural epiphytotic conditions can be used in breeding programmes to achieve broad spectrum and durable ALS resistance.

Canpolat and Maden (2020) studied reaction of seventeen common bean cultivars against ten randomly selected most aggressive isolates of *P. griseola* and found that out of nine bush type bean cultivars, two varieties viz., Bourgondia and Yalova 17, showed resistant reaction against all of ten isolates while three climbing types, Fabio, Burayşe and Selvi, were found resistant against all the isolates of *P. griseola*.

Chapter-3

MATERIALS AND METHODS

The present investigation entitled "Characterisation of angular leaf spot pathogen and identification of source(s) of resistance in common bean." was carried out during Kharif 2020 in the lab/field of the Division of Plant Pathology of Faculty of Agriculture, SKUAST-K, Wadura, Sopore. The experiment details are proposed as under.

3.1 Isolation and morpho-cultural characterization of the pathogen

Common bean leaf samples showing typical symptoms of angular leaf spot were collected and brought to the laboratory for pathogen isolation. The pathogen isolation was done from sporulating lesions on infected leaves (Plate 1). The fungal synemma on infected leaves were picked up with a fine needle containing a small piece of agar and then placed in two drops of sterile water on water agar (WA) medium, spread on the agar surface and incubated at 22-24^{o}C for 24 to 48 hr for germination of conidia. Single spore isolation was done by picking up a single germinated conidium by the aid of a microscope and then transferred to V-8 juice agar plates. Five or six transfers of single spores were made in Petri dishes. The plates are then wrapped in aluminium foil and incubated at 22-24^{o}C for 14 to 21 days or until cultures reached to a diameter of 2.5 to 10 mm.

Purified isolates were maintained on water agar slants at 26±1^{o}C in BOD incubator for further use (Plate 1).

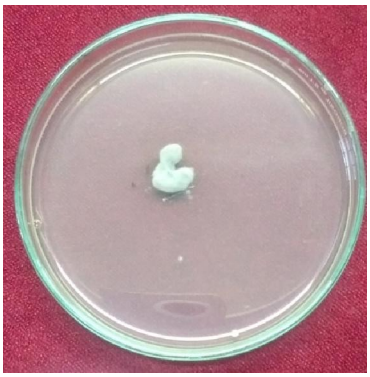
Morphological characteristics of the pathogen such as shape, size, septation and colour of mycelium, synnemata and conidia were studied and compared with standard description given in authentic literature for identification of the pathogen.

(a)

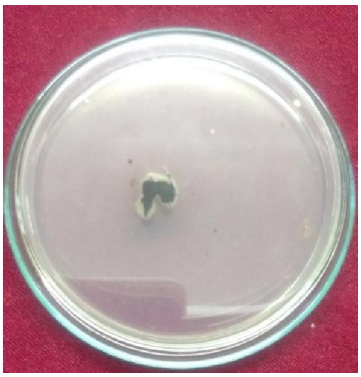
(b)

(c)

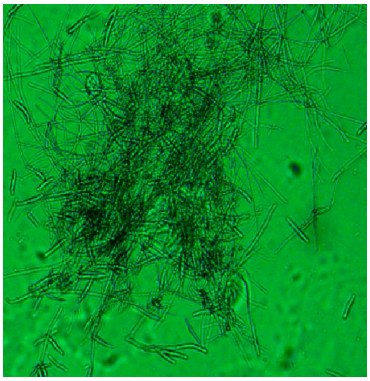
Plate-1: Isolation, Identification and Maintenance of the pathogen (a) Isolation (b) Purification (c) Maintenance



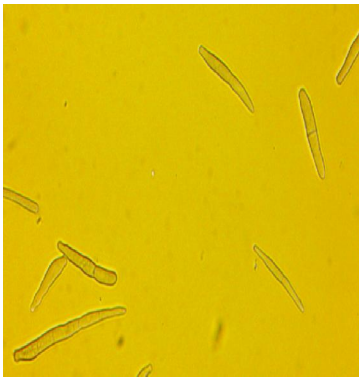
(a)



(b)

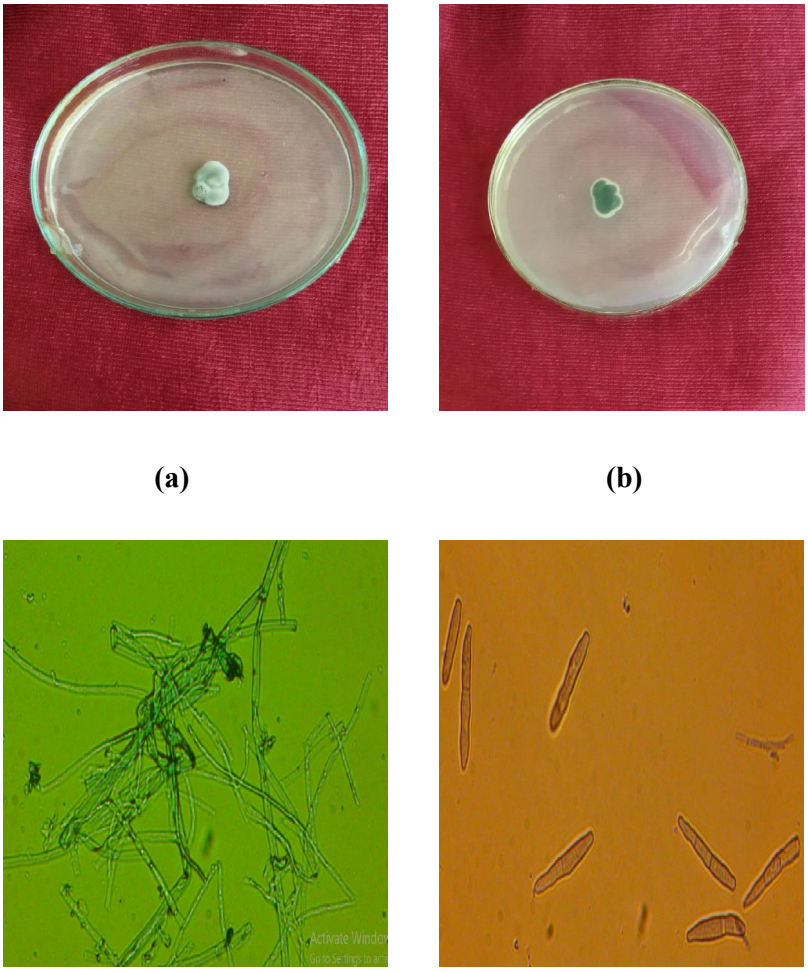


(c)



(d)

Plate-2: Morpho-cultural characteristics of *Pseudocercospora griseola* isolate Pg01 on V8 agar medium at 24±1°C (a) Upper side of colony (b) Underside of colony (c) Mycelium at 100X (d) Conidia at 400X



(a) **(b)**

(c) **(d)**

Plate-3: Morpho-cultural characteristics of *Pseudocercospora griseola* isolate Pg02 on V8 agar medium at 24±1°C (a) Upper side of colony (b) Underside of colony (c) Mycelium at 400X (d) Conidia at 400X Conidia at 400X

3.2 Molecular characterisation of the pathogen

3.2.1 Extraction of Genomic DNA

The isolates Pg01 and Pg02 were grown on potato dextrose broth in 150 ml Erlenmeyer flasks. After fifteen days these cultures were used for DNA isolation. Mycelia of both the isolates was taken from flasks by filtration through a double layered sterilized filter paper. The resultant mycelia was used for isolation of total genomic fungal DNA using modified CTAB (Cetyl trimethyl ammonium bromide) method (Murray and Thompson, 1980). From each isolate dried mycelium was ground into fine powder by constant crushing using autoclaved and pre-chilled mortar and pestle in liquid nitrogen. Fine powdered tissue of about 40-50 mg was transferred into 1.5 ml polypropylene centrifuge tube containing about 700µl pre-heated (65 ºC) 2X CTAB extraction buffer (100 mM Tris HCl Ph 8.0, 1.4 M NaCl, 20 Mm EDTA Ph 8.0, 2% CTAB, 1% PVP and 0.5% β-mercaptoethanol (added just before use)). The fine powder of tissues was suspended in the buffer by inverting and rotating the tubes properly. The samples were incubated at 65 ºC for 50-60 minutes in a water bath. The tubes were mixed occasionally while maintaining at 65 ºC. After 50-60 minutes of incubation in water bath 700µl of chloroform: isoamyl alcohol (24:1) was added and the tubes were swirled to form emulsion. The samples were centrifuged at 10,000 rpm for 10 minutes at room temperature. The supernatant of tubes was transferred to a sterilized 1.5 ml polypropylene centrifuge tube using large bore tip and then again 700µl mixture of chloroform isoamyl alcohol in the same ratio was added and that step was repeated. Then supernatant was transferred to another tube and same volume of about 600-700 µl pre-chilled isopropyl alcohol was added and the tubes were inverted gently several times and kept at - 20 ^{o}C. These tubes were centrifuged at 10,000 rpm for 10 minutes at 4 ^{o}C and then supernatant decanted. The DNA was then rinsed twice with 70 per cent ethanol for five minutes in order to remove any residual salts followed by re-centrifugation. Pellet was collected and the leftover ethanol was dried up completely by turning down microfuge

tubes on a blotting paper and allowed to air dry (at room temperature). The pellet was dissolved in 200µl of 1X TE (Tris EDTA buffer-10Mm Tris HCl, 1mM EDTA, Ph 8.0). The tubes were left at room temperature for few hours to dissolve the DNA. Heat-treated RNase (Fermentas) was then added to a final concentration of 10µg/ml. It was mixed and then incubated at 37 ^{o}C for 30 minutes. DNA of both the isolates was obtained in similar way and stored at -80 ^{o}C for further use.

