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Morphological and molecular identification of stem rot pathogen in berseem (*Trifolium alexandrinum* L.)

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Abstract
Berseem is an annual leguminous multicut fodder crop of India and grown during *rabi* season. Stem rot disease was observed in seed production fields. The etiological agent causing symptoms of stem rot was identified by morphological and molecular criteria. The fungal pathogen was isolated on potato dextrose agar (PDA) medium and development of whitish mycelia with numerous black colored sclerotia was observed throughout the colony. The sclerotia were initially brown and turned black upon maturation. Effect of different temperatures and pH on growth of stem rot pathogen was determined at 48, 72 and 96 h. The pathogen was able to grow at a temperature range 5 °C to 30 °C and pH range 5 to 8 and showed maximum growth at 20±3 °C and 6.5±0.5, temperature and pH, respectively. The number of sclerotia, fresh weight, size (length x width) and thickness was maximum at a temperature range 20 °C to 25 °C and pH range 6 to 6.5. PCR amplification of ITS region of rDNA with primer pair produced a fragment size of 550 bp. The rDNA sequence analysis of pathogen showed only 1 to 2 SNP difference with reference isolates of *S. trifoliorum* and *S. sclerotiorum*, which resulted 99% homology with reference sequences of *S. trifoliorum* and deposited in NCBI, GenBank. The phylogenetic analysis confirmed molecular relationship of berseem stem rot pathogen (MF035963) with different strains/ isolates of *S. trifoliorum* and closely related to *S. sclerotiorum* (KJ744364).

Keywords: Berseem, Fodder crop, Sclerotia, *Sclerotinia trifoliorum*, Stem rot

Introduction

Berseem (*Trifolium alexandrinum* L.) is an annual legume fodder crop native to Egypt and famously known as Egyptian clover (Zayed, 2013). It is popular among the livestock farmers of the world due to its multicut nature, highly nutritious quality (20% crude protein and 62% total digestible nutrient) and most potent milk multiplier (Iqbal and Iqbal, 2014; Manjunatha *et al*., 2017). Apart from providing quality green fodder, berseem being a legume crop is having high nitrogen fixation ability ensuing the improvement of soil fertility (Faruqui *et al*., 2002). In India it is major fodder crop of *rabi* season in central, northern and north-west regions (Satyapriya *et al*., 2013). Green fodder yield potential of berseem crop ranged from 62 to 105 t ha^{-1} and dry fodder yield from 9 to 18.8 t ha^{-1} with seed yield potential of 0.3 to 0.78 t ha^{-1} (Vijay *et al.,* 2016). However, the crop is affected by pests and diseases at various stages of its growth and development (Rathi *et al*., 2007b). Root rot complex (Fungi: *Macrophomina* sp., *Fusarium* sp. and nematode; *Tylenchorhynchus vulgaris*) and stem rot (*Sclerotinia trifoliorum*) are considered as serious biotic constraints in berseem growing regions of India (Faruqui *et al*., 2002). Although the incidence of disease is common all over India but it is a severe problem in north and N-W regions of India (Manjunatha *et al*., 2017). Among these, stem rot has become potential threat to berseem fodder and seed production since few years due to prevalence of favorable environmental conditions especially mean temperature (< 25 °C) during winter months of the year (Rathi *et al*., 2010). *Sclerotinia* sp. is a serious necrotrophic plant pathogenic fungus that causes disease on wide variety of crop plants including cool season fodder crops such as *Medicago* and *Trifolium* sp. (Gargouri *et al*., 2017). The pathogen is known to infect plant stem near or at the soil surface during vegetative stage. Symptoms of disease occurrence are visible during January and February when temperature is low (Rathi *et al*., 2010). Disease often occurs in patches in field and upon infection water-soaked lesions on stem are developed, which rapidly progress around the stem of infected plant. Under favorable environmental conditions the disease causes total failure of crop (Vijay *et al*., 2016). The fungus perpetuates in soil by producing a large number of sclerotia in infected tissues and on plant debris (Vleugels *et al*., 2013). *S. trifoliorum* as an etiological agent for stem rot disease in berseem was reported by few researchers from India

