LR-4856
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RESEARCH ARTICLE Legume Research- An International Journal, Volume Issue : ()

Distribution and Identification of *Colletotrichum* Species Associated with Soybean Anthracnose/Pod Blight in Different Geographical Locations of Uttarakhand

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10.18805/LR-4856

ABSTRACT

Background: The anthracnose/pod blight disease caused by *Colletotrichum* species, is considered as a major limitation in soybean cultivation worldwide, causing significant yield losses. This study was conducted to determine the prevalence of soybean anthracnose/ pod blight in different geographical locations of Uttarakhand and to identify the *Colletotrichum* species associated with the disease.

Methods: Roving survey was carried out in various soybean growing areas of Uttarakhand, covering 82 villages in 11 districts during kharif 2017 to 2019. In order to record the prevalence of the disease, incidence and severity were recorded periodically in soybean fields. From different surveyed locations, samples of anthracnose and pod blight were collected, isolated and identified on the basis of genetic analysis of the ITS region of rDNA.

Result: The survey study revealed that disease was evident in all surveyed districts of Uttarakhand except district Haridwar. The pooled mean incidence and severity of pod blight infection in different districts ranged from 3.96 to 27.0 per cent and 2.04 to 15.69 per cent, respectively, maximum in the U. S. Nagar district and minimum in the Almora district. It was observed that disease incidence and severity decreased with rises in altitude with highly significant negative correlation of -0.89 and -0.89, respectively. Three distinct *Colletotrichum* species *i.e.*, *C. truncatum, C. cliviae* (=*C. cliviicola*) and *C. chlorophyti* were identified through molecular characterization of the ITS-5.8S rDNA region (BLASTn queries), which were associated with soybean anthracnose/pod blight disease in Uttarakhand.

Key words: BLAST, *Colletotrichum,* ITS-rDNA, Pod blight, Soybean.

INTRODUCTION

Soybean [*Glycine max* (L.) Merril] is a high value crop with uncommon chemical composition besides 40 % protein and 20% oil. In India, remarkable growth has been reported in the area under cultivation of soybean, which was just 0.03 m ha in 1970 and has now reached 11.33 million ha in 2018 (SOPA, 2018) and is ranked fifth after USA, Brazil, Argentina and China (SOPA, 2020; USDA, 2020). As soybean cultivation has increased, the incidence of numerous diseases has increased as well (Gupta and Paul, 2002). Among numerous diseases, anthracnose/pod blight caused by *Colletotrichum* species, is a major limitation on soybean cultivation worldwide, resulting in significant yield losses (Amrate *et al.,* 2021; Nene and Srivastava, 1971; Sharma *et al.*, 2011).

Soybean anthracnose is mainly associated with falcate-conidiated *C. truncatum* (Schw.) Andrus and Moore (syn. *C. dematium* var. *truncata*; sexual stage *Glomerella truncata* Armstrong and Banniza) (Armstrong-Cho and Banniza, 2006; Hyde *et al.*, 2009; Sharma *et al.*, 2011). In recent years, two species with curved conidia, *C. chlorophyti* Chandra and Tandon and *C. incanum* Yang, Haudenshield and Hartman, have been identified as the cause of this disease (Yang *et al.*, 2012; Yang *et al.*, 2014). Finally, the species with cylindrical conidia, namely; *C. cliviae* (=*C. cliviicola*) Yang, Liu, Hyde and Cai was recently recognized as another member of the soybean anthracnose complex (Barbieri *et al.*, 2017; Dias *et al.*, 2018). Different species of *Colletotrichum* have different ITS and 5.8S-rDNA gene sequences, which are frequently used to distinguish between them (Kendal and Rygiewiicz, 2005). In present study we have surveyed different geographical areas of Uttarakhand, to know the prevalence and distribution of soybean anthracnose/pod blight disease and identification of associated *Colletotrichum* spp. with ITS.

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How to cite this article: Bhatt, P., Singh, K.P. and Aravind, T. (2022). Distribution and Identification of *Colletotrichum* Species Associated with Soybean Anthracnose/Pod Blight in Different Geographical Locations of Uttarakhand. Legume Research. DOI: 10.18805/LR-4856.