3.2.2 Assessment of DNA quality and quantity

DNA quantity was checked by Agarose gel electrophoresis. In this 0.8 g of agarose was dissolved in 100 ml of 0.5X Tris acetate EDTA (Ethylene diamine tetra acetic acid) (TAE) electrophoresis buffer. The whole mixture was heated till the agarose was dissolved completely. Then mixture was cooled down to 60 ºC with constant stirring. Then Ethidium bromide was added to a final concentration of 0.5µl /mg of buffer. This agarose solution was poured into an already prepared gel mould with combs and was left for 20-30 min for solidification. DNA samples for loading were prepared by adding loading dye (2µl) (6X) (0.25% W/V bromophenol blue, 50% glycerol in sterile water) to 8 µl DNA so that the final concentration of loading dye was 1X. The samples of DNA were loaded into wells with the help of micropipette. Along with the DNA samples, marker of known concentration was also loaded. The agarose gel was run for about 1-2 hours and visualized under UV transilluminator using photo gel documentation system (Alfa Imager EC, Protein Simple, USA) and DNA samples were photographed. The intensity of fluorescence of each sample was compared with that of a standard marker and then concentration of each DNA sample was ascertained. The quality samples of DNA was judged based on whether DNA formed a single high molecular weight band (good quality) or a smear (degraded/poor quality). The DNA of both the samples was diluted to 25 ng/µl by adding double distilled sterile water and was used for PCR.

Two ITS primers viz., ITS1 and ITS4 (Table 3.1) were used for amplification of ribosomal DNA sequence of the test pathogen (White *et al*., 1990).

Table 3.1: ITS Primers, their sequence and annealing temperatures for PCR analysis

S.No.	Primer	Sequence	Annealing Temperature
1	ITS1	5'TCCGTAGGTGAACCTGCG3'	58^{o}C
2	ITS4	5'TCCTCCGCTTATTTGATATGC3'	58^{o}C

3.2.3 PCR amplification

In vitro amplification using polymerase chain reaction (PCR) (Saiki *et al*., 1988) was performed in 0.2 ml PCR tubes in a T-Gradient Whatman Biometra thermal cycler using 50-60 ng of genomic DNA for ITS analysis of each isolate in a final volume of 25µl per reaction. The stock and the final concentration of different components used in PCR for ITS primers are given (Table 3.2).

Table 3.2: Stock and final concentration of various components of reaction mixture used in PCR for ITS analysis

Components	Stock Conc.	Volume (µl)	Final Conc.
Sterile distilled water	..	15.8	..
PCR buffer	10X	2.5	1.0X
$Mgcl_2$	25mM	1.5	1.5mM
DNTPs	2.5mM	2.0	0.2mM
Primer	10pmol	1.0	0.4pmol
Taq Polymerase	5U/µl	0.2	1.0 Unit
DNA template	25 ng	2.0	50.0 ng
	Total	**25**	

*10X PCR buffer: 10mM Tris HCl, Ph 8.3, 50Mm KCl

The reaction mixture in PCR tubes was given short spin in microfuge (Thermo Scientific, Thermo Electron Corporation) and placed in 96 well thermal cycler. PCR amplification was performed in thermal cycler programmed for initial denaturation at 94 ^{o}C followed by 35 cycles at 94 ^{o}C for 1 minute, at annealing temperature for 1 minute, 72 ºC for 2 minutes and a final extension of 10 minutes.

3.2.4 Visualization of PCR products

Loading dye of 4µl of 6X was added to 25µl of the amplified product so as to make the final concentration of the loading buffer in the reaction samples to 1X. The PCR products were resolved on 1.2 per cent agarose gel. The gel was prepared in 0.5X TAE buffer. Ethidium bromide was added to gel at concentration of 0.5µg/µl. The gel was run at 5 V/cm, visualized under UV light and photographed using Alfa Imager gel documentation system (Alfa Imager EC, protein Simple, USA).

3.2.5 Sequencing of the pathogen isolates

After PCR amplification using ITS primers, 5µl of PCR product of both the isolates was electrophoresed to ensure successful amplification and remaining 20µl PCR products of both the isolates were sent for custom sequencing (Biokart India Pvt Ltd Banglore-43).

3.2.6 Data analysis

After custom sequencing of two isolates of the pathogen, the sequences were retrieved from chromatograms using BioEdit version 7.0 (Hall, 1999) and compared against those sequences already available in the databases using programme BLASTn (http://www.ncbi.nlm.nih.gov/BLAST). The consensus sequence reconfirmed by comparing it with the original data output. The sequences were aligned using CLUSTAL W software (Thompson *et al.*, 1994) and submitted to gene bank National Centre for Biotechnological Information (NCBI).

For sequence analysis based on ITS regions of *Pseudocercospora griseola*, the sequences were compared with nine *Pseudocercospora mesoamericana* f .sp.

mesoamericana sequences, three *Pseudocercospora griseola* f.sp. griseola sequences, one *Pseudocercospora rhamnellae* and one *Pseudocercospora proteae* sequence. All nucleotide sequences were aligned using CLUSTAL X 1.8 multiple alignment programme (Thompson *et al.,* 1997) and refined manually. The GENEDOC package (www.psc.edu/biomed/genedoc/gdpf.html) (accession numbers **MZ413342** and **MZ452242)** was used for formatting the sequences to make them compatible with the desired software.

3.3 Phenotypic analysis for angular leaf spot resistance

The screening of common bean genotypes for resistance against Angular leaf spot was carried out during Kharif 2020 at Faculty of Agriculture, Wadura. A set of 88 diverse common bean lines from two different gene pools (Andean and Meso-American) were used for evaluation

3.3.1 Phenotypic analysis under field conditions

Common bean lines were raised over an area of 1000 m^2 in an Augmented Block Experimental Design during kharif 2020 at Faculty of Agriculture, Wadura (Plate-4). These lines were assessed for disease resistance under natural epiphytotic conditions and disease intensity was recorded at R_7-stage using the scale given by Van Schoonhoven and Pastor-Corrales, (1987**)** described in Table 3.3.

Table 3.3: Standard rating scale for assessment of disease severity in field

Score	Description
1	No visible symptoms
3	Upto 2% of leaf and pod surface affected with non sporulating lesions
5	5% of pod and leaf surface affected with small lesions having limited sporulation
7	10% of pod and leaf surface affected with large sporulating lesions
9	25 % or more area of the leaf or pod is covered by large sporulating lesions.

3.3.2 Phenotypic analysis under artificial inoculation conditions in greenhouse

To estimate the usefulness of potential sources of ALS resistance, common bean genotypes that displayed resistance in the field were further studied in the greenhouse using pathogen race *Phaeoisariopsis griseola* f.sp *griseola* (Pg02). The experiment was conducted in a completely randomised design with three replications (Plate-4). The soil was sterilized by autoclaving and was put in pots. Fifty-four genotypes were raised in greenhouse using garden soil in plastic pots (200 $inch^3$) at the rate of three plants per pot. Seedlings were inoculated with a conidial suspension ($2.0x10^{4}$ conidia mL^{-1}) of the pathogen at first trifoliate stage. The suspension was sprayed on both the surfaces of leaf with the help of a hand atomiser. Disease severity was assessed after 15 days of inoculation using the scale given by Librelon *et al*. (2015) as described in Table 3.4.

Table 3.4: Scale for assessment of disease severity under greenhouse conditions

Score	Leaf area covered
1	0%
2	2%
3	4%
4	7%
5	7-16%
6	16-26%
7	26-32%
8	32-38%
9	>38%

Data Analysis

In both the experiments, genotypes scoring 3 or less were considered as resistant, 3.1-6 were intermediate and those scoring greater than six were susceptible (Mahuku *et al*., 2003).

(a) **(b)**

(c) **(d)**

Plate-4: Experimental trials for screening of common bean genotype against angular leaf spot disease. (a) Field trial (b) Disease scoring of common bean genotypes in field (c) Greenhouse trial (d) Disease scoring of common bean genotypes in greenhouse

Chapter-4

EXPERIMENTAL FINDINGS

The findings of present investigation entitled "Characterisation of angular leaf spot pathogen and identification of source(s) of resistance in common bean are presented as under:

4.1 Morphological characterization of the pathogen

Angular leaf spot infected common bean leaf samples were collected and brought to laboratory for isolation of pathogen using standard methods as discussed in the materials and methods. In total, two isolates viz., Pg01 and Pg02 were purified using single spore technique (Tuite, 1969) and different morpho-cultural characteristics were recorded (Table-4.1 & 4.2). The isolates of the pathogen Pg01 and Pg02 were slow growing with about 2.0 mm and 2.5 mm colony diameter, respectively on V-8 agar juice medium after incubation of 15 days at 25±1^{o}C. Colony colour of isolate Pg01 was initially greyish which later became black and colony colour of isolate Pg02 was initially greyish white which later turns dark olivaceous. The mycelium of both the isolates Pg01 and Pg02 was septate, irregularly branched, hyaline with a diameter of 3.47-4.01 µm (Av.3.7 µm). Both the isolates were characterized by having synnematous and pigmented conidiophores. The size of conidiophores was 90-158 × 3.26-4.6 µm (Av.138×3.6 µm) in isolate Pg01 and 91.5-156 × 3.22-4.5 µm (Av.139 × 3.2 µm) in isolate Pg02. The conidia of both the isolates were cylindrical in shape and septate (3 to 8 septa). The conidia of both the isolates were olivaceous grey to olive brown in colour. Conodial size ranged from 38.10-49.04 × 3.1-6.29 µm in Pg01 and 38.16-49.0 × 3.2-6.26µm in Pg02. Based on these morpho-cultural characters, the isolates were identified as *Pseudocercospora griseola* (Sacc.) Crous & U. Braun.