(Kumar *et al*., 2003; Rathi *et al*., 2007a; Pande *et al*., 2008). However, Mehboob *et al.* (2016) identified *S. sclerotiorum* as a causal agent for berseem stem rot from Pakistan through molecular and morphological characterization. Berseem stem rot pathogen was identified as *S. trifoliorum* based on morphological characters in India. The morphological characteristics *viz*., growth rate, sclerotial characters, ascospore dimorphism have been used for the identification and differentiation of *Sclerotinia* species (Ekins *et al*., 2005). But morphological characters driven interaction between genetic and environmental factors which might lead difficulties to differentiate within the fungal species (Longo *et al*., 2014). Host specificity is used primarily to describe the restriction of host range of *S. trifoliorum* to *Trifolium* species (Held and Haenseler, 1953). However, the overlapping host ranges, host specificity does not provide reliable marker for identification and differentiation of the species (Gargouri *et al*., 2017), therefore, researchers have used other techniques. Molecular markers such as, random amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP) (Ekins *et al*., 2005; Manjunatha *et al*., 2014) and sequence and phylogenetic analysis of Internal Transcribed Spacer (ITS) region (nuclear ribosomal DNA) were used for identification and differentiation of *Sclerotinia* species (Mahadevakumar *et al*., 2016; Gargouri *et al*., 2017). As per our knowledge, only few reports are available from India, on berseem stem rot pathogen and they were pertaining mainly to management aspects, while scanty information is available on etiological agent and its characterization in India as well as berseem growing countries. Hence, this study was carried out with the objective to identify berseem stem rot pathogen through morphological and molecular methods.

Materials and Methods

Isolation and pathogenicity of pathogen

Sampling: In March 2016, symptoms of stem rot were observed in berseem seed production fields at Central Research Farm, ICAR-Indian Grassland and Fodder Research Institute (IGFRI), Jhansi, India. Infected plants were collected in sterilized plastic polythene bags for pathogen isolation.

Isolation and pathogenicity test: Symptomatic stem were separated from plants and thoroughly washed with distilled water and cut into small pieces, approximately 1 cm in size. The spieces were surface-sterilized with 70% (v/v) ethanol for 1 min, washed 3 times with sterile distilled water and dried with sterilized filter paper. The sterilized pieces were placed in Petri plates containing potato dextrose agar (PDA) medium and incubated at 20±1 °C for 5 days. The colonies that developed from the infected stem were sub-cultured onto PDA for purification. The pure culture of the pathogen was maintained for further studies. To determine the pathogenicity of the fungus, one mycelial disc (5 days old, 5 mm) was placed into a small cut of berseem stem and put in to a petri plate with two moist filter papers. This was then incubated at 20±1 °C, under laboratory conditions.

Morphological characterization

Morphological characteristics: Mycelial plugs (5 mm diameter) from a 3-day-old culture was placed in the center of Petri plate (9 cm diameter) containing 15 ml of PDA amended with streptomycin (100 mg L^{-1}), pH 5.6, and incubated at 20±1 °C in the darkness. Colony diameter was recorded after 24, 48, 72 and 96 h. and mean growth rate was estimated (mm 24 h^{-1}). After 30 days, the sclerotia of each Petri plate were separated by using camel hair brush and the number of sclerotia of each Petri plate was counted manually. Ten sclerotia per plate were arbitrarily chosen to record fresh weight. Thickness and size (length and width) of scletrotia were measured by digital vernier caliper (Aerospace-digital).

Cultural characteristics

Effect of temperature on growth: The effect of temperatures, *viz*., 5, 10, 15, 20, 25, and 30 °C on the growth and sclerotia production was studied. *Sclerotinia trifoliorum* was cultured on potato dextrose medium amended with streptomycin (100 mg L^{-1}). After incubation at above said different temperatures, the radial growth was recorded after 48, 72 and 96 h. The sclerotial characters were recorded by the method as described earlier.

Effect of pH on growth: Seven pH levels *viz*., 5.0, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0 were taken as treatments. One hundred ml of PDA was taken on 250 ml conical flask. Different pH were adjusted by adding either 0.1 N HCl or 0.1 N NaOH and measured by pH meter (pH System 36, Systronics). Advanced hyphae of 72 h old culture grown on PDA at pH 6.0 was cut by flame sterilized 5 mm cork borer and used as inoculum. Inoculated petriplates were kept in growth chamber at a temperature of 20±1 °C. The radial growth and sclerotial observations were determined by the methods as described earlier.

