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Developmental biology and infection cycle of *Sclerotinia sclerotiorum* causing stem rot of carnation in India

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Carnation (*Dianthus caryophyllus* L.) is a cut flower with greater stipulation in the world cut flower market. In India, carnations are cultivated under polyhouses in Nilgiris and Kodaikanal districts in the state of Tamil Nadu. Carnations cultivation is impeded by various diseases, among them stem rot caused by *Sclerotinia sclerotiorum* (Lib.) de Bary was found to be predominant in all varieties. Survey among commercially cultivated varieties of carnation during 2013, revealed the occurrence of stem rot incited by *Sclerotinia sclerotiorum*, for the first time in India. The pathogen was identified as *Sclerotinia sclerotiorum* on the basis of phenotypic and genotypic characteristics. Carpogenic germination was induced artificially, and life cycle of the fungi was studied. Microscopic studies of the apothecium revealed the presence of spermatia, croziers, paraphyses as well as monomorphic ascospores specific to *S. sclerotiorum*.

Key words: Apothecium, carnation, croziers, India, *Sclerotinia,* spermatia.

INTRODUCTION

Floriculture is a persuasive field in trade with high potential returns per unit area. Because of this large number of farmers are attracted towards cut flower cultivation. In India, conditions prevailing in hilly regions are highly favorable for the cultivation of various cut flowers. Area under cut flower production is increasing constantly (Indian Horticulture Database, 2015). Carnations are one of the most preferred cut flower varieties next to rose owing to their shelf life and greater degree of available colours. Karnataka is the leading state in India accounting for carnation production. Total production of carnation all over India is 800 MT (Indian Horticulture Database, 2015). Even though carnation cultivation is increasing, it's development is hampered by various diseases. Among them, stem rot incited by *S. sclerotiorum* was found to be highly destructive. *Sclerotinia* has been previously reported as a polyphagous fungi infecting more than 148 plant species

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(Saharan and Mehta, 2008). *Sclerotinia* is a facultative parasitic fungus belonging to *Ascomycota* (Kirk et al., 2008). It is a robust pathogen having greater rate of survival even under adverse environmental conditions and survives up to eight years in soil as sclerotial bodies (Adams and Ayers, 1979). Yield losses have been reported to be hundred per cent in susceptible crops (Purdy, 1979). Three well documented species of *Sclerotinia* (*S. sclerotiorum*, *S. minor* and *S. trifoliorum*) are considered to be much important and likely to have wide host range and reported with huge economic loss (Saharan and Mehta, 2008). In India, stem rot of carnation was identified for the first time in 2013. (Vinod Kumar et al., 2015). The results of our study on survey, morphological, molecular characterization and apothecium induction in *S. sclerotiorum* are presented in this communication.

MATERIALS AND METHODS

Survey

Commercially cultivated carnation varieties like Charmant pink, Pudding yellow, Castor purple, Baltico white, White liberty, Golem purple, Yellow liberty, Bizet, Gaudina red and Farida were surveyed for the occurrence of stem rot in kothagiri, kodumudi, kunnur regions of Nilgiris district in Tamil Nadu, India. The disease incidence was calculated by using the formula proposed by Wheeler (1969).

$$\text{Percent Disease Incidence} = \frac{\text{No.of infected plants}}{\text{Total No.of plants}} \times 100$$

Isolation

The pathogen was isolated from both infected stem tissue and sclerotial bodies. Infected stem tissues and sclerotial bodies were surface sterlized with 0.1% mercuric chloride ($HgCl_2$) for 60 s and then the chemical traces were removed by rinsing thrice in sterile distilled water. Surface sterlized tissues and sclerotia were plated on sterile Petri plates containing potato dextrose agar (PDA) medium, amended with 100 µg/ml of streptomycin sulphate and incubated at 20 ± 2°C for 7 days. After emergence of fungal growth, the pathogen was pure cultured by single hyphal tip technique (Tutte, 1969).