Submitted: 17-12-2021 **Accepted:** 24-03-2022 **Online:** 12-05-2022

MATERIALS AND METHODS

Survey

Roving surveys were carried out in various soybean growing areas, covering 82 villages in 11 districts of Uttarakhand

(Udham Singh Nagar, Nainital, Almora, Champawat, Bageshwar, Pithoragarh, Haridwar, Rudraprayag, Tehri Garhwal, Pauri Garhwal and Dehradun) during *kharif* 2017 to 2019. In order to record the occurrence of the disease, incidence and severity were recorded periodically in soybean fields at different localities surveyed. Two fields were randomly selected at each location and the number of infected plants were counted in a 1 m^2 area comprising ten plants in each of the four corners and the centre of the field. Per cent disease incidence (DI) is defined as a measure of infected plants from total plants and calculated using the given formula:

Per cent disease incidence =

$$\frac{\text{Number of diseased plants}}{\text{Total number of plants observed}} \times 100$$

The data on the severity was recorded on the scale according to Mayee and Datar (1986) as: 0=No symptoms on the leaves; 1= 1% of the leaf area covered; 3= 1.1 to 10 %; 5= 10.1 to 25%; 7=25.1 to 50 % and 9 = > 50% of the area covered by lesions. These scales were further converted to per cent disease index (PDI) using the formula given by Wheeler (1969).

$$PDI = \frac{\text{Sum of individual disease rating}}{\text{Number of plants assessed} \times \text{Maximum disease rating}} \times 100$$

Collection, Isolation and purification of cultures

From different surveyed geographical locations, the infected samples of anthracnose and pod blight symptoms were collected and enclosed in a blotter sheet and placed separately in an envelope with location details. These samples were stored in a refrigerator (4°C) and deep freezer (-20°C) for further studies.

Further lab experiments were carried out in the Epidemiology and Forecasting Laboratory in the Department of Plant Pathology, GBPUAT, Pantnagar. For isolation, diseased samples were washed thoroughly in tap water and cut into small pieces (with half healthy part) of about half centimetre by using a sterilized blade. Surface sterilization of the cut pieces was done by dipping them in one per cent sodium hypochlorite (NaOCl) solution for 30-60 seconds followed by three consecutive washing with sterilized distilled water and then placed on pre-sterilized blotting paper to remove excess moisture. The surface sterilized diseased pieces were then aseptically transferred to PDA plates and incubated at 28±1°C in a B.O.D. Incubator. After 48-72 hours of incubation, the marginal mycelial growth was aseptically transferred on to PDA plates for purification. All cultural studies were conducted in an aseptic environment under laminar air flow.

DNA extraction and PCR amplification

Pure cultures of all collected isolates were sub-cultured in potato dextrose broth (PDB) medium. Three to four mycelial discs were taken from margins of actively growing cultures and placed in 100 ml volumes of PDB in 250 ml flasks incubated at 25°± 2°C for 8-10 days. The mycelium was then harvested by filtration through a pre-sterilized fine muslin cloth and then washed with sterile-distilled water. Total genomic DNA was extracted from all isolates using a slight modification of the CTAB method described by Lee and Taylor (1990). The molecular identification was done by using fungal-specific primers ITS4 (TCCTCCGCTT ATTGATATGC) and ITS5 (GAAGTAAAAGTCGTAACAAGG) (White *et al*., 1990**),** synthesized by the Gene-i Laboratories, Pvt. Ltd. (Bengaluru). Polymerase Chain Reaction (PCR) was performed using a PCR master mix from Gene-i Laboratories Pvt. Ltd., which contains Taq DNA polymerase, dNTPs, MgCl2 and reaction buffers at optimum concentrations. For PCR amplification, a final reaction volume of 25 µl was prepared, containing 1X PCR master mix (12.5 µl), 1 µM forward primer (1 µl), 1 µM forward primer (1 µl), 100 ng of template DNA (2 µl) and molecular biology grade water (8.5 µl). The template DNA was added at last in the mixture. PCR was carried out in a programmable thermal cycler (BioRad-iCycler) as follows: 94°C for 3 min; 35 cycles of denaturing at 94°C for 50 s, annealing at 54°C for 30 s and elongation at 72°C for 1 min; and a final extension step of 72°C for 7 min. The PCR amplified products (mixed with gel loading dye) were run along with 100 bp ladder on 2.0 per cent agarose gel in 0.5X TBE buffer at 80V for 2 hrs. For staining, ethridium bromide (EtBr) solution was added to the gel. The gel image was visualized on a gel documentation system.

DNA sequencing and sequence alignment

PCR products were sent for purification and sequencing to Gene-i Laboratories Pvt. Ltd. The obtained sequences were analysed for construction of consensus sequences by using the BioEdit sequence alignment editor (Hall, 1999) and then individually aligned using the BLASTn sequence alignment tool at NCBI to identify the sequence homology with database sequences of pre deposited *Colletotrichum* spp. Sequences were submitted to NCBI GenBank for getting accession numbers.