Molecular characterization

DNA extraction and PCR amplification: The fungus was cultured in potato dextrose broth (PDB) to obtain mycelia mat for DNA extraction. Agar discs were cut out from an actively growing fungal colony with a 5 mm diameter cork borer and placed into 250 ml conical flask containing 100 ml of PDB. These cultures were incubated at 20±1 °C in an incubator for 10 days. After 10 days mycelial mat was harvested by filtration through Whatman No.1 filter paper and washed repeatedly with distilled water and stored at -20 °C for genomic DNA extraction. Approximately 250 mg of fungal mycelia was taken and ground into fine powder using liquid nitrogen. Total DNA was extracted from mycelial powder by CTAB method (Murray and Thompson, 1980) with slight modification. The primer pair ITS1 (5'CGGATCTCTTGGTTCTGGGA3')/ ITS4 (5'GACGCTCGAACATGCC3') described by White *et al*. (1990) were used to amplify ITS region (18S-28S) of rDNA. The PCR conditions included an initial denaturation at 95 °C for 3 min, 40 cycles of denaturation at 94 °C for 1 min, primer annealing at 55 °C for 45 s, followed by primer extension for 1 min at 72 °C and final extension at 72 °C for 10 min. PCR was performed using Thermocycler (BIOER, XP cycler). Amplified PCR products (550 bp) were separated on 1% agarose gel and examined with the help gel documentation unit (Syngene).

Sequence and phylogenetic analysis: Amplicons of the ITS1/ITS4 PCR (550 bp) were submitted to the AgriGenome Labs (Calicut, India) for sequencing. The resulting nucleotide sequences were aligned with ITS sequences obtained from NCBI data base by CAP3 sequence assembly program (Huang and Madan, 1999). Assembled sequence was deposited in GenBank, National Center for Biotechnology Information (NCBI) data base (MF035963). Assembled nucleotide sequences of *S. trifoliorum* berseem isolate was subjected to BLASTn analysis to find out homology with other *Sclerotinia* sp. sequences from DNA database available at NCBI. Sequences showing higher homology were retrieved for phylogenetic analysis. Using MEGA 7.0 software, phylogenetic relationship was determined by Neighbor-Joining (NJ) method at 1000 bootstrap value (Kumar *et al*., 2016).

Results and Discussion

Symptoms and pathogenicity test: Diseased plants with water-soaked lesions, resulting in wilting, bleaching and eventually leading to stem rot were observed in all berseem seed production plots. Fluffy, white mycelium characteristic of *Sclerotinia* species developed from rotten tissue. The fungus isolated from rotten stem of berseem and its association as an etiological agent was proved by pathogenicity test. The artificial inoculation of the fungus produced white mycelia with morphological characteristics as those of the original isolate. *Scleorotinia* sp. causes disease on a wide range of economically important crops including legume fodder crops throughout the world (Gargouri *et al*., 2017). This disease is a serious limiting factor for green fodder and seed production in berseem (Manjunatha *et al*., 2017). Pathogenicity test reproduced symptoms as originally observed on field conditions and results of our study were clearly supported by earlier reports of *S. trifoliorum* and *S. sclerotiorum* association in different host species (Vleugels *et al*., 2013; Mehboob *et al*., 2016).

Morphological characterization: The pathogen produced fluffy white mycelial growth on PDA medium 5 days after incubation at 20±1 °C (Fig. 1a). Mycelia were hyaline and septate. Sclerotia formed on delicate and smooth *S. trifoliorum* mycelium over entire surface of the plate in 8-10 days and often two or more sclerotia were fused in a large stroma. Later, it converted into black, cylindrical or irregular sclerotia (Fig. 1b). Sclerotia of berseem stem rot pathogen were firmly attached to the medium and were difficult to remove. Morphological features of pathogen were recorded and summarized in Table 1. Similarly, Kim and Cho (2003) and Vleugels *et al*. (2013) observed a variation in the number and size of sclerotia in *Sclerotinia* species and within species. Effect of different temperatures and pH on radial growth of fungus was evaluated (Fig. 2a & 2b) at 48, 72 and 96 h after incubation. The pathogen was able to grow at a temperature range 5 °C to 30 °C and pH range 5 to 8, and maximum growth of fungi was observed at 20±3 °C and 6.5±0.5, temperature and pH respectively. Similarly, effect of temperatures and pH on sclerotial characters was evaluated (Fig. 3a & 3b). The pathogen mycelia able to form sclerotia at a temperature range 5 °C to 30 °C and pH range 5 to 8. The number of sclerotia, fresh weight, size (length x width) and thickness was maximum at a temperature range 20-25 °C and pH range 6-6.5. The sclerotial formation and their weight, size (length x width) was found minimum at 5 °C temperature and at pH 5 and 7.5 to 8. There was no significant difference in thickness of sclerotia at different temperature and pH range. The fungus associated with stem rot of berseem is very serious in northern parts of the India during winter months where mean temperature was less than 20 °C and soil pH from 5.5 to 6.5 (Rathi *et al*., 2010; Manjunatha *et al*., 2017). Therefore, effect of different temperatures