Table 4.1: Morpho-cultural characteristics of *Pseudocercospora griseola* isolate Pg01 on V8 agar medium at 24±1°C

Propagule	Shape	Colour	Size (µm)	Septation
Colony	Slow growing, regular	Initially greyish finally becomes black	1-2 mm after 5 days on V-8 agar juice media at 25± 1 ºC	No
Mycelium	Branched and smooth	Hyaline	3.47-4.01 (diameter)	Septate
Conidiophore	Filliform, branched	Dark brown	90-158 × 3.26-4.6	Septate
Conidia	Cylindrical	Olivaceous brown	38.10-49.04 × 3.1-6.29	Septate

Table 4.2: Morpho-cultural characteristics of *Pseudocercospora griseola* isolate Pg02 on V8 agar medium at 24±1°C

Propagule	Shape	Colour	Size (µm)	Septation
Colony	Slow growing, Irregular	Initially greyish white later became black	2.5 mm after 5 days on V-8 agar juice media at 25± 1 °C	No
Mycelium	Branched and smooth	Sub-hyaline	3.1-4.42 (diameter)	Septate
Conidiophore	Filliform, branched	Dark brown	91.5-156 × 3.22-4.5	Septate
Conidia	Cylindrical	Olivaceous brown	38.16-49.0 × 3.2-6.26	Septate

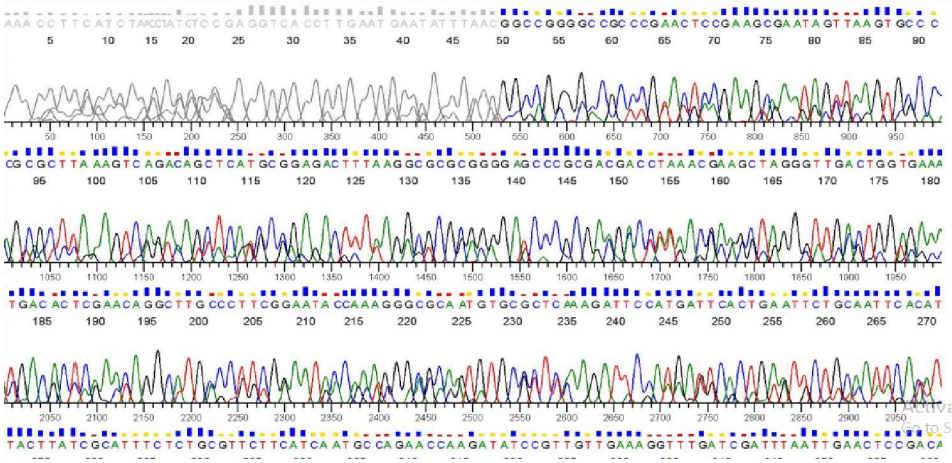
4.2 Molecular characterization

4.2.1 Sequence analysis of ribosomal DNA (rDNA) region

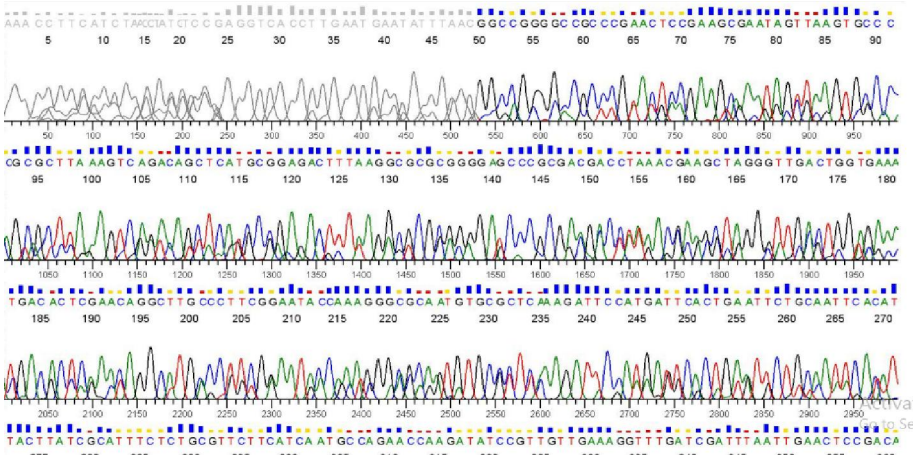
Internal transcribed spacer (ITS) regions of rDNA of two isolates were amplified using ITS1 and ITS4 primers and amplified products were custom sequenced. The sequences were retrieved from chromatograms (Plate-5) and the sequence information was analysed through BLASTn programme which indicated that the sequences showed maximum similarity with *Pseudocercospora griseola* f.sp. *griseola* sequences available at NCBI (Table 4.3). Two sequences were submitted to NCBI and Accession Numbers were obtained (Table 4.3)

4.2.2 Phylogenetic Analysis

Two sequences of *Pseudocercospora griseola* (present study) were compared with nine *Pseudocercospora mesoamericana* f .sp. *mesoamericana* sequences, three *Pseudocercospora griseola* f.sp. *griseola* sequences, one *Pseudocercospora rhamnellae* and one *Pseudocercospora proteae* sequence available in NCBI database by molecular evolutionary genetic analysis (MEGA) software v 5.05. An optimal tree was generated by using Kimura-2-parameter substitution model and different taxa were clustered together in a bootstrap test with 1000 replicates (Fig. 1). The phylogenetic analysis grouped 2 sequences of *Pseudocercospora griseola* Pg01 and Pg02 (present study) and 3 *Pseudocercospora griseola* sequences (retrieved from NCBI database) in a single clade and the Mesoamerican types were grouped separately which indicated that ITS sequences differentiate two pathogen races as Andean and Mesoamerican types. Other two *Pseudocercospora rhamnellae* sequence and *Pseudocercospora proteae* sequence were clustered separately as an out group, thereby indicating that ITS sequence analysis was a powerful tool for authentic identification and genetic diversity studies.



(a) Chromatogram of *Pseudocercospora griseola* isolate Pg01



(b) Chromatogram of *Pseudocercospora griseola* isolate Pg02

Plate-5: Chromatogram of *Pseudocercospora griseola* isolates

Table 4.3: *Pseudocercospora griseola* and other fungal isolates used for phylogenetic analysis based on sequences of rDNA region

Isolate	Host	Isolate code	Accession no.	Similarity Score in BLAST search
1.	Common bean	Pg01	MZ413342	*Pseudocercospora griseola* f. sp. *griseola* (98.24%)
2.	Common bean	Pg02	MZ452242	*Pseudocercospora griseola* f. sp. *griseola* (96.71%)

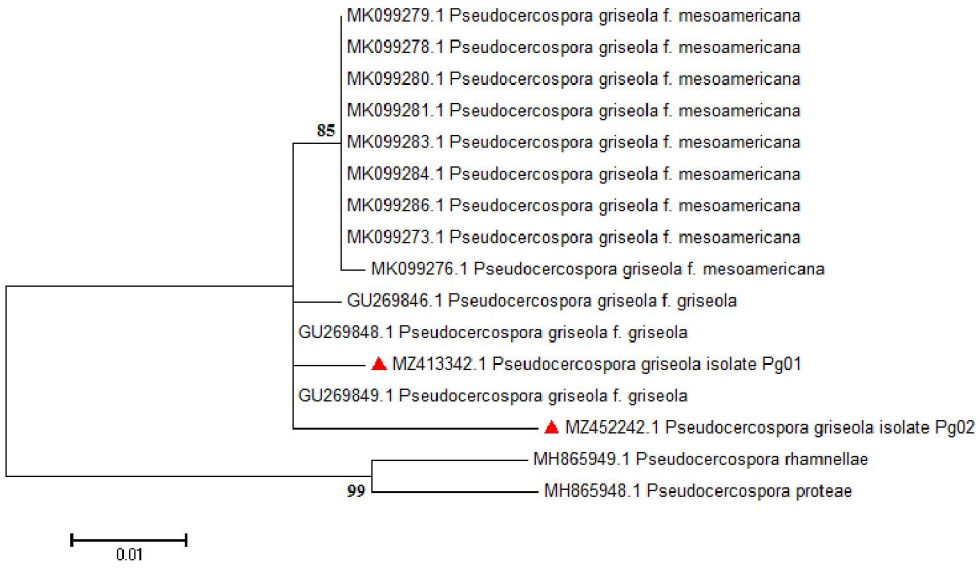


Fig. 1: Phylogenetic relationship among *Pseudocercospora griseola* and other fungal isolates

4.3 Phenotypic analysis for identification of source(s) of resistance against angular leaf spot

4.3.1 Evaluation of Common bean genotypes under natural epiphytotic conditions of field

Eighty-eight genotypes were screened for resistance to Common bean Angular leaf spot under natural epiphytotic conditions at experimental plots of Faculty of Agriculture, Wadura. The genotype showed varied response to disease (Table 4.4, Plate-6). The disease reaction score varied from 1 to 9 with lowest in genotype WB-1677, WB-22 and highest in N-89.

