Genetic Diversity Studies Based on Morphological Variability, Pathogenicity and Molecular Phylogeny of the *Sclerotinia sclerotiorum* Population From Indian Mustard (*Brassica juncea*)

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White mold or stem rot disease are ubiquitously distributed throughout the world and the causal organism of this disease *Sclerotinia sclerotiorum* (Lib.) de Bary, is known to infect over 400 plant species. Sclerotinia stem rot is one of the most devastating fungal diseases and poses a serious threat to the worldwide cultivation of oilseed *Brassica* including India. *S. sclerotiorum* pathogen usually infects the stem but in severe cases leaves and pods also affected at different developmental stages that deteriorate not only the oil quality but also causing the seed and oil yield losses up to 90% depending on the severity of the disease infestation. This study investigated the morphological and molecular characterization of pathogenic *S. sclerotiorum* (Lib) de Bary geographical isolates from oilseed *Brassica* including *Brassica juncea* (Indian mustard). The aim of this study was to compare isolates of *S. sclerotiorum* originated from different agro-climatic conditions and to analyse similarity or differences between them as well as to examine the virulence of this pathogen specifically in *Brassica* for the first time. The collection of *S. sclerotiorum* isolates from symptomatic *Brassica* plants was done and analyzed for morphological features, and molecular characterization. The virulence evaluation test of 65 isolates on four *Brassica* cultivars has shown 5 of them were highly virulent, 46 were virulent and 14 were moderately virulent. Phylogenetic analysis encompassing all the morphological features, SSR polymorphism, and ITS sequencing has shown the existence of high genetic diversity among the isolates that categorized all the isolates in three evolutionary lineages in the derived dendrogram. Further, genetic variability analysis based on sequences variation in ITS region of all the isolates has shown the existence of either insertions or deletions of the nucleotides in the ITS region has led to the interspecies variability and observed the variation were in a clade-specific manner. Together this analysis observed the existence of higher heterogeneity and genetic variability in *S. sclerotiorum* isolates collection and indicates the presence of clonal and sexual progenies of the pathogen in the mustard growing regions of India surveyed in this study. With a higher level of genetic variability and diversity among the *S. sclerotiorum* population needs robust screening approaches to identify the donor parent and utilize them in resistance breeding program for effectively counter the menace of stem rot disease in *Brassica*.

Introduction

Globally India continues to be at a 3rd position after Canada and China in acreage (19.3%) and after China and Canada in production (11.1%) of rapeseed-mustard. In India, among nine edible oilseed crops, the share of rapeseed-mustard is about one-fourth of total area and one-third of total oil production in the country. During 2015–2016, production (6.82 mt) and productivity (1184 kg/ha) was achieved (Anonymous, 2016). Rapeseed-mustard is the major source of income especially for the marginal and small farmers in rainfed areas which are about 25% of the total cultivated area. In spite of its increase in demand for the year the production of oilseed Brassica remains to stagnate over the year and most of the demands are being met through import from outside the India. The main reason behind productivity stagnation in Indian Brassica is its susceptibility and damages caused to the crop by various insect pests and disease infestation in addition to the other yield-limiting factors. Out of thirty diseases known to infest the Brassica crops in India, stem rot has been found one of the most devastating diseases that heavily damages the crops during the flowering stage of development. The stem rot disease which is caused by fungal pathogens, *Sclerotinia sclerotiorum* (Lib) de Bary, ubiquitously found throughout the world is a polyphagous, soil-borne plant pathogen that infects more than 400 plant species of diverse phylogenetic origin (Boland and Hall, 1994; Saharan and Mehta, 2008; Sharma et al., 2015). In India, during the eighties and nineties, the stem rot (SR) disease in rapeseed-mustard was of a minor importance, because of its seldom appearance over the ground level of the isolated plants after mycelial infection. A widely adopted monocropping practices and cultivation of rapeseed-mustard under irrigated condition has significantly increased the sclerotial population in the soil that has made SR very serious disease of oilseed *Brassica* crops in states including Rajasthan, Haryana, Punjab, Uttar Pradesh, Bihar, Assam, West Bengal and Madhya Pradesh (Sharma et al., 2015). This fungus has been long considered as prototypical necrotrophs as it begins highly pathogenic phase by releasing oxalic acids and cellulolytic enzymes immediately upon host cuticle penetration followed by mycelial proliferation inside the host cell followed by a saprophytic phase that supports the sclerotia formation (Hegedus and Rimmer, 2005). However, the recent studies decipher the fact of evidence for the occurrence of a brief biotrophic phase just within the apoplastic space next after the establishment of the host-pathogen connection and hence based on these it is more appropriately classified as a hemibiotroph (Kabbage et al., 2015). The information related to the genetic diversity of the pathogen and their effective virulence over the target crop is the foremost requirement for taking the breeding program for development of pathogen resistance in the release of the region-specific cultivars. Various diversity analysis tools based on the molecular methods like microsatellite haplotype (Aldrich-Wolfe et al., 2015), SSR (simple sequence repeat or microsatellite-based marker; Meinhardt et al., 2002), AFLP (amplified fragment length polymorphism; Cubeta et al., 1997), and SRAP (sequence-related amplified polymorphism technique; Li et al., 2009) have been used in analyzing the genetic diversity of the pathogen *S. sclerotiorum* from different host species. Very limited variability was observed in ITS (internal transcribed spacer) sequences in *S. sclerotiorum* isolates from various host species (Njambere et al., 2008) and thus a universal barcode markers were developed from the nearly conserved nature of the nuclear ribosomal internal transcribed spacer (ITS) region for imparting the individual identity to the fungus up to genus level (Schoch et al., 2012). Furthermore, MCGs is another diversity analysis method based on the mycelial compatibility grouping (MCG) has been used in establishing the kinship among *S. sclerotiorum* isolates from chickpea (Kull et al., 2004; Li et al., 2008). In addition to it, the