Pathogenicity

Pathogenicity experiments were conducted on 30 days old potted cuttings of carnation variety, Charmant pink as per method described by Kim and Cho (2003). The cuttings were grown in pots with sterilized pot mixture. Mycelial discs and sclerotial bodies of the pathogen were inoculated to cuttings separately. Mycelial discs (9 mm) were placed in stem portion, 2 cm above ground and covered with moist cotton. Sclerotial bodies (3-5) covered in muslin cloth were buried in soil in proximity with the collar region of the healthy cuttings. Both the tests were replicated thrice with three cuttings per replication. After inoculation the pots were covered with polybags and incubated, ambient inside the polyhouse maintained at Elkhil Agrotech Pvt Ltd at Udhagamandalam. Healthy control was also maintained subsequently. After expression of symptoms the pathogen was re- isolated to confirm pathogenicity.

Identification

The pathogen was subjected to both morphological and molecular characterization. The study fungus was compared with well documented species of *Sclerotinia viz*., *S. sclerotiorum*, *S. minor* and *S. trifoliorum.* Morphological discrimination was based on ascospore morphology and size of sclerotia. Molecular identification was carried out by sequencing the 18S-28S rRNA gene.

Morphological characterization

Species differentiation was possibly made between them by studying the colony character, sclerotia and ascospore morphology (Ekins et al., 2005). Apothecium was induced as per the procedure proposed by Cobb and Dillard (2004).

Induction of apothecium

Before induction of apothecium the sclerotial bodies were subjected for a conditioning process. Medium to large sclerotial bodies (7.4 to 15.5 mm) were selected for better per cent rate of carpogenic germination. Sclerotial bodies were tied in cheese cloth bags and immersed in a container filled with tap water and incubated at 6^0 C. Fresh air was continuously circulated by using an aquarium pump. In this conditioning process, water was replenished once in a week till stipe initiation.

After conditioning process, sclerotia with stipe initials were transferred to Petri plates containing clean, sterilized, dry sand. Sand was moistened and plates were overlaid with lids in order to prevent desiccation. Moisture was maintained with sterile distilled water. Plates were incubated at 20°C in alternate dark and light periods with Bright Boost cool white fluorescent light-18W/840 (Phillips, China make), till apothecia were produced. The conditioning treatment was replicated thrice and a control was also maintained. Apothecium was subjected for ultramicrotome, and observed under microscope to study the ascus and ascospore morphology.

Microtome sectioning

In order to study the ultra-structures, apotheium was subjected to ultra microtome studies as demonstrated by Johanson (1940). The specimen was soaked for a minimum of 12 h in Formalin: Alcohol: Acetic acid: Water in the ratio of 10:50:5:35. Later the specimen was washed in a series of 60, 70, 80, 90 and 100 per cent Teritary Butly Alcohol. Then the specimens were embedded in molten wax at 52-54°C. Then after drying 12 micron thick sections were made with Spencers rotary microtome. After sectioning the specimens were subjected to de waxing with xylene: ethanol (1:1) for 30 min. After de waxing, the specimens were stained with safranin. Excess stains were washed with series of 50, 70 and 90 per cent ethanol. Later the specimens were dried and then mounted on glass slides with DPX mount.

Molecular characterization

The fungus was cultured on potato dextrose broth at 20 ± 2^0C for 4-5 days. Then the mycelium was collected, dried and powdered by freezing in liquid nitrogen. The genomic DNA was extracted by Cetyl Trimethyl Ammonium Bromide (CTAB) method as described by Chakraborty et al. (2010).

PCR amplification of 18S-28S rRNA gene

The genomic DNA was used as a template and subjected for the

Table 1. Prevalence of stem rot in different varieties of carnation in Nilgiris district of Tamil Nadu.

Variety	Colour	Percentage of stem rot incidence		
		Kothagiri	Kodumudi	Kunnur
Yellow liberty	Yellow	12.60	3.60	4.3
Gaudina red	Red	8.60	2.90	3.70
Farida	Pink	11.5	3.80	3.50
Bizet	Light pink	14.70	4.40	4.70
Castor purple	Purple	24.60	6.60	6.80
Golem purple	Purple	18.30	5.50	5.60
White liberty	White	13.50	4.50	5.00
Charmant pink	Light Pink	38.50	9.60	10.4
Pudding yellow	Yellow	22.20	7.60	7.20
Baltico white	White	28.40	8.60	8.30
Mean incidence		18.29	5.81	5.95