Based on the reaction score the genotypes were categorized as resistant, intermediate and susceptible (Table 4.5), out of 88 different genotypes twenty two genotypes viz**.** WB 1255,WB 222,WB1677,WB 83,WB 352,WB 643,WB 22,WB 1607,WB 952,N-3,WB 371,WB 216,WB 185,WB 1129,Arka anop,SR-1,N-3,G-716,WB 45,WB 1006,WB 1199 and WB 413 were found resistant while forty-nine viz. WB 1634, WB 4709, WB 249, WB 1131, WB 967, WB 923, WB 6,WB 1319,WB 429,WB 1643,WB 492, WB 258,GLP-1,N-2,WB 966,SFB-1,WB 195-1,WB 811,WB 832,UK-2, WB 1187,WB 257,WB 489,WB 1682,WB 1455,WB 451,N-11,WB 1466,N-10,GLY-1,WB 1282,WB 1189,WB 206,WB 112,WB 473,WB 603,WB 947,WB 934,WB 258,N-1,WB-195-2,WB 1310,WB 651,N-9,WB 4564,GL-3,WB 1694,N-10,WB 435 showed intermediate reaction and seventeen genotypes viz. WB 1644, N-7, WB 1644, N-7, WB 662, WB 341,WB 1492,WB 956,WB 1518, N-4, Arka komal, N-5,WB 1185,WB 341,WB 642,WB 401, N-89, N-5 and WB 1249 were found susceptible. Phenotypic evaluation in field revealed that most of the genotypes showed intermediate reaction (Fig 2).

WB-371(R) **WB-1677(R)** **WB-216(I)**

N-89 **WB-1492** **WB-1644**

Plate-6: Field reaction of different common bean genotypes to angular leaf spot

Table 4.4: Disease reaction of common bean genotypes to angular leaf spot disease under naturally occurring field conditions during Kharif 2020

S.No.	Genotype	Score	Reaction
1.	WB 1634	5	Intermediate
2.	WB 4709	5	Intermediate
3.	WB 249	3.8	Intermediate
4.	WB 1131	4.2	Intermediate
5.	WB 967	3.8	Intermediate
6.	WB 1255	3	Resistant
7.	WB 222	3	Resistant
8.	WB 923	4.2	Intermediate
9.	WB 6	5.4	Intermediate
10.	WB 1319	5	Intermediate
11.	WB 429	4.6	Intermediate
12.	WB 1643	3.8	Intermediate
13.	WB 492	3.8	Intermediate
14.	WB 258	5	Intermediate
15.	WB1677	1	Resistant
16.	WB 83	1.4	Resistant
17.	WB 1644	8.2	Susceptible
18.	WB 352	2.2	Resistant

19.	WB 643	2.2	Resistant
20.	WB 22	1	Resistant
21.	GLP-1	4.2	Intermediate
22.	N-2	5.4	Intermediate
23.	N-7	6.6	Susceptible
24.	WB 662	6.6	Susceptible
25.	WB 966	5.8	Intermediate
26.	SFB-1	5.8	Intermediate
27.	WB 341	6.2	Susceptible
28.	WB 195-1	4.2	Intermediate
29.	WB 1607	1.4	Resistant
30.	WB 952	3	Resistant
31.	WB 811	3.4	Intermediate
32.	WB 832	5.4	Intermediate
33.	N-3	3	Resistant
34.	WB 371	2.6	Resistant
35.	UK-2	5	Intermediate
36.	WB 1187	5	Intermediate
37.	WB 1492	6.2	Susceptible
38.	WB 257	3.4	Intermediate
39.	WB 489	5	Intermediate

40.	WB 1682	3.8	Intermediate
41.	WB 216	1.8	Resistant
42.	WB 1455	5.8	Intermediate
43.	WB 451	4.6	Intermediate
44.	WB 185	3	Resistant
45.	WB 1129	3	Resistant
46.	N-11	5	Intermediate
47.	WB 956	7	Susceptible
48.	WB 1466	4.2	Intermediate
49.	WB 1518	6.6	Susceptible
50.	N-4	7.4	Susceptible
51.	N-10	5.8	Intermediate
52.	Arkakomal	6.6	Susceptible
53.	GLY-1	4.6	Intermediate
54.	N-5	8.6	Susceptible
55.	WB 1185	6.2	Susceptible
56.	WB 341	6.2	Susceptible
57.	WB 642	7.4	Susceptible
58.	WB 1282	4.6	Intermediate
59.	WB 1189	5.4	Intermediate
60.	Arka anop	1.8	Resistant

61.	SR-1	1.4	Resistant
62.	N-3	2.2	Resistant
63.	WB 206	3.4	Intermediate
64.	G-716	1.8	Resistant
65.	WB 45	1.4	Resistant
66.	WB 1006	3	Resistant
67.	WB 112	3.4	Intermediate
68.	WB 473	4.2	Intermediate
69.	WB 1199	3	Resistant
70.	WB 603	4.2	Intermediate
71.	WB 947	3.4	Intermediate
72.	WB 934	4.6	Intermediate
73.	WB 413	3	Resistant
74.	WB 258	5	Intermediate
75.	N-1	5	Intermediate
76.	WB-195-2	5	Intermediate
77.	WB 1310	5.4	Intermediate
78.	WB 651	5.8	Intermediate
79.	N-9	3.8	Intermediate
80.	WB 4564	3.8	Intermediate
81.	GL-3	3.8	Intermediate

82.	WB 401	6.2	Susceptible
83.	N-89	9	Susceptible
84.	N-5	8.6	Susceptible
85.	WB 1249	6.2	Susceptible
86.	WB 1694	5	Intermediate
87.	N-10	5.8	Intermediate
88	WB 435	3.4	Intermediate

Table 4.5: Categorization of common bean genotypes on the basis of reaction to Angular Leaf Spot disease under field conditions

Disease Scale	Reaction	Genotypes	No.
≤3	RESISTANT	WB -1255, WB- 222, WB- 1677, WB -83, WB -352, WB -643, WB -22, WB -1607, WB -952, N-3, WB- 371, WB -216, WB -185, WB -1129, Arka- anop, SR-1, N-3, G-716, WB- 45, WB- 1006, WB- 1199, WB -413	22
3.1-6	INTERMEDIATE	WB -1634, WB- 4709, WB -249, WB -1131, WB -967, WB- 923, WB -6, WB- 1319, WB- 429, WB- 1643, WB- 492, WB- 258, GLP-1, N-2, WB- 966, SFB-1, WB 195-1, WB -811, WB -832, UK-2, WB -1187, WB- 257, WB- 489, WB -1682, WB- 1455, WB- 451, N-11, WB- 1466,N-10, GLY-1, WB -1282, WB- 1189, WB -206, WB- 112, WB- 473, WB -603, WB- 947, WB -934, WB -258, N-1, WB-195-2, WB -1310, WB -651, N-9, WB -4564, GL-3, WB -1694, N-10, WB- 435	49
≥6	SUSCEPTIBLE	WB- 1644 ,N-7, WB 1644,N-7, WB 662, WB -341, WB- 1492, WB -956, WB -1518, N-4, Arka komal, N-5, WB- 1185, WB -341, WB- 642, WB- 401, N-89, N-5, WB -1249	17

4.3.2 Germplasm screening under artificial inoculation conditions of greenhouse

Fifty four common bean genotypes were screened in greenhouse under artificial inoculation conditions with the isolate Pg02 (Table 4.6). The genotypes showed varied response to Andean race of *P. griseola* (Plate 7). The disease scores ranged from 1 to 9. Highest disease score of 9 was recorded in genotype WB-371 whereas, lowest was recorded in genotype WB-1129.

Based on disease reaction score, the genotypes were categorized as resistant, intermediate and susceptible (Table 4.7). Among fifty four genotypes screened in greenhouse, eleven genotypes viz. WB -1677, WB -83, WB- 643, WB -22, WB- 952 and WB -371 were resistant, thirteen genotypes viz. WB- 4709, WB- 249,WB-1131,WB-1255,WB-429, WB-352,GLP-1,WB-257, WB-1455,WB-185, WB- 1518,N-3 and WB -206 were intermediate while rest of the genotypes viz. WB-967,WB-923,WB- 1319,WB- 1643,WB- 492, WB 195-1,WB -1607,WB-811,WB-832,WB- 1492, WB-489,WB-1682,WB-1129,WB -1466,WB -1185, WB-642,WB-1282,WB-1189,WB-112,WB-473,WB-1199,WB-603,WB-947,WB-934,WB-1310,WB-651,WB-1249,WB 1694, N-10,WB-435 were susceptible. Most of the genotypes that were screened in greenhouse showed susceptible reaction (Fig 3).