diversity based on the morphological appearance of sclerotia, mycelial growth, and ascospores formation have also been reported in analyzing the genetic diversity of *S. sclerotiorum* isolates in previous studies (Li et al., 2008; Sharma et al., 2013). However, polymorphism and genetic diversity of the *S. sclerotiorum* isolates at the morphological and DNA sequence level has not been comprehensively studied so far especially for the isolates from Brassica species of India.

Being a polyphagous nature, *S. sclerotiorum* pathogen is usually infecting not only the majority of the economically important dicotyledonous species but also serve as the major pathogen for several monocotyledonous plant species (Boland and Hall, 1994). The yield loss estimated with the *S. sclerotiorum* infestation has exceeded hundred million dollars annually because of the lack of resistance cultivar of the crop species and also because of lack of the effective management practices (Tok et al., 2016). In general, the management of *S. sclerotiorum* borne disease is not much easy because of its widespread existence, irregular incidence, and the long-term survival by producing huge numbers of sclerotia in the soil. Although several control measures like chemical and cultural methods have been devised and adopted for countering the Sclerotinia stem rot menace (Rousseau et al., 2007) none of them were found fully effective in preventing either the process of disease infection or pathogenesis progression after infection. The extent of genetic diversity of the pathogen and their widespread distribution among host species across the growing regions play an important role in determining and devising the control strategies to efficiently control the diseases in the more effective way. Hence, the availability of the genetic variability information related to the target pathogen is the foremost requirement for designing the management means to counter the disease incidence more effectively. In plant-pathogen interactions, development of new pathogenic races, and the breakdown of host resistance are the limiting factors in resistance deployment against plant diseases. The pathogen's life history characteristics and evolutionary potential are major factors leading to the pathogen overcoming host resistance. Therefore, major efforts should be focused not only in understanding the genetic structure of the fungal populations but also to determine how populations will evolve in response to different control strategies (McDonald and Linde, 2002).

In recent past, India observed the frequent incidence of the *S. sclerotiorum* infestation on cereals and horticultural species that draws the wide attention of the researcher over this fungal pathogen. In pursuance of basic understanding about pathogenicity, diversity and distribution pattern of the *S. sclerotiorum* were extensively studied on isolates collected from various host species like chickpea (Mandal and Dubey, 2012), vegetable crops (Choudhary and Prasad, 2012), cumin (Prasad et al., 2017), carnation (Kumar et al., 2015), oilseed *Brassica* (Sharma et al., 2013), and their identity were established on the basis of morphological features and cultural conditions. However, major variation in the growth characteristics has been reported in the growing collection of *S. sclerotiorum* isolates even as they belong to the same *Sclerotinia* species. Indeed, as projected with diversity analysis *S. sclerotiorum* isolates has been reported of possessing the variation in morpho-physiological, biochemical properties, molecular features and pathogenicity in terms of virulence because of the presence of clonal and sexual progenies together even in the same crop and in same region (Atallah et al., 2004; Sexton and Howlett, 2004; Irani et al., 2011). For establishing the console features of the pathogen especially in *Brassica* growing regions of India, the present study was aimed at determining the genetic diversity within *S. sclerotiorum* population from various *Brassica* species based on

morphological characteristics, genotyping with simple sequence repeats (SSR) markers and molecular phylogeny by ITS sequence analysis.

Materials and Methods

Sclerotinia sclerotiorum Isolates

Brassica growing areas in 10 states of India (Rajasthan, Haryana, Punjab, Delhi, U.P., Bihar, Uttarakhand, Himachal Pradesh, Jammu & Kashmir, and Jharkhand) were surveyed and stem rot disease infected plants were collected from 65 different locations (Table 1). The sclerotia obtained from the stem rot infested Indian mustard plant samples were first washed with sterile water than surface sterilized with 70% ethanol for 2 min and again washed two times with sterile water. The drained sclerotia over pre-sterilized filter papers were placed on nutrient media (Potato Dextrose Agar; PDA) plates supplemented with 50 μg/ml tetracycline antibiotics to prevent the growth of bacterial contamination. The samples were wrapped in a brown envelope and kept for incubation at 20° ± 2°C in dark for 4–5 days. After the development of the white fluffy mass of mycelial growth of *S. sclerotiorum*, the mycelial plaques were used to sub-culture the isolates on PDA slants and pure culture of them was stored at 4°C for future use. Morphological identification of the isolates was based on cultural characteristics of the *S. sclerotiorum* and morphology of the mycelial mat and sclerotia formation traits.