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ORIGINAL RESEARCH ARTICLE

Bacillus cereus ApKb1 associated with the damping-off disease on *Andrographis paniculata* in India

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ABSTRACT

During the early rainy season (June-July) of 2018 and 2019, seedlings in A. paniculata nursery at CIMAP experimental field were found dying due to damping-off disease. The symptoms comprised basal stem lesions spreading upward, making the stem become wired at a later stage and falling off due to premature death. The damping-off disease incidence on A. paniculata nursery was estimated as 25% and 30% during 2018 and 2019, respectively. A few bacteria were isolated from the diseased plantlets, and pathogenicity assays were performed by spray inoculation of bacterial suspension (2 ×10^4 CFU/ml) on 7-10 day old plantlets of A. paniculata. Our results indicated a strong association of ApKb1 isolate to damping-off symptoms on A. paniculata, and the microscopic observations revealed that the pathogen was Gram-positive, rod-shaped, and 0.30-0.38 µm in size. Further identification of the pathogen, based on 16S rDNA sequencing and BLASTn homology search of partial 16S rDNA sequence (1374bp), showed 99% similarity with Bacillus cereus strain DD123. This is the first report of the damping-off disease of A. paniculata associated with Bacillus cereus ApKb1 in India, to the best of our knowledge.

INTRODUCTION

Andrographis paniculata (Burm. F.) Wall. Ex Nees, commonly known as Kalmegh, is a highly valued Ayurvedic medicinal shrub within the family Acanthaceae and is native to the Indian subcontinent. Besides, it is also distributed in different regions of Southeast Asia, China, America, and the West Indies (Okhuarobo *et al.*, 2014).

For centuries, different parts of the Kalmegh plant were extensively used to treat a range of diseases such as dysentery, cancer, ulcer, diabetes, bronchitis, skin diseases, flatulence, influenza, dyspepsia, malaria, and a variety of other chronic and infectious diseases (Akbar 2011; Jarukamjorn *et al.*, 2008; Mishra *et al.*, 2007; Gupta *et al.*, 1990; Burkill *et al.*, 1966). In traditional medicinal system *A. paniculata* has been accepted as a potent medicinal herb, and over the past three decades, many types of research have validated its efficacy in the modern therapeutic area (Kumar *et al.*, 2004; Nanduri *et al.*, 2004; Tan *et al.*, 2004; Misra *et al.*, 1992; Sharma *et al.*, 1991). Around 26 ayurvedic medicines and preparations are reported to incorporate herbal materials from *A. paniculata*; hence it has been categorized as an essential medicinal plant in Indian pharmacopeia (Verma *et al.*, 2019; Kumar *et al.*, 2014; Niranjan *et al.*, 2010). Enormous medicinal values and broad geographical distribution of this plant throughout the country have attracted Indian

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farmers to commercialize the Kalmegh crop. No major insect pest and disease infestation has been reported on Kalmegh. However, during field surveys conducted in the early rainy season (June-July) of two consecutive years- 2018 and 2019 – at CSIR-CIMAP experimental fields, incidences of seedling damping-off disease in nurseries of *A. paniculata* were observed. This emerging disease is a major concern due to the plant's high utility and commercial cultivation aspects. Therefore, the present study explores the microbial association of damping-off disease of *A. paniculata,* which can be helpful for crop protection against premature death.

MATERIALS AND METHODS

Survey and isolation of pathogens

The nurseries of *A. paniculata* at CSIR-CIMAP experimental fields (26.8922171°N, 80.9839458°E), Lucknow, India, were surveyed during the months of June-July in 2018 and 2019. The disease incidence (%) was estimated (no. infected plants/ no. of healthy plants × 100) in a block (1×1 ft), and four blocks per bed and at least two beds in a nursery were observed. Dead or infected seedlings of *A. paniculata* showing typical damping-off symptoms were collected and subjected for isolation of causative agents. The infected seedlings were cut into 3-5 mm pieces, surface-sterilized with ethanol (70%) for the 30s, followed by 1% sodium hypochlorite for 1 min, then rinsed thrice in sterile distilled water (SDW), air-dried under Laminar Air Flow, and placed onto different culture media such as potato dextrose agar (PDA) and nutrient agar (NA) plates. The plates were incubated for growth at 28 ±2°C for fungi and 35 ±2°C for bacteria. The microbial cultures were sub-cultured onto fresh media, preserved in slants as well as in glycerol stock, and stored at 4°C for further use. The infected/dead plantlets were further subjected for preparation of dried specimens and preserved at Microbial Germplasm Collection (MGC) at Plant Pathology department, CSIR-CIMAP, Lucknow, India under accession no. MSApDf180703.