WB-216(R)

WB-952(R)

WB-371(R)

GLP-1(I)

WB-832(S)

WB-923(S)

Plate-7: Reaction of Common bean genotypes to Angular leaf spot in greenhouse

Table 4.6: Disease reaction of common bean genotypes under controlled conditions in greenhouse during kharif 2021

S.No.	Genotype	Score	Reaction
1.	WB- 4709	6	Intermediate
2.	WB- 249	5.6	Intermediate
3.	WB -1131	5.6	Intermediate
4.	WB -967	6.6	Susceptible
5.	WB- 1255	1.3	Resistant
6.	WB- 923	7	Susceptible
7.	WB -1319	6.3	Susceptible
8.	WB- 429	5.3	Intermediate
9.	WB- 1643	6.3	Susceptible
10.	WB -492	6.6	Susceptible
11.	WB-1677	2.6	Resistant
12.	WB- 83	3	Resistant
13.	WB -352	6	Intermediate
14.	WB -643	2	Resistant
15.	WB -22	6	Intermediate
16.	GLP-1	4.6	Intermediate
17.	WB 195-1	7.6	Susceptible
18.	WB- 1607	7.3	Susceptible
19.	WB- 952	3	Resistant
20.	WB- 811	6.3	Susceptible
21.	WB- 832	8.3	Susceptible
22.	WB -371	1	Resistant
23.	WB -1492	7.6	Susceptible
24.	WB- 257	5.6	Intermediate
25.	WB -489	6.3	Susceptible
26.	WB -1682	7.3	Susceptible
27.	WB -216	2.3	Resistant

28.	WB -1455	6	Intermediate
29.	WB- 185	5.6	Intermediate
30.	WB- 1129	9	Susceptible
31.	WB -1466	7.3	Susceptible
32.	WB- 1518	5.6	Intermediate
33.	WB -1185	8.3	Susceptible
34.	WB -642	7.3	Susceptible
35.	WB -1282	8.3	Susceptible
36.	WB -1189	6.3	Susceptible
37.	N-3	3.6	Intermediate
38.	WB -206	6	Intermediate
39.	G-716	1.6	Resistant
40.	WB- 45	1.3	Resistant
41.	WB -1006	2.6	Resistant
42.	WB -112	6.6	Susceptible
43.	WB -473	7.6	Susceptible
44.	WB -1199	6.6	Susceptible
45.	WB- 603	7.3	Susceptible
46.	WB -947	7.6	Susceptible
47.	WB -934	6.6	Susceptible
48.	WB- 1310	7.6	Susceptible
49.	WB- 651	7.6	Susceptible
50.	GL-3	1.6	Resistant
51.	WB- 1249	7.3	Susceptible
52.	WB- 1694	7.6	Susceptible
53.	N-10	8.3	Susceptible
54.	WB -435	7	Susceptible

Table 4.7: Categorization of Common bean genotypes on the basis of reaction to Angular Leaf Spot disease under field conditions

ANGULAR LEAF SPOT SCALE	REACTION	GENOTYPES	No.
≤3	RESISTANT	WB- 1677,WB- 83,WB -643,WB -22,WB -952,WB- 371, WB -216, G-716,WB- 45,WB- 1006,GL-3	11
3.1-6	INTERMEDIATE	WB -4709, WB -249, WB -1131, WB -1255, WB -429, WB -352, GLP-1, WB -257, WB -1455, WB -185, WB -1518,N-3,WB -206	13
6.1-9	SUSCEPTIBLE	WB- 967, WB -923, WB -1319, WB 1643, WB 492, WB 195-1, WB 1607, WB 811, WB- 832, WB- 1492, WB 489, WB 1682, WB 1129, WB -1466, WB 1185, WB 642, WB 1282, WB -1189, WB 112, WB 473, WB- 1199, WB 603, WB 947, WB 934, WB 1310, WB -651,WB- 1249,WB -1694,N-10,WB 435	30

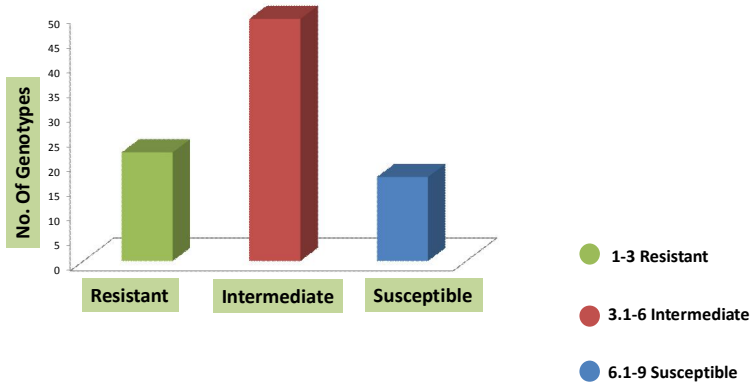


Fig.2: Reaction of common bean genotypes to angular leaf spot under natural epiphytotic condition in field

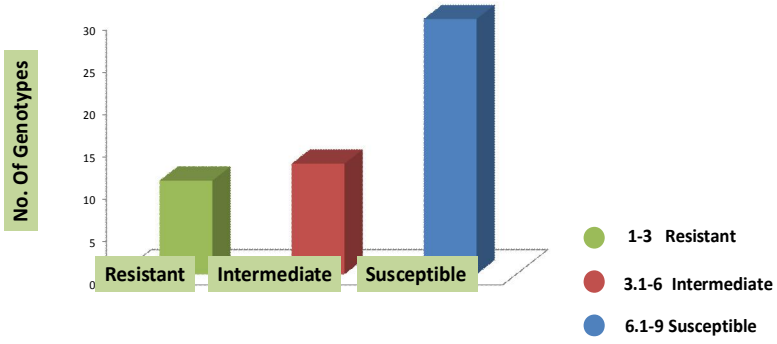


Fig.3: Reaction of common bean genotypes to angular leaf spot under controlled conditions of green house

Chapter-5

DISCUSSION

Common bean (*Phaseolus vulgaris* L.) is the most important edible pulse crop worldwide after soybean and peanut in the production (Mongi, 2016), and is popularly known as nutritional powerhouse due to its quality proteins and nutritional balance. However common bean cultivation also faces serious economic threats due to various biotic stresses. Angular leaf spot is one of the most serious diseases of common bean and causing up to 90 per cent yield losses. Although the disease can be managed with frequent use of fungicides, but the most effective and environmentally safe strategy to control this disease is the use of improved varieties with resistant genes of both Andean and Mesoamerican origin. However, co-evolution of the pathogen with its host has made breeding for ALS resistance even more complex. It was therefore, worthwhile to conduct present investigation with objectives as outlined in the introduction chapter.

The findings of the present investigation entitled "Characterisation of angular leaf spot pathogen and identification of source(s) of resistance in common bean" are briefly discussed as under:

The pathogen was isolated from naturally infected common bean plants and the morphological and cultural characters were studied. In culture the isolates of pathogen Pg01 and Pg02 were slow growing with about 2 mm and 2.5 mm colony diameter respectively on V-8 agar juice medium after incubation of 5 days at 25±1^{o}C. Colony colour of isolate Pg01 was initially greyish which later becomes black and colony colour of isolate Pg02 initially greyish white which later on turned dark olivaceous. Crous *et al.,* 2006 reported the similar colony characteristics of *P.griseola*. The mycelium of both the isolates Pg01 and Pg02 was septate, irregularly branched, hyaline with a diameter of 3.47-4.01 µm (av.3.7 µm). Both the isolates were characterized by having synnematous and pigmented conidiophores. The size of conidiophores was 90-158 × 3.26-4.6 µm with an

average of 138×3.60 µm in isolate Pg01 and 91.5-156 × 3.22-4.50 µm with an average of 139 × 3.20 µm in isolate Pg02. Conidial dimensions varied from 38.10-49.04 × 3.1-6.29 µm in Pg01 and 38.16-49.0 × 3.2-6.26 µm in Pg02. Similar, morpho-cultural characteristics of the pathogen for *P. griseola* had been observed earlier from Kashmir (Rashid, (2018) and from different parts of the world (Lianos, 1957; Hocking, 1967; Ellis, 1971; Bose and Sindhan, 1972; Karanja *et al*., 1994; Staglein *et al*., 2003; Adikshita, 2012,). Based on these morphological characters, both the isolates were identified as *Pseudocercospora griseola* (Sacc.) Crous & U. Braun.

In order to delineate the forma species, the two pathogen isolates were characterized based on sequence analysis of ITS region. Two sequences of *Pseudocercospora griseola* of present investigation showed maximum similarity with *Pseudocercospora griseola* f.sp. *griseola* sequences available at NCBI using BLASTn programme. Thus, sequence analysis of ITS region is a powerful tool for authentic identification of fungal species as this region is highly conserved within species but varies from species to species. Two sequences of *Pseudocercospora griseola* obtained in present study were submitted to NCBI and accession numbers obtained are MZ452242 and MZ413342. Phylogenetic analysis led to the grouping of isolates Pg01 and Pg02 together with the sequences *Pseudocercospora griseola* f.sp. *griseola* retrieved from NCBI GenBank. However, the sequences of *Pseudocercospora griseola* f. sp. *mesoamericana* were clustered separately. Canpolat and Maden (2021) characterized Turkish isolates with ITS sequence analysis and found that all the twenty one isolates in their study corresponded to *Pseudocercospora griseola* f. sp. *griseola*. It is therefore speculated that either of the two forms (Andean and Mesoamerican) may dominate the pathogen population in a particular geographical area. However in depth studies of pathogen population structure are warranted to get more insights into the pathogen diversity of *P.griseola* in Jammu and Kashmir.