RESULTS AND DISCUSSION

Symptomatology

The symptoms of soybean anthracnose/pod blight were observed on nearly all above-ground plant parts, including leaves, petiole, stems and pods (Fig 1). On leaves, irregular brown necrotic lesions were observed. In later stages, the middle necrotic portion withered away, resulting in a shot hole-like appearance. Petiole symptoms included sunken necrotic spots. When the host reached maturity, the fungus attacked the pods and stems severely. Numerous fruiting structures (acervuli) formed rings on the pod surface, eventually cause blackening of the pod and preventing it from filling. The affected pods either produced no seed or if produced were shrivelled and smaller in size. The foremost obvious impact of the disease is the generation of withered, infected, destitute quality grains which are useless as a seed material.

Prevalence and severity of soybean anthracnose/pod blight in Uttarakhand

Systematic surveys conducted in eleven districts of Uttarakhand during *kharif* 2017 to 2019 revealed variable occurrence and distribution of the disease, except in the Haridwar district, where no soybean cultivation was recorded during three years of study. This variable distribution may be attributed due to the variation in agro-climatic conditions prevailing at different geographical locations. The foliage anthracnose outbreak was widespread in Uttarakhand's foothill districts *viz.*, U. S. Nagar, Nainital and Dehradun districts. Whereas, in the mid hills and high hills region of the state, symptoms of foliage anthracnose were unclear due to the high prevalence of frog eye spot disease. As a result, only observations of pod blight were included in the survey studies.

Fig 1: Typical symptoms of *C. truncatum* infection on different plant parts of soybean.

Pod blight disease was prevailed in all the surveyed districts except Haridwar with pooled mean incidence and severity ranged from 3.96 to 27.0 per cent and 2.04 to 15.69 per cent, respectively at farmers field (Table 1). Mean pod blight incidence and severity was found maximum in the U. S. Nagar (27.70 and 15.69%) and Nainital district (18.14 and 7.49%). Among all surveyed districts, least disease incidence and severity was recorded for Pithoragarh (4.80 and 2.50%) and Almora (3.96 and 2.04%). As the disease was majorly clustered at *tarai* regions of U. S. Nagar and Nainital district (Pantngar and nearby places), where during September and October moderate temperature (ranging from 27°C to 33°C) and high relative humidity (>90%) were recorded during 2017 to 2019. The weather data was obtained from the agrometeorology observatory, Pantnagar. These environmental conditions are found very congenial for the development of anthracnose/pod blight disease in soybean as described by Sinclair and Backman (1989). Singh *et al.* (2001) also observed maximum disease incidence of soybean anthracnose after the second fortnight of September when average temperature of about 28.4°C, average relative humidity of 76 percent and average rainfall of 92.5 mm prevailed. Similar results were obtained by Aggarwal *et al.* (2017), who reported that temperature ranged from 22°C to 29°C, relative humidity >80 per cent and optimum rainfall was favourable for anthracnose disease development.

The variation of disease intensity among different surveyed places might be due to differences in local situations like cropping pattern, varietal status, growth stages, altitude/latitude and also microclimatic conditions (Chavan and Dhutraj, 2017). The survey study covered

Table 1: District wise pod blight incidence and severity during 2017-2019.

District	Altitude (m)	Mean per cent disease incidence (%)	Mean per cent disease index (%)
Bageshwar	1153.43	16.08	4.65
Nanital	407.44	18.14	7.49
Udham Singh Nagar	259.43	27.70	15.69
Pithoragarh	1530.87	4.80	2.50
Almora	1489.23	3.96	2.04
Champawat	1734.69	7.28	2.93
Pauri Garhwal	1181.02	10.74	5.33
Tehri Garhwal	1497.96	6.74	2.72
Dehradun	451.20	16.11	9.30
Chamoli	1259.10	12.58	2.59
Rudraprayag	805.97	15.42	9.28

*Mean of per cent disease incidence and PDI at a different locations of a district.