PCR amplification of 18S-28S rRNA gene using the primer pairs, ITS 1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS 4 (5'-TCCTCCGCTTATTGATATGC-3') (White et al., 1990). The PCR reaction was performed with ready to use Taq DNA Polymerase 2x master mix supplied from Ampliqon, comprising Tris-HCl pH 8.5, $(NH_4)_2So_4$, 3mM $MgCl_2$, 0.2% Tween 20, 0.4mM dNTPs, 0.2units/µl Ampliqon Taq DNA polymerase and inert red dye. The programming cycle was accomplished as follows: initial denaturation at 94^{0}C for 2 min, 30 cycles of denaturation at 94^{0}C for 30 s, annealing at 57^{0}C for 30 s, extension at 72^{0}C for 1 min and final extension at 72^{0}C for 7 min (Saitoh et al., 2006).

Life cycle assessment

Different stages of the pathogen associated with its life cycle were studied by inoculating mycelium and sclerotia of the pathogen on seedlings. Subsequently, the developmental biology of apothecium and their components were studied.

RESULTS AND DISCUSSION

Survey

This is the first record of *sclerotinia* rot of carnations in India. Survey results revealed that the variety Charmant pink was highly susceptible to stem rot. In survey the stem rot incidence was relatively greater in Kothagiri compared to that of Kunnur and Kodumudi. Among the varieties surveyed, pink coloured variety Charmant pink was highly susceptible with 38.50 per cent disease incidence followed by Baltico white (28.40%) and Castor purple (24.60%). Least incidence was observed in the variety Gaudina red (8.60%). However the same was severely susceptible to fairy ring spot and blossom blight. Mean stem rot incidence in Kothagiri was 18.29 per cent whereas in kunnur and kodumudi stem rot incidence was comparatively less *viz.,* 5.81 and 5.94, respectively (Table 1). Consistent relative humidity and relatively lower temperature, prevailing in Kothagiri region have resulted in greater loss compared to other regions.

Symptomatology

Symptoms were found to be associated from seedling to maturity stage. However, the plants are highly susceptible at seedling stage. Initial symptoms include paleness of the plant accompanied with drooping leaves (Figure 1a). However, the most typical symptom is the presence of cottony white mycelial growth on root zone (Figure 1b), as well as collar region of the plant. Subsequently, the plant dries to straw yellow and finally dies. Besides, longitudinal splitting of the infected stems revealed plenty of dark, black, sclerotial bodies (12-20) of varying shapes and sizes (Figure 1c). Saharan and Mehta (2008) described that symptom includes, cottony white mycelium on the root zone and collar region accompanied with dark, black, irregular sclerotia inside the stem, followed by, complete drying of the plant to straw yellow. Similar descriptions were made by Purdy (1979).

Isolation

On PDA, pure cultured study fungus produced fluffy white mycelial growth. The hyphae was hyaline, septate and metamorphosed into irregular, black sclerotial bodies of various size following mycelia aggregation as per the previous reports (Colotelo, 1974; Purdy, 1979). In this study, the pathogen produced dense cottony white mycelium on PDA and fungal mass covered the entire Petriplate within 5 days. After 8 days large, dark, black, irregular sclerotial bodies appeared in a circular fashion along the corners of the Petri plate (Figure 2a). The hyphae were hyaline and septate. Sclerotia were produced, superseding the accumulation of nutrients in

Figure 1. (a) Pale drooping infected plant, (**b**) Root zone colonized by mycelium of *S. sclerotiorum*, **(c)** Sclerotial bodies inside the infected stem.

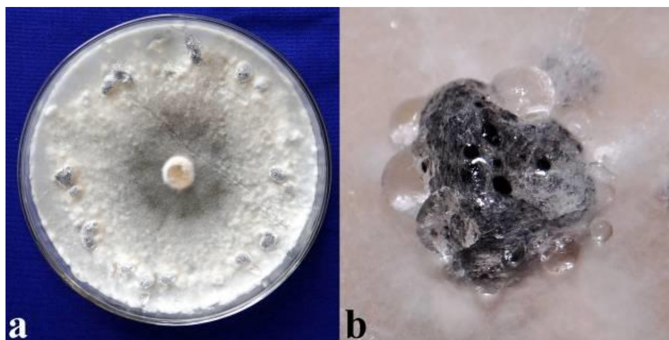

Figure 2 . (a) Colony morphology of *S. sclerotiorum,* **(b)** Dew drops on developing sclerotia.