Other researchers have also reported the importance of ITS sequence analysis for species identification and also for discrimination of Andean and Mesoamerican races of *Pseudocercospora griseola* (Crous *et al*., 2006; Landeras *et al*., 2015; Chilagane *et al*., 2016; Serrato-Diaz *et al*., 2020).

Eighty-eight genotypes were screened for resistance to Common bean Angular leaf spot under natural epiphytotic conditions at experimental plots of FoA, Sher-e-Kashmir University of Agricultural Sciences & Technology of Kashmir Wadura. The genotypes showed varied response to angular leaf spot. Out of eighty-eight different genotypes screened twenty two genotypes were resistant, forty nine showed intermediate reaction and seventeen were susceptible. Adikshita and Sharma (2016) screened 18 common bean germplasm/lines for ALS resistance under natural epiphytotic and artificial inoculation conditions and found that none of the lines were resistant. In contrast, twenty two genotypes were found resistant when screened under natural epiphytotic conditions in present study which is obviously due to the fact that number of genotypes screened in present study was more.

Most of the genotypes showed moderate or susceptible reaction and only lesser proportion of genotypes were found resistant indicating that field screening for angular leaf spot resistance is reliable and the lines showing field resistance shall be used in breeding programmes for durable angular leaf spot resistance as reported by Rezene and Mekonin (2019). Several researchers from different countries have identified different sources of resistance to Angular leaf spot (Pastor- Corrales *et al*., 1998; Mahuku *et al*., 2003; Aggarwal *et al*., 2004; Padua *et al*., 2012; Sanglard *et al*., 2013; Ddamulira *et al*., 2014; Kijana *et al*., 2017; Pereira *et al*., 2019; Rodriguez *et al*., 2019).

A set of fifty-four genotypes was screened in greenhouse under artificial inoculation conditions with Andean race Pg02. Among fifty-four genotypes screened in greenhouse, eleven genotypes were resistant, thirteen genotypes showed intermediate reaction while rest of the genotypes were susceptible. In

greenhouse experiment, most of the genotypes were found susceptible. It is possibly due to the fact that most of the genotypes used in present study belong to Andean gene pool (Banoo *et al*., 2020) that have succumbed to the Andean race Pg02. The presence of admixtures was also reported in the same study. Therefore, the genotypes that were found resistant may possibly belong to Mesoamerican gene pool and/or the admixtures. However, further studies are needed to categorize the genotypes as Andean and Mesoamerican types on individual basis using Phaseolin gene marker. Several other studies have also reported the co-evolution between common bean and *P. griseola* wherein Andean isolates of *P. griseola* infect varieties belonging to Andean gene pool and Mesoamerican isolates may infect genotypes from both the gene pools (CIAT, 1995; Mahuku, 2003; Sartorato 2002; Ddamulira *et al*., 2014; Ddamulira *et al.,* 2015; Mukamuhirwa *et al*., 2017).

Chapter - 6

SUMMARY AND CONCLUSION

Pathogen characterization plays a key role in disease management. High genetic diversity in the pathogen makes it difficult to achieve durable resistance in the host; hence the need arises to identify new sources of resistance from time to time. The present investigation was, therefore carried out to characterize common bean angular leaf spot pathogen based on morphology and Internal Transcribed Spacer (ITS) region and to identify the sources of resistance in common bean. The results obtained during the present investigations are summarized as under:

Common bean is an important legume crop of the world and is most widely consumed grain both as green vegetable and as a pulse. Angular leaf spot disease is one of the major constraints in common bean cultivation. It reduces the quality and marketability of common bean seed across bean-growing regions of the world (Pastor-Corrales *et al.* 1998). The pathogen associated with bean angular leaf spot in Kashmir was isolated from disease leaves. The isolates of pathogen viz. Pg01 and Pg02 were slow growing with 2.0 mm and 2.5 mm colony diameter respectively on V-8 agar juice medium after incubation of 5 days at 25 ± 1^{0}C. Colony colour of isolate Pg01 was initially greyish which later became black and colony colour of isolate Pg02 was initially greyish white which later became dark olivaceous. The mycelia of both the isolates Pg01 and Pg02 were septate, irregularly branched, hyaline with a diameter of 3.47-4.01 µm (Av.3.7 µm) in Pg01 and 3.1-4.42 µm (Av. 3.87) in Pg02. Both the isolates were characterized by having synnematous and pigmented conidiophores. The size of conidiophores varied from 90-158 × 3.26-4.6 µm with an average of 138×3.60 µm in isolate Pg01 and 91.5-156 × 3.22-4.5 µm with an average of 139 × 3.20 µm in isolate Pg02. The conidia of both the isolates were cylindrical in shape and with 3 to 8 septa and olivaceous grey to olive brown in colour. Conidial dimensions varied from 38.10-49.04 × 3.1-6.29 µm in isolate Pg01 and 38.16-49.0 × 3.2-6.26 µm in Pg02. The isolates were identified as *Pseudocercospora griseola* (Sacc.)

Crous & U. Braun based on its morpho-cultural characteristics as described by Crous *et al*. (2006).

Two ITS sequences of *Pseudocercospora griseola* obtained in present study (Pg01 and Pg02) showed maximum similarity (98.24 per cent for Pg01 and 96.71 per cent for Pg02) with *Pseudocercospora griseola* f.sp. *griseola* Saccardo, Crous and Braun) sequences available in NCBI database, depicting usefulness of ITS region in authentic identification of fungal species. Both the sequences were submitted to NCBI and accession numbers (MZ452242 and MZ413342) were obtained. Phylogenetic analysis was carried out to find the relationship between the isolates obtained in present study with those retrieved from NCBI GenBank. Persual of phylogenetic tree revealed that both the isolates (Pg01 and Pg02) were grouped together with *P.griseola* f.sp. *griseola* sequences whereas the sequences of *P.griseola* f.sp *mesoamericana* were clustered separately. It indicates the ITS sequence data could clearly discriminate between the two forms (Andean and Mesoamerican) of *P.griseola*. Furthermore, *Pseudocercospora rhamnellae* sequences and *Pseudocercospora proteae* sequences were clustered separately as an out group, thereby indicating ITS sequence analysis was found to be a powerful tool for authentic identification of plant pathogenic fungi.

Eighty eight diverse common bean genotypes were screened in field under natural epiphytotic conditions. Out of eighty-eight different genotypes twenty-two genotypes were resistant, forty-nine genotypes showed intermediate reaction and seventeen genotypes were susceptible. The genotypes WB-1677, WB-22 showed highly resistant reaction with a score of one and genotype N-89 showed highly susceptible reaction with a score nine. Among fifty four genotypes screened in greenhouse under artificial inoculation conditions with isolate Pg02, eleven genotypes were resistant, thirteen genotypes were intermediate while as rest of the genotypes were susceptible. In greenhouse the genotype WB-371 showed highly resistant

reaction with a score of 1.3 and genotype WB-1129 showed highly susceptible reaction with a score of nine.

CONCLUSION

- The Pathogen associated with Common bean Angular Leaf Spot was identified as *Pseudocercospora griseola* on the basis of morphological characteristics.
- Sequence analysis revealed that both the isolates viz., Pg01 and Pg02 belong to Andean group i.e, *Pseudocercospora griseola* f. sp. *griseola*
- For germplasm evaluation under field conditions, out of 88 different genotypes twenty two genotypes were resistant, forty nine genotypes showed intermediate reaction and seventeen were susceptible.
- Among fifty four genotypes screened in greenhouse eleven genotypes were resistant, thirteen genotypes showed intermediate reaction while rest of the genotypes were susceptible.
- This study has identified new sources of resistance to angular leaf spot that could be utilized in future breeding programmes.

LITERATURE CITED

Abadio, A. K. R., Lima, S. S., Santana, M. F., Salomao, T. M. F., Sartorato, A., Mizubuti, E.S.G., Araujo1, E. F. and Queiroz, 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.

Adikshita. 2012. Epidemiology and management of angular leaf spot of French bean caused by *Phaeoisariopsis griseola* (Sacc.) Ferraris. M.Sc. thesis submitted to Dr. Yashwant Singh Parmar University of Horticulture and Forestry, Nauni, Solan. pp. 63

Adikshita. and Sharma, M. 2016. Screening of French bean germplasm against angular leaf Spot (*Phaeoisariopsis griseola*). *International Journal of Science and Environment* **5**(6): 4307-4311.

Aggarwal, V. D., Pastor-Corrales, M. A., Chirwa, R.M. and Buruchara, R.A. 2004. Andean beans (*Phaseolus vulgaris* L.) with resistance to the angular leaf spot pathogen (*Phaeoisariopsis griseola*) in southern and eastern Africa. *Euphytica* **136**(2):201-210.

Allorent, D. and Savary, S. 2005. Epidemiological characteristics of angular leaf spot of bean: A systems analysis. *European Journal of Plant Pathology* **113**(4):329-341.