Table 1. Morphological and culture characteristics of stem rot pathogen isolated from berseem

Characteristics	Berseem stem rot pathogen
Colony	
Color	White to gray
Mean growth rate	20 mm/ day
Sclerotia bodies	
Shape	Globular to irregular
Pattern	Scattered
Color	Initially dark brown, at maturity it turns black color
Mean fresh weight(mg)	430
Number of sclerotia	36 /plate
Mean size	1.7 to 3.6
Length (mm)	2.4 to 6.5
Width (mm)	1.0 to 3.6
Thickness (mm)	1.1 to 2.14
Cultural	
Temperature	20 ± 2 ^{0} C
pH	6 to 6.5

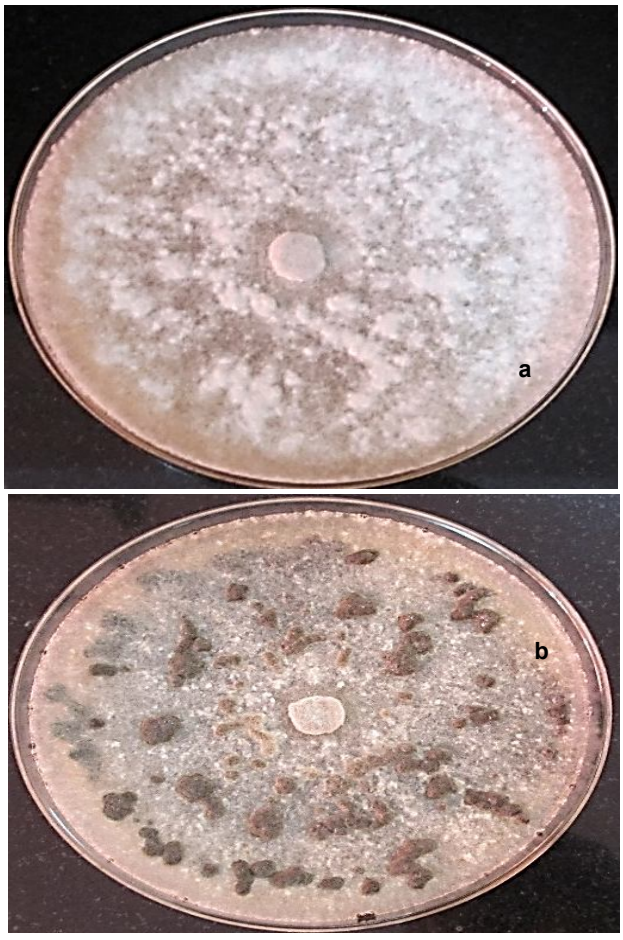

Fig 1. Growth of *S. trifoliorum* on PDA medium. a) Colony growth 5 days after incubation; b) Sclerotia formed on culture plate 30 days after incubation