Figure 3. Pathogenicity with typical drooping and sclerotial bodies in stem.

the form of dew drops (Figure 2b). The morphology and development of our isolate resembled *S. sclerotiorum* as described by Kohn (1979) and Wang et al. (2008). Sclerotial size and number were greater in Petri plates exposed to light, compared to that incubated under complete darkness.

Pathogenicity

Inoculation of mustard plants with 6 mm mycelial disc of the pathogen *S. sclerotiorum* expressed the symptom of stem rot disease after 10 days when incubated at 100% relative humidity at 22°C (Kim and Cho, 2003). Pathogenicity in *Brassica* sp, Canola, peanut and Bell pepper has been established successfully (Young et al., 2012; Khangura and Macleod, 2013; Faske et al., 2014; Gonzalez et al., 1998). Similarly, in our study pathogenicity was proved by inoculating mycelial discs and sclerotial bodies. The typical symptoms were expressed by the plants after 25 days of inoculation. The plant turned pale and showed drooping symptom (Figure 3a) and, typical rot was observed in the stem portion accompanied with small, black sclerotial bodies (Figure 3b). Hundred per cent infection was observed in the plants inoculated with mycelia disc and sclerotial bodies. The pathogen was re-isolated several times and morphological characters were akin to that of the previously isolated pathogen. Thus Koch postulates were established.

Developmental biology of *S. sclerotiorum* and morphological characterization

Identity of the pathogen was confirmed by morphological characterization mainly based on sclerotial size and ascospore morphology. Morphology of study fungus was compared with three well documented commonly occurring species of *Sclerotinia*. Species differentiation was confirmed among three plant pathogenic species *viz., S. sclerotiorum, S. minor* and *S. trifoliorum* by morphologically characterization as proposed by Ekins et al. (2005). The most distinguishing parameter was the