Anonymous 1999. Meeting demand for beans for sub-Saharan Africa in sustainable ways: Annual report Project IP-2. CIAT, Cali, Colombia

Arx, J. A., von 1983. *Mycosphaerella* and its anamorphs. Proceedings van de Koninklijke Nederlandse Akademie van Wetenschappen, Series C **86**(1): 15-54

Banoo, A., Nabi, A., Rasool, R. S., Shah, M. D., Ahmad, M., Sofi, P. A. and Padder, B. A. 2020. North-western Himalayan common beans: population

structure and mapping of quantitative anthracnose resistance through genome wide association study. *Frontiers in Plant Science* **11:**1459.

Bhat, T. A., Bhat, N. A., Bhat, H. A., Ahanger, R. A., Wani, S. H. and Dar, S. A. 2017. Status of bacterial blight of common beans (Phaseolus vulgaris L.) in Kashmir. Journal of Pharmacognosy and Phytochemistry 6: 376-379.

Bose, S. K. and Sindhan, G. S. 1972. Leaf spot of French beans caused by *Isariopsis griseola* Sacc. and its control. *Progressive Horticulture* **4:**69-75.

Braun, U. 1992. Taxonomic notes on some species of the *Cercospora* complex. Nova Hedwigia **55**: 211–221.

Braun, U. 1995a. A monograph of *Cercosporella*, *Ramularia* and allied genera (Phytopathogenic Hyphomycetes). Vol. 1. IHWVerlag, Eching.

Braun, U. 1995b Miscellaneous notes on phytopathogenic hyphomycetes (II). *Mycotaxon* **55**: 223–241.

Broughton, W. J., G. Hernández, M. Blair, S. Beebe, P. Gepts, and J. Vanderleyden. 2003. Beans (*Phaseolus spp.*); model food legumes. *Plant Soil* **252**: 55–128.

Buruchara, R. A. 1983. Determination of pathogenic variation in *Isariopsis griseola* Sacc. And *Pseudomonas syringae pv. Phaseolicola* (Burk, 1926), Young, Dye and Wilkie 1978. Ph.D. dissertation submitted to University of Nairobi, Kenya [**cf**: RPP 88 : 1348].

Busogoro, J., Jijakli, M. and Lepoivre, P. 1999. Virulence variation and RAPD polymorphism in African isolates of *Phaeoisariospis griseola* (Sacc.) Ferr., the causal agent of angular leaf spot of common bean. *European Journal of Plant Pathology* **105:** 559–569.

Camara, C. R. S., Urrea, C. A. and Schlege, V. 2013. Pinto Beans (*Phaseolus vulgaris* L.) as a Functional Food: Implications on Human Health. *Agriculture* **3**: 90-111.

Canpolat, S. and 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.

Canpolat, S. and Maden, S. 2021. "Characterization of Turkish isolates of *Pseudocercospora griseola* the causal agent of angular leaf spot of common beans". *Journal of Plant Protection Research* **61**(1): 95-102.

Chilagane, L. A., Nchimbi-Msolla, S., Kusolwa, P. M., Porch, T. G., Diaz, L. M. S. and 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.

Correa-Victoria, F. J., Pastor-Corrales, M. A. and Saettler, A. W. 1989. Angular leaf spot pp. 59-75. **In**: Bean Production Problems in the Tropics. *International Centre of Tropical Agriculture.*

Crous, P. W., Liebenberg, M. M., Braun, U. and 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.

Damasceno-Silva, K. J., Souza, E. A., Sartorato, A. and Freire, C. N. S. 2008. Pathogenic variability of isolates of *Pseudocercospora griseola*, the cause of common bean angular leaf spot, and its implications for resistance breeding. *Journal of Phytopathology* **156**: 602-606.

Ddamulira, G. 2015. Characterization of *Pseudocercospora griseola*, identification of local sources of resistance and effectiveness of gene pyramiding in controlling angular leaf spot in common bean. PhD dissertation submitted to Department of Agricultural Production, Makerere University, Kampala, Uganda, 112 p

Ddamulira, G., Mukankusi, C., Ochwo-Ssemakula, M., Edema, R., Sseruwagi, P. and Gepts, P. 2014. Identification of new sources of resistance to angular leaf spot among Uganda common bean landraces. *Canadian Journal of Plant Breeding* **2**(2): 55-65.

Deighton, F. C. 1990. Observations on *Phaeoisariopsis*. *Mycological Research* **94**: 1096-1102.

Ellis, J. B. 1881. New species of North American fungi. *Bulletin Torrey Botanical Club* **8**: 64-66.

Ellis, J. B. and Everhart, B. M. 1893. New species of North American fungi from various localities. In: *Proceedings of Academy of National Sciences*, Philadelphia, pp 128-172.

Ellis, M. B. 1971. *Dematiaceous hyphomycetes*. Common Wealth Mycological Institute, Kew, UK, p. 608.

FAO, 2017. Food and Agriculture Organisation. http://faostat3.fao.org

Ferraris, T. 1909. Osservazioni micologiche su specie del gruppo Hyphales (Hyphomycetae). *Annales Mycologicum* **7**: 273-86.

Gepts, P., Osborn, T. C., Rashka, K. and Bliss, F. A. 1986. Phaseolin-protein variability in wild forms and landraces of the common bean (*Phaseolus vulgaris*): evidence for multiple centres of domestication. *Economic Botany* **40**:451-468.

Guzman, P., Gepts, P., Temple, S., Mkandawire, A. B. C. and Gilbertson, R. L.1999. Detection and differentiation of *Phaeoisariopsis griseola* isolates with the polymerase chain reaction and group-specific primers. *The American Phytopathological Society* **83**:37-42.

Guzmán, P., Gilbertson, R. L., Nodari, R., Johnson, W. C., Temple, S. R., Mandala, D., Mkandawire, A. B. C. and Gepts, P. 1995. Characterization of variability in the fungus *Phaeoisariopsis griseola* suggests co-evolution with the common bean (*Phaseolus vulgaris*). *Phytopathology* **85**: 600-607.

Hall, T. A. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* **41**: 95-98.

Harter, L. and Zaumeyer, W. J. 1944. A monographic study of bean diseases and methods of their control. *United States Department of Agriculture Technical Bulletin* **868**: 255.

Hazra, P. and Som, M.G. 2005. *Vegetable Science*. Kalyani Publishers, Ludhiana, India pp. 491.

Hocking, D. 1967. A new virulent form of *Phaeoisariopsis griseola* causing circular leaf spot of French bean. *Plant Disease Reporter* **51**: 276-278.

Jesus-Junior, W. C., Vale, F. X. R., Martinez, C. A., Coelho, R. R., Costa, L. C., Hau, B. and Zambolim, L. 2001. Effects of angular leaf spot and rust on leaf gas exchange and yield of common bean (*Phaseolus vulgaris*). *Photosynthetica* **39**: 603–606.

Karanja, T. M., Ombe, A. W. and Mibey, K. 1994. The effect of media and light regimes on cultural and morphological characteristics and sporution of *Phaeoisariopsis griseola* Deighton. *East African Agriculture Forestry Journal* **59**: 241-251.

Kijana, R., Abang, M., Edema, R., Mukankusi, C. and Buruchara, R. 2017. Prevalence of angular leaf spot disease and sources of resistance in common bean in Eastern Democratic Republic of Congo. *African Crop Science Journal* **25**: 109-122.

Landeras, E., Traoiella, E., Brana, M. and Gonzalez, A.J. 2015. Occurrence of angular leaf spot caused by *Pseudocercospora griseola* in *Phaseolus vulgaris* in Asturias, Spain. *Spanish Journal of Agricultural Research* **15**(3) e10SC03.

Lianos, C. 1957. Pathogenicity of *Isariopsis griseola* Sacc. on bean (*Phaseolus vulgaris* L.). *Acta Agronomica* **7**: 164-190.

Librelon, S. S., Souza, E. A., Pereira, R., Pozza, E. A., Abreu. and A. F. B. 2015. Diagrammatic scale to evaluate angular leaf spot severity in primary leaves of common bean. *Australasian Plant Pathology* **44**: 385-395.

Liebenberg, M. M. and Pretorius, Z. A. 1997. A review of angular leaf spot of common bean (*Phaseolus vulgaris* L.). *African Plant Protection* **3**: 81-106.

Mahuku, G. S., Jara, C., Cajiao, C. and Beebe, S. 2003. Sources of resistance to angular leaf spot (*Phaeoisariopsis griseola)* in common bean core collection, wild *Phaseolus vulgaris* and secondary gene pool. *Euphytica* **130:** 303-313.

Mahuku, G. S., Jara, C., Cuasquer, J. B. and Castellanos, G. 2002. Genetic variability within *Phaeoisariopsis griseola* from Central America and its implications for resistance breeding of common bean. *Plant Pathology* **51**:594–604.

Miles, E. 1917. Some diseases of economic plants in Puerto Rico. *Phytopathology* **7:** 345-348.

Monda, E. O., Sanders, F. E. and Hick, A. 2001. Infection and colonization of bean leaf by *Phaeoisariopsis griseola*. *Plant Pathology* **50**: 103-110.