Pathogenicity assay

Pathogenicity of morphologically distinct isolates ApKb1, Apb2, Apb19a, Apb19b, and Apb19c obtained from the infected seedlings of *A. paniculata* nursery was determined on 7-10 days old plantlets of *A. paniculata* cv CIM-Megha growing under glasshouse conditions. *A. paniculata* plantlets (100 seedlings/pot) were raised in sterilized earthen pots (15×33) having autoclaved garden soil and sand (2:1) mixture. The pots, having one-week-old plantlets of *A. paniculate,* were inoculated with bacterial suspension (2×10^4 CFU/ml) by spraying at the lower stem and soil surface, while control plants were sprayed with SDW. The plantlets were irrigated regularly and maintained at 28±2°C, 80% relative humidity with 12hrs light/dark photoperiod in the glasshouse. The inoculated seedlings were observed for damping-off symptoms, and readings were recorded up to 15 days after inoculation (DAI). The diseased and infected seedlings were subjected to re-isolation and confirmation with the originally inoculated pathogen.

Morphological and molecular identification

The bacterial isolate ApKb1, which proved Koch's postulates in the pathogenicity assay, was subjected to morphological and molecular characterization. The morphological characterization of the bacterial isolate ApKb1 was performed on the basis of colony morphology, size, shape, and Gram's staining, and microscopic observations were recorded with the help of a compound microscope (Carl Zeiss Microscopy, GmbH Germany).

Total genomic DNA of bacterial pathogen ApKb1 was extracted using the DNeasy Ultraclean Microbial kit (Qiagen, Germany). The partial 16S rDNA region was amplified using the universal primer set 27F and 1492R as described by Fredriksson NJ *et al.*, 2013 and the purified amplicons were subjected for direct sequencing using 3130 Genetic Analyzer (Applied Biosystems, USA), with Big Dye™ Terminator v3.1 Cycle Sequencing kit, following the manufacturer's instructions. Molecular identification of the isolate ApKb1 was made through homology search using BLASTn tool against the GenBank database at NCBI, and the partial 16S rDNA sequence was deposited to GenBank accession no. (MN93404). Multiple sequence alignment and phylogenetic tree construction were performed with MEGA7 software using the neighbor-joining method (Kumar *et al.*, 2016).

RESULTS AND DISCUSSION

Survey and pathogen isolation

The seedlings in *A. paniculata* nursery at CSIR-CIMAP experimental field, Lucknow, India, were found to be affected by the damping-off disease, and the disease incidence under natural conditions was estimated at 25% and 30% during the months of June-July in 2018 and 2019, respectively. The average temperatures during two successive years (2018-2019) in the early rainy season (June-July) were 40/24°C (day/night) with a relative humidity between 70% and 100%. The soil in nursery beds was wet; however, waterlogging conditions were not observed during the post-emergence stages of the *A. paniculata* seedling nursery. The bacterial isolates such as ApKb1, Apb2, Apb19a, Apb19b, and Apb19c were obtained from the infected samples, while no fungal growth was observed on PDA plates.

Pathogenicity assay

Pathogenicity tests on 7-10 days old plantlets of *A. paniculata* revealed that damping-off symptoms were observed at 4 DAI on the plantlets inoculated with ApKb1 isolate. The disease initiated with the appearance of water-soaked lesions on the basal stem region, spreading in the upward direction, and mainly the area above the seedling leaves became compressed; however, in some plants, the lower stem below seedling leaves was also affected and became wired. At the later stage, plantlets fell off due to premature death (Fig. 1a-b). Around 80% incidence was recorded within 7 DAI, and 100% incidence was observed up to 15 DAI, while control plants and the plantlets inoculated with other bacterial isolates such as Apb2, Apb19a, Apb19b, and Apb19c remained healthy and symptomless. All the symptoms were similar to those infected under natural conditions, and the same bacterium was re-isolated from the dead plantlets.