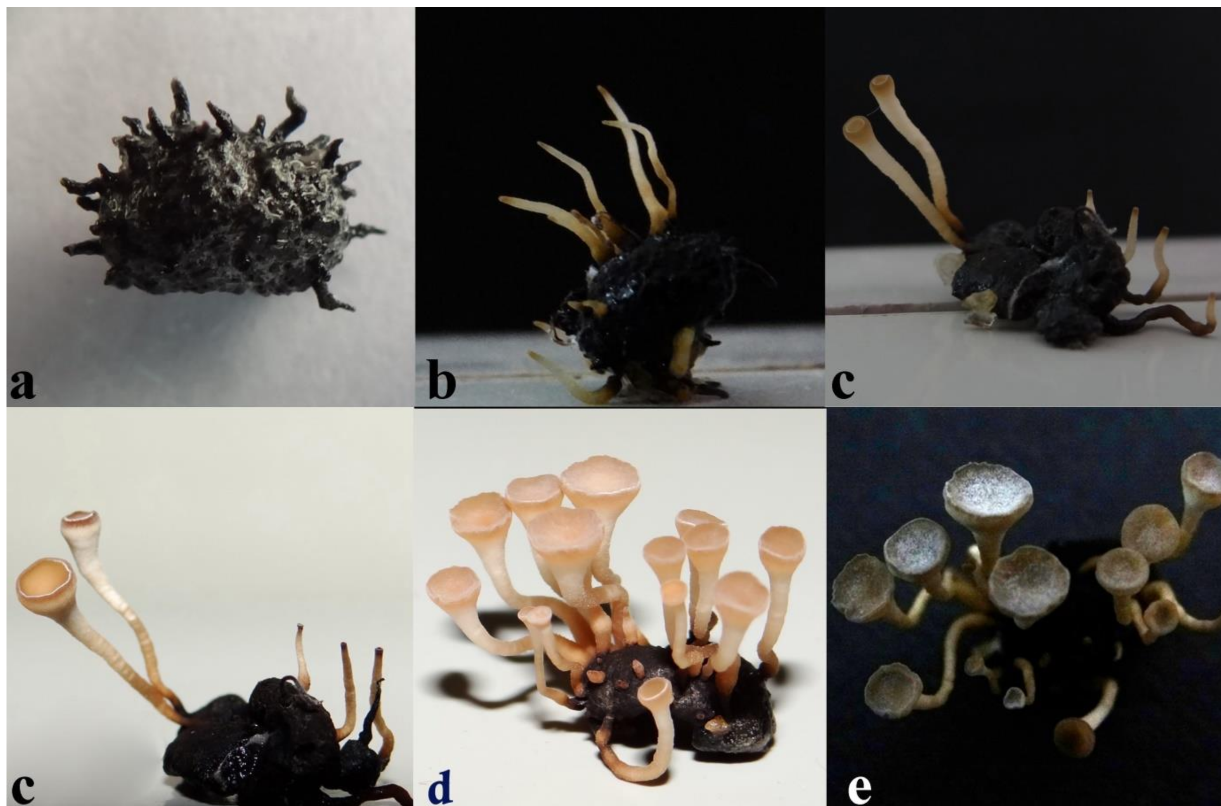

Figure 4. (a) Sclerotia with stipe initials, **(b)** Melanization and elongation of stipe initials, **(c)** Initiation of disc differentiation, **(d)** Growing disc, **(e)** Completely matured apothecium, **(f)** Apothecial disc loaded with ascospores.

sclerotial size that differentiated *S. minor* (1-2 mm dia) from other two species *viz., S. trifoliorum* and *S. sclerotiorum* that produced larger sclerotial bodies (3-100 mm dia). *S. minor* with comparatively smaller sclerotia enables separation from *S. trifoliorum* and *S. sclerotiorum* (Jagger, 1920; Willets and Wong, 1971; Wong, 1979; Tariq et al., 1985). Ascospore morphology enabled further separation between the other two species *viz., S. sclerotiorum* and *S. trifoliorum*. Monomorphic ascospores were observed in *S. sclerotiorum*, whereas *S. trifoliorum* is known to produce dimorphic ascospores (Kohn, 1979; Uhm and Fujii, 1983; Ekins et al., 2005). According to previous studies (Saharan and Mehta, 2008; Pellegrini et al., 1989) *S. sclerotiorum* produced larger sclerotia compared to other species and produced monomorphic ascospores. In our study, the pathogen produced, large, dark, black irregular, sclerotial bodies ranging from 7x4 to 15x5 mm under *in vitro*. This confirmed the identity between *S. sclerotiorum* and *S. trifoliorum* eliminating the chance to be *S. minor*.

In order to study the ascospore morphology, sclerotia were subjected for carpogenic germination. Sanogo and Puppala (2007) reported that *S. sclerotiorum* produced tan to beige coloured apothecial discs. In our study, conditioned sclerotia produced stipe initials after 2 months. After fifteen days of incubation, the black stipe initials got melanised, grew in length and cup shaped; ochraceous apothecium were produced from the stipe initials at the rate of 10-20 per sclerotia. The apothecia formed were of varying sizes ranging from 1.0 to 1.5 cm. The developmental stages of apothecium *viz.,* stipe initiation, melanisation and elongation of stipe initials, disc differentiation and maturation were observed and recorded (Figure 4). Disc differentiation of apothecium was observed only under the exposure of stipe initials to light. Under darkness, length of the stipe increased without disc differentiation. This revealed the importance of cool white fluorescent light in the development of apothecium. All the three replications subjected to conditioning process produced apothecium @ 10-15 apothecium/sclerotia; however, control remained the same.

The ascocarp was made up of four layers *viz*., hymenium, subhymenium, medullary excipulum and ectal excipulum. A row of vertically arranged asci constituted the fertile hymenial layer (Figure5a) which is the outer most and open part of apothecial disc. The subhymenium was composed of closely packed longitudinal cells immediately below hymenium (Figure 5b). Balloon like elongated parenchyma formed the medullary excipulum (Figure 5c). The ectal excipulum was composed of compactly arranged globose pseudoparenchymatous