Mongi, R. J. 2016. Breeding for resistance against angular leaf spot disease of common bean in the Southern Highlands of Tanzania. Ph.D dissertation submitted to College of Agriculture, Engineering and Science University of Kwazulu-Natal, Republic of South Africa. pp. 205.

Mukamuhirwa, F., Mukankusi, M. C., Tusiime, G., Butar, L., Musoni, A., Ngaboyisonga, C., Gahakwa, D., Gibson, P. and Kelly, K. 2017. Resistance Levels to Root Rot and Angular Leaf Spot Diseases in Selected High Iron Bean Genotypes. *Advances Crop Science Technology* **5**: 274.

Murray, M. G. and Thomson, W. F. 1980. Rapid isolation of high molecular weight plant DNA. *Nucleic Acid Research* **8:** 4321-4325.

Nay, M. M., Souza, T. L. P. O., Raatz, B., Mukankusi, C. M., Vidigal, M. C. G., Abrcu, A. F. B., Mclo, L. C. and Pastor-Corrales, M. A. 2019. A review of angular leaf spot resistance in common bean. *Crop Science* **59**: 1376-1391.

Nietsche, S., Borem, A., Carvalho, G. A., Paula, Junior, T. J., Fortes-Ferreira, C., GonCalves de Barros, E. and Moreira, M. A. 2001. Genetic diversity of *Phaeoisariopsis griseola* in the State of Minas Gerais, Brazil. *Euphytica* **117**:77-84.

Padua, P. F., Barcelos, Q. L., Souza, E. A., Abreu, A. F. B. 2016. Identification of common bean resistant sources to angular leaf spot disease in a Brazilian germplasm collection. Annual Report of the Bean Improvement Cooperative **59**: 115-116.

Pastor-Corrales, M. A., Jara, C. E. and Singh, S. P. 1998. Pathogenic variation in, sources of, and breeding for resistance to *Phaeoisariopsis griseola* causing angular leaf spot in common bean. *Euphytica* **103**: 161-171.

Pereira, R., Abreu, A. D. F. B., Nalin, R. S. and Souza, E. A. D. 2019. Phenotyping for angular leaf spot severity and its implication in breeding common bean for resistance. *Scientia Agricola* **76**:415-423.

Pereira, R., Souza, E. A., Barcelos, Q. L., Abreu, A.F.B. and Librelon, S.S. 2015. Aggressiveness of *Pseudocercospora griseola* strains in common bean genotypes and implications for genetic improvement. *Genetics and Molecular Research* **14**:5044-5053.

Rashid, U. 2018. Status and Management of angular leaf spot of beans (*Phaseolus vulgaris* L.) caused by *Phaeoisariopsis griseola* (Sacc.) Ferraris. M. Sc. thesis submitted to Faculty of Agriculture, Sher-e-Kashmir University of Agricultural Sciences and Technology of Kashmir, Wadura, Sopore pp. 1-72.

Rezene, Y. and Mekonin, S. 2019. Screening common bean (*Phaseolus vulgaris* L.) germplasm for resistance against angular leaf spot (*Pseudocercospora griseola*) disease under field condition. *Journal of Plant Studies* **8**: 30-34

Rezene, Y., Tesfaye, K., Clare, M. and Gepts, P. 2018. Pathotypes characterization and virulence diversity of *Pseudocercospora griseola*, the causal agent of angular leaf spot disease collected from major common bean (*Phaseolus vulgaris L.*) growing areas of Ethiopia. *Journal of Plant Pathology and Microbiology* **9**: 445.

Rodriguiz, D., Beaver, J., Jenson, C. E. D. and Porch, T. 2019. Identification of resistance sources of common bean (*Phaseolus vulgaris* L.) to angular leaf spot (*Pseudocercospora griseola*). *Revista Facultad Nacional de Agronomia* **72**: 8785-8791.

Romero-Arenas, O., Huato, D. M. A., Tapia R. J. A., Simón B. A., Lara H. M. and Huerta, C. E. 2013. The Nutritional value of Beans (*Phaseolus vulgaris* L.) and its importance for Feeding of Rural communities in Puebla Mexico. *International Research Journal of Biological Sciences* **2**(8): 59-65.

Saccardo, P. A. 1878. Fungi veneti novi vel critici. 8. *Michelia* **1**: 239-275.

Saccardo, P. A. 1886. *Sylloge Fungorum* **4**: 631.

Saettler, A. W. and Correa, F. J. 1988. Transmission of *Phaeoisariopsis griseola* by bean seeds. *Journal of Seed Technology* **12**: 133-142.

Saettler, A. W. 1991. Angular Leaf Spot. In: Compendium of Bean Diseases. Hall R. (Ed.). APS Press, St. Paul, M.N. pp. 15-16.

Saiki, R. K., Gelfand, D. H., Stofel, S., Scharf, S. J., Higushi, R., Hom, G. T., Mullis, K. B. and Erlich, H. A. 1988. Primer detected enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* **239**: 487-491.

Sanglard, D. A., Ribeiro, C. A. G., Balbi, B. P., Arruda, K. M. A., de Barros, E. G., & Moreira, M. A. 2013. Characterization of the angular leaf spot resistance gene present in common bean cultivar Ouro Negro. *Journal of Agricultural Science* **5**(2):19.

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.

Schwartz, H. F. and Harveson, R. M. 2015. Bacterial wilt of dry edible beans in the central high plains of US. *Plant Disease* **99**: 1665-1677.

Schwartz, H. F., Correa, V. F., Pineda, D. P. A., Otoya, M. M. and Katherman, M. J. 1981. Dry bean yield losses caused by Ascochyta, angular and white leaf spots in Columbia. *Plant Disease* **65**: 494-496.

Serrato-Diaz, L. M., Navarro-Monserrat, E. D., Rosas, J. C., Chilagane, L. A., Bayman, P. and Porch, T. G. 2020. Phylogeny of *Pseudocercospora griseola* from Puerto Rico, Central America and Tanzania confirms the existence of an Afro-Andean clade. *European Journal of Plant Pathology* **157**(11) 533-547.

Sharma, R. D. and Sohi, H. S. 1978. Evidence of a new strain of *Phaeoisariopsis griseola* Sacc. from India. *Current Science* **47**: 348-349.

Silva, K. J. D. E., De Souza, E. A., Sartorato, A. and De Souza Freire, C. N. 2008. Pathogenic variability of isolates of *Pseudocercospora griseola*, the cause of common bean angular leaf spot, and its implications for resistance breeding. *Journal of Phytopathology* **156**:602-606.

Sindhan, G. S. and Bose, S. K. 1972. Leaf spot of French beans caused by *Isariopsis griseola* Sacc. and its control. *Progressive Horticulture* **4**: 69-75.

Singh, A. K. and Saini, S. S. 1980. Inheritance of resistance to angular leaf spot *(Isariopsis griseola* Sacc.) in French bean (*Phaseolus vulgaris* L.). *Euphytic*a **29**: 175-176.

Singh, S. P. 2001. Broadening the genetic basis of common bean cultivars: A Review. *Crop Science* **41**: 1659-1675.

Stenglein, S. A. and Balatti, P. A. 2006. Genetic diversity of *Phaeoisariopsis griseola* in Argentina as revealed by pathogenic and molecular markers. *Physiological and Molecular Plant Pathology* **68**(4-6):158-167.

Stenglein, S., Ploper, L. D., Vizgarra, O. and Balatti, P. 2003. Angular leaf spot: A disease caused by the fungus *Phaeoisariopsis griseola* (Sacc.) Ferraris on (*Phaseolus vulgaris* L.). *Advances in Applied Microbiology* **52**: 209.

Sutton, B. C. 1980. *The coelomycetes.* Commonwealth Mycological Institute, Kew, UK, pp 696.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. and Higgins, D. G. 1997. The Clustal X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* **25**: 4876-4882.

Thompson, J. D., Higgins, D. G. and Gibson, T. J. 1994. CLUSTALW: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. *Nucleic Acids Research* **22**: 4673-4680.

Tuite, J. 1969. Plant pathological methods-fungi and bacteria. Burgess Publ. Co., Minneapolis, Minnesota, pp. 239.

Van-Schoonhoven, A. and Pastor-Corrales, M.A. 1987. Standard system for the evaluation of bean Germplasm. CIAT, Cali, Colombia.

Wagara, I. N., A. W., Mwang-ombe, J. W., Kimenju, R.A., Buruchara, R. Jamnadass and Majiwa, P.A.O. 2004. Genetic diversity of *Phaeoisariopsis griseola* in Kenya as revealed by AFLP and group-specific primers. *Journal of Phytopathology* **152**: 235–242.

White, T. J., Bruns, T. Lee S. and Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics pp. 315-322. **In**: PCR Protocols: A Guide to Methods and Applications (Eds. MA, Innis., D. H., Gelfand., J. J. Sninsky., T. J. White, editors.). New York Academic Press.

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CERTIFICATE

Certified that all the corrections/amendments as suggested by external examiner **Dr. Amrish Vaid, Professor & Head, Division of Plant Pathology, SKUAST-J**, Chatha during viva-voce examination held on 03-11-2021 have been incorporated in the manuscript entitled **"Characterisation of angular leaf spot pathogen and identification of source(s) of resistance in common bean"** submitted by **Ms. Saima Gani (Regd. No. MSA/2019/1297)**.

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