altitudes ranging from 259 to 1734 metres above mean sea level and it was observed that disease incidence and severity decreased with rises in altitude (Fig 2). Variations in altitude affect temperature and rainfall, as increasing altitude is associated with a decline in temperature (Minda *et al.*, 2018; Siles *et al.*, 2016), which also affects the prevalence and distribution of the disease. Highly significant negative correlation of -0.89 and -0.89 was recorded between altitude and disease incidence and severity, respectively. The lowest disease incidence and severity were observed at high altitudes of 1489.23 to 1734.69 m in Almora, Pithoragarh and Champawat districts, while the maximum disease occurrence was observed at lower altitudes of 259.43 to 805.97 m. The high value of coefficient of determination (R^2) showed a 79 percent contribution of selected altitude and ultimately environmental conditions of different surveyed locations towards disease development in farmer's field. Our results are in agreement with the findings of Tsedaley *et al.* (2016) who also found that altitudinal gradients influenced sorghum anthracnose disease severity with a negative correlation. Similarly, Olatinwo *et al.* (1999) observed the effect of altitude on the prevalence of *Stenocarpella macrospora* (Earle) leaf blight of maize. They have found the highest disease incidence in the mid altitude zone with a moderate positive correlation.

Identification of *Colletotrichum* species

A total of 24 isolates of *Colletotrichum* spp. were collected from different geographical locations in 10 districts of Uttarakhand (Table 2). Genomic DNA of each isolate was extracted and amplified with an ITS primer. The PCR amplification of ITS-5.8S rDNA of all isolates using ITS4 and ITS5 primers resulted in an amplified product at 480-610 bp region (mostly at 560 bp). On the basis of sequencing and molecular characterization of ITS-5.8S rDNA region (BLASTn queries) it was found that the majority of *Colletotrichum* isolates (n=17) had ITS sequences matching to the *C. truncatum*. The 4 isolates from Pithoragarh (Ct-Bis, Ct-Gur, Ct- Egy and Ct-Sta) and two isolates of Champawat district (Ct- Loh1, Ct-Loh2) were found to belong to the *C. cliviae* (= *C. cliviicola*) and one isolate of Almora district (Ct-Gag) showing similarity with *C. chlorophyti.* NCBI accession numbers for all of the isolates are listed in Table 3. ITS analysis of 24 isolates of *Colletotrichum* spp. showed that *C. truncatum* was the most dominant species (71%),

Table 2: List of *Colletotrichum* spp. isolates collected from different geographical regions of Uttarakhand.

Isolate name	Place	District	Altitude (m)
Ct-Kot	Kotdwar	Pauri Garhwal	421.74
Ct-Pad	Padli (Karnaprayag)	Chamoli	789.79
Ct-Mar	Marghat	Rudraprayag	855.85
Ct-The	Koti	Tehri Garhwal	1444.00
Ct-Gar	Garur	Bageshwar	1124.29
Ct-Kha	Khamedi	Bageshwar	968.21
Ct-Gas	Haldwani	Nainital	430.11
Ct-Himt	Himmatpur	Nainital	361.78
Ct-Hal	Halduchaur	Nainital	301.95
Ct-Poj	Pojai	Nainital	331.02
Ct-Maj	Majhera	Nainital	901.17
Ct-Bhi	Bhimtal	Nainital	1370.00
Ct-Pant	Pantnagar	Udham Singh Nagar	244.09
Ct-Bin	Bindukhatta	Udham Singh Nagar	241.04
Ct-Kic	Kichha	Udham Singh Nagar	293.16
Ct-Egy	Egyardevi	Pithoragarh	1442.59
Ct-Gur	Gurna	Pithoragarh	1323.92
Ct-Bis	Bishar	Pithoragarh	1460.99
Ct-Sta	Dhamora	Pithoragarh	1400.57
Ct-Loh1	Sui	Champawat	1754.00
Ct-Loh2	Lohaghat	Champawat	1700.34
Ct-Alm	Haulbhag	Almora	1204.73
Ct-Gag	Ranikhet	Almora	1107.12
Ct-Bam	Bamsyun	Almora	1175.10