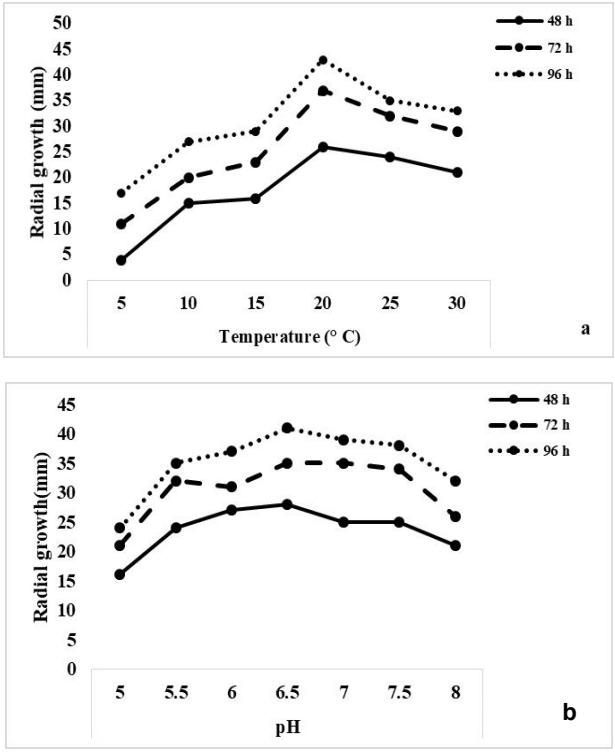

Fig 2. Effect of temperature (a) and pH (b) on radial growth of *S. trifoliorum*

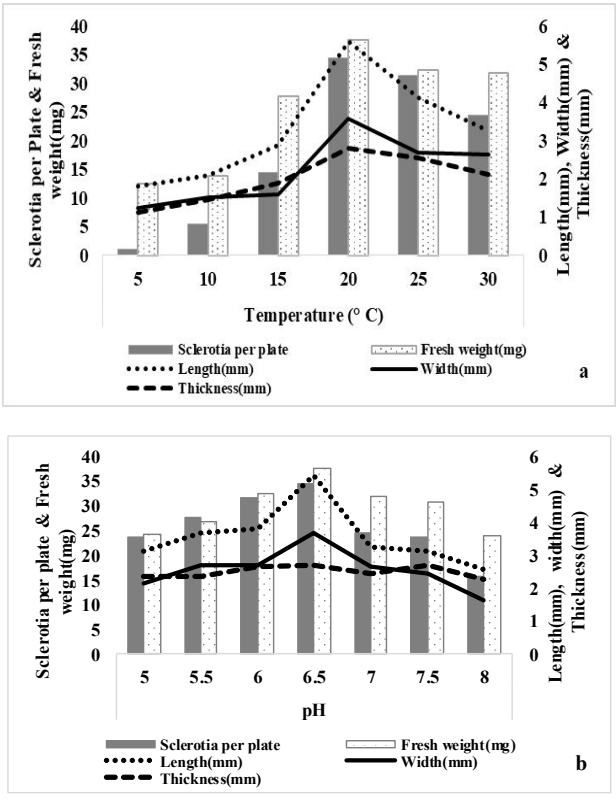

Fig 3. Effect of temperature (a) and pH (b) on sclerotia of *S. trifoliorum*

and pH on growth was evaluated *invitro*. In the current study, maximum mycelial growth by berseem stem rot pathogen was observed at 18-25 °C and 6.5±0.5, temperature and pH respectively. At temperature < 15 °C or > 25 °C and pH < 5.5 or > 7.0, least radial and sclerotial development was observed. Mahadevakumar *et al*. (2016) and Mehboob *et al*. (2016) reported temperature of 20-25 °C as optimum for growth and sclerotial production by *Sclerotinia* sp. Mycelial plugs of *S. sclerotiorum* inoculated on PDA had the highest growth rate at 25 °C. No growth was observed at 35, 40 and 45 °C (Mansour *et al*., 2008).