Morphological and molecular identification

The morphological features of isolate ApKb1 showed milky-white, granular colonies of 2-5 mm in size with a wavy edge and smooth consistency on nutrient agar plates, and large, pink-colored, smooth colonies were observed on MacConkey agar (Fig. 1c). The microscopic observations of isolate ApKb1 revealed that the pathogen was Gram-positive, rod-shaped bacillus, and 0.30-0.38 µm in size (Fig.1d).

A partial 16S rDNA sequence of about 1374bp was obtained, and the BLASTn analysis at the NCBI GenBank database revealed that the 16S rDNA sequence has 99% similarity with *Bacillus cereus* DD123(KR822280). Multiple sequence alignment of the 16S rDNA region of closely related *Bacillus* spp. was carried out. The phylogenetic tree indicated that the isolate ApKb1 was clustered within the same clade comprising the reference strain of *Bacillus cereus* (Fig. 2). Based on the disease symptoms on naturally infected nursery plantlets, cultural features, morphological characteristics, molecular data, and pathogenicity, the bacterial pathogen ApKb1was identified as *Bacillus cereus*, and a partial 16S rDNA sequence of the isolate ApKb1 was submitted to the GenBank under the accession MN934104.

Damping-off is a very destructive disease encountered in raising nursery seedlings of several crops caused by diverse soil-borne and seed-borne microbes. The extensive reports on damping-off incidences and their severity represent that this is one of the most challenging problems in nurseries and fields, associated with several abiotic and biotic factors. The abiotic factors play an essential role in damping-off disease incidence and severity (Foster *et al.*, 2017; Rojas *et al.*, 2016). During the early rainy season, a decrease in temperature interspersed with rains resulting in the rise of soil moisture and humidity level favor the seed germination and increases the soil-borne microbial populations (You *et al.*, 2017; Barbetti *et al.*, 2007). The effect of environmental conditions on many soil-borne fungi has been associated with damping-off. In contrast, the association of bacterial pathogens with the damping-off disease on crops is comparatively less reported.

In the present study, a disease causative agent, *Bacillus cereus* strain ApKb1, was potentially associated with the post-emergence damping-off disease of *A. paniculata*. Although several reports indicate microbial communities as causal agents of damping-off disease due to their synergistic interactions (Lamichhane *et al.* 2015; Liu *et al.*, 2016), few reports indicate the complex disease also being

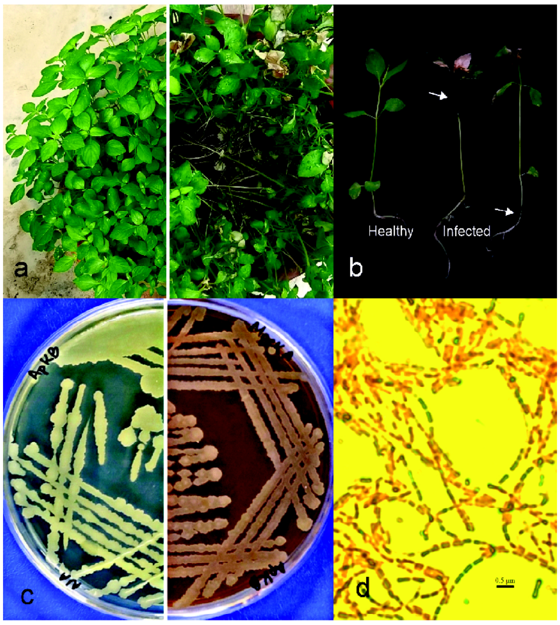

Figure 1: Damping-off disease symptoms: a. indicating healthy (left) and infected (right) nursery of *A. paniculata*, b. Post-emerging seedlings were showing damping-off symptoms caused by *B. cereus* ApKb1, c. Pure culture of *B. cereus* ApKb1 on Nutrient agar and MacConkey agar media, and d. they were indicating rod-shaped *B. cereus* ApKb1, showing Gram-positive reaction.

caused by mono-culture inoculations (Navi *et al.*, 2019; Matoba *et al.*, 2008). The present study's findings also co-relate with the mono-culture inoculations in damping-off disease. About 25% and 30% damping-off disease incidences were observed successively during 2018 and 2019 on emerging seedlings of *A. paniculata* nursery under natural field conditions. The occurrence of damping-off disease on *A. paniculata* seedlings was re-established artificially with single microbial inoculations, and up to 100% mortality was observed on mono-culture inoculations under glasshouse conditions. Mono-culture inoculations of *Bacillus cereus* ApKb1 showed significant involvement in damping-off disease, which causes the death of post-emergence seedlings under artificial glasshouse conditions and natural field conditions.