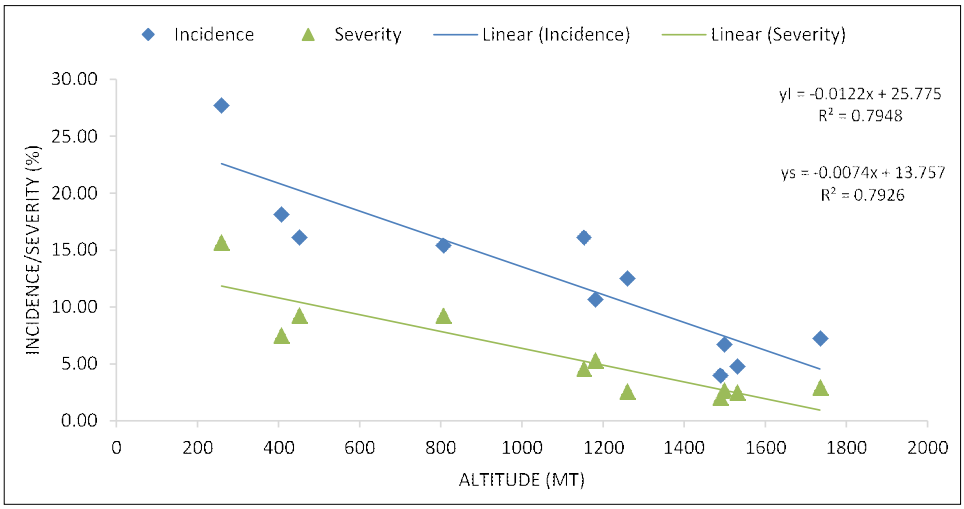

Fig 2: Effect of altitude on the incidence (a) and severity (b) of pod blight disease of soybean.

Table 3: NCBI GenBank accession number of ITS gene sequence of 24 isolates of *Colletotrichum* species.

Isolates designation	NCBI accession number
Ct-Gar	MZ262465
Ct-Kha	MZ262466
Ct-Gau	MZ262467
Ct-Himt	MZ262469
Ct-Hal	MZ262468
Ct-Poj	MZ262470
Ct-Maj	MZ262473
Ct-Bhi	MZ262478
Ct-Alm	MZ262471
Ct-Gag	MZ242078
Ct-Bam	MZ262477
Ct-Egy	MZ262452
Ct-Gur	MZ262453
Ct-Bis	MZ262454
Ct-Sta	MZ262455
Ct-Loh1	MZ262451
Ct-Loh2	MZ262450
Ct-Pant	MZ348830
Ct-Bin	MZ262472
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Ct-Pad	MZ262475
Ct-Mar	MZ262476
Ct-The	MZ262479

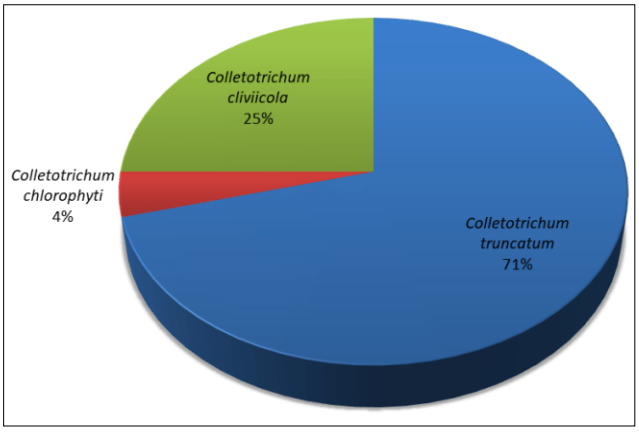

Fig 3: The proportion of *Colletotrichum* species associated with soybean anthracnose/pod blight in Uttarakhand.

followed by *C. cliviae* (= *C. cliviicola*) (25%) and *C. chlorophyti* (4%) that is associated with the anthracnose/pod blight disease in Uttarakhand (Fig 3). This disease is mainly associated with *C. truncatum* (Schw.) Andrus and Moore (Armstrong-Cho and Banniza, 2006; Hyde *et al.*, 2009; Sharma *et al.*, 2011), in recent years, *C. cliviae* and *C. chlorophyte* were also found as a novel incitant (Yang *et al.*, 2012; Barbieri *et al.*, 2017). In a study done by Rogério *et al.* (2016), molecular analysis of 51 *Colletotrichum* isolates revealed sequence similarity with *C. truncatum* reference species and until 2007, it was the sole cause of soybean anthracnose in Brazil. Among the three *Colletotrichum* species identified in the present study, *C. truncatum* reported in wider range of altitudes, while the *C. cliviae* and *C. chlorophyte* were reported in higher altitudes (> 1100 mt). These finding indicates that adaptability to various altitudes or environmental conditions were found to be important factors in the distribution of *Colletotrichum* species (Han *et al.*, 2016).

CONCLUSION

The present study demonstrated that disease intensity varied across the different survey locations in Uttarakhand, which could be explained by variations in altitude. It was observed that disease incidence and severity decreased with rises in altitude. The maximum disease pressure was observed in the low-altitude *tarai* zone, where congenial environmental conditions prevailed during the course of disease development. Altitude shifts had a significant impact on disease distribution as well as its causal agent. Three different *Colletotrichum* spp. were identified to be associated with soybean anthracnose/pod blight disease, of which *C. truncatum* was reported in a wider range of altitudes, while *C. cliviae* and *C. chlorophyte* were reported in higher altitudes only.

Conflict of interest: None.

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