Molecular characterization: The PCR amplification of berseem stem rot pathogen ITS region (rDNA) using primer pair, ITS1 and ITS 4 yielded a single fragment of an approximately 550 bp. Amplified product was sequenced and sequence data was deposited in NCBI data base (MF035963). Nucleotide Blast analysis (BLASTn) of stem rot (Jhansi isolate) showed only 1 to 2 single nucleotide polymorphism (SNP) and shared 99% homology with most of *S. trifoliorum* and *S. sclerotiorum* strains / isolates available in GenBank. Moreover, results of sequence analysis showed that berseem stem rot pathogen is a member of *Sclerotinia* sp. Our results were in accordance with findings of Baturo-Ciesniewska *et al*. (2017), who reported Kura clover stem blight pathogen, *S. trifoliorum* had 99% homology with most of *S. trifoliorum* and strains in GenBank. Similarly, Gargouri *et al*. (2017) showed ITS sequences of *S. trifoliorum* which showed homology with reference sequences of *S. trifoliorum* and *S. sclerotiorum.* Phylogenetic tree (Fig. 4) was constructed based on Neighbour-Joining [NJ] method using MEGA7 software (Kumar *et al*., 2016). *S. trifoliorum* and *S. sclerotiorum* formed two clusters with very less diversity. Most of *S. sclerotiorum* strains / isolates were grouped under cluster I and the present berseem stem rot isolate (MF035963) was closely associated with strain of *S. sclerotiorum* (KJ744364). It indicated that ITS region in the *Sclerotinia* genus is not diverse enough to distinguish between *Sclerotinia* sp. Hence, the observation of ascospores from apothecia grown from sclerotia as well as molecular identification based on β-tubulin gene (Vleugels *et al*., 2012), or species-specific primers (Baturo-Ciesniewska *et al*., 2017) could provide reliable information on exact etiology of berseem stem rot. Thus, considering all morphological and ITS based molecular data together, it was identified that the pathogen isolated from naturally infected berseem is *Sclerotinia* sp. and closely related to *S. sclerotiorum* (KJ744364).

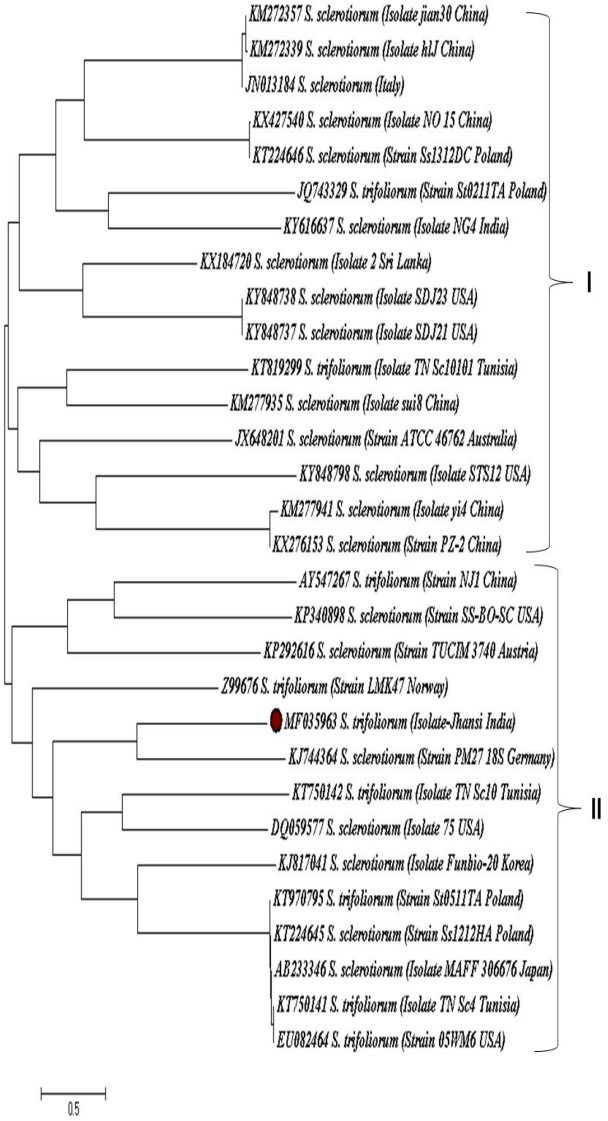

Fig 4. Neighbor-Joining (NJ) tree showed phylogenetic relationship between 32 sequences of *S. trifoliorum* based on the ITS rDNA sequences

Conclusion

Both morphological and molecular characterization confirmed *Sclerotinia* sp. is responsible for causing stem rot in berseem crop and it is closely related to *S. trifoliorum* and *S. sclerotiorum.* Further, our studies failed to distinguish present isolate from *S. trifoliorum* and *S. sclerotiorum* based ITS markers. Hence, ascospores characteristics and sequencing of β-tubulin gene may provide more insight in identifying exact species of *Sclerotinia*. It is concluded that our findings has given a base to further study the epidemiological aspects and biology of stem rot pathogen in berseem.

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