CONCLUSION

Bacillus cereus is widely distributed in soil, vegetables, and leaves and is also reported as an endophyte in several plants (McDowell *et al.*, 2019). The pathogen spreads through contaminated raw and processed foods and is associated with food poisoning, contributing to a range of human diseases (Bottone, 2010). The current study highlighted the association of *Bacillus cereus* ApKb1 with the damping-off disease in both natural field conditions and under controlled glasshouse conditions, thereby indicating the significance of environmental

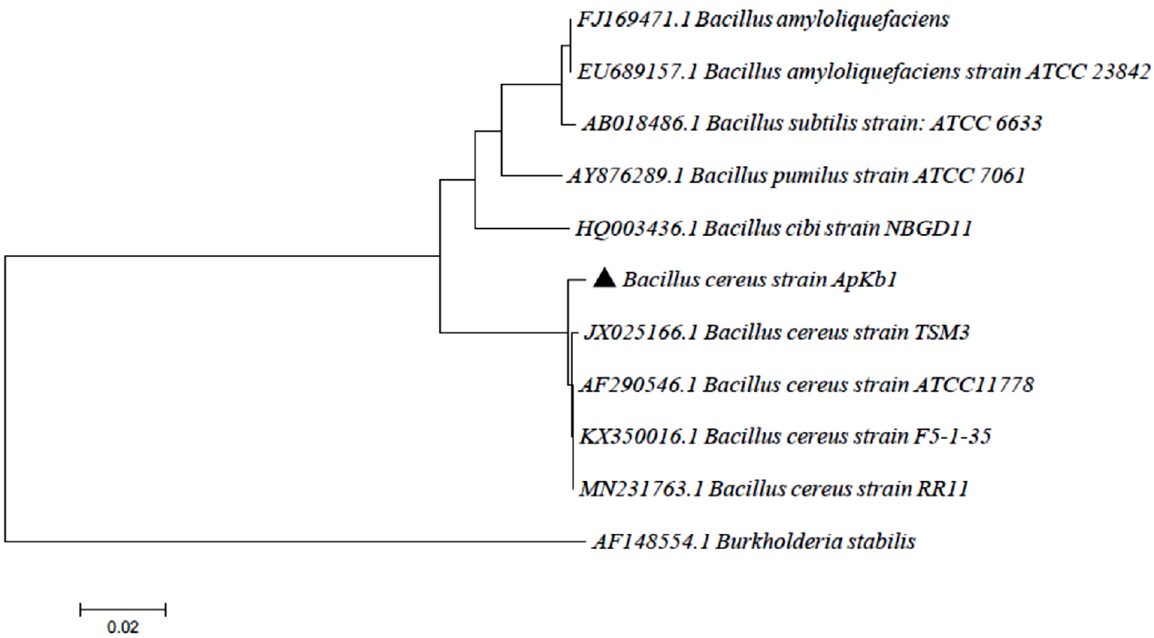

Figure 2: The Neighbour-joining (NJ) tree based on partial 16S rDNA sequences, showing relatedness of *Bacillus cereus* ApKb1 with other *Bacillus* species. *Burkholderia stabilis* was used as an outgroup; the scale bar represents two substitutions per 100 bases. The evolutionary analysis was conducted in MEGA 7.

distribution and occurrence of these bacteria. Several integrated pest management (IPM) approaches, combining preventive and curative strategies (Lamichhane *et al.*, 2017), have already been defined to control the damping-off disease. The multifaceted damping-off condition requires more research to generate further knowledge towards durable and sustainable management. To our knowledge, the current study is the first report on the association of emerging bacterial pathogen *Bacillus cereus* ApKb1 with the damping-off disease on *A. paniculata* and highlights the need for future studies on the interaction between host and environment towards the management of the damping-off disease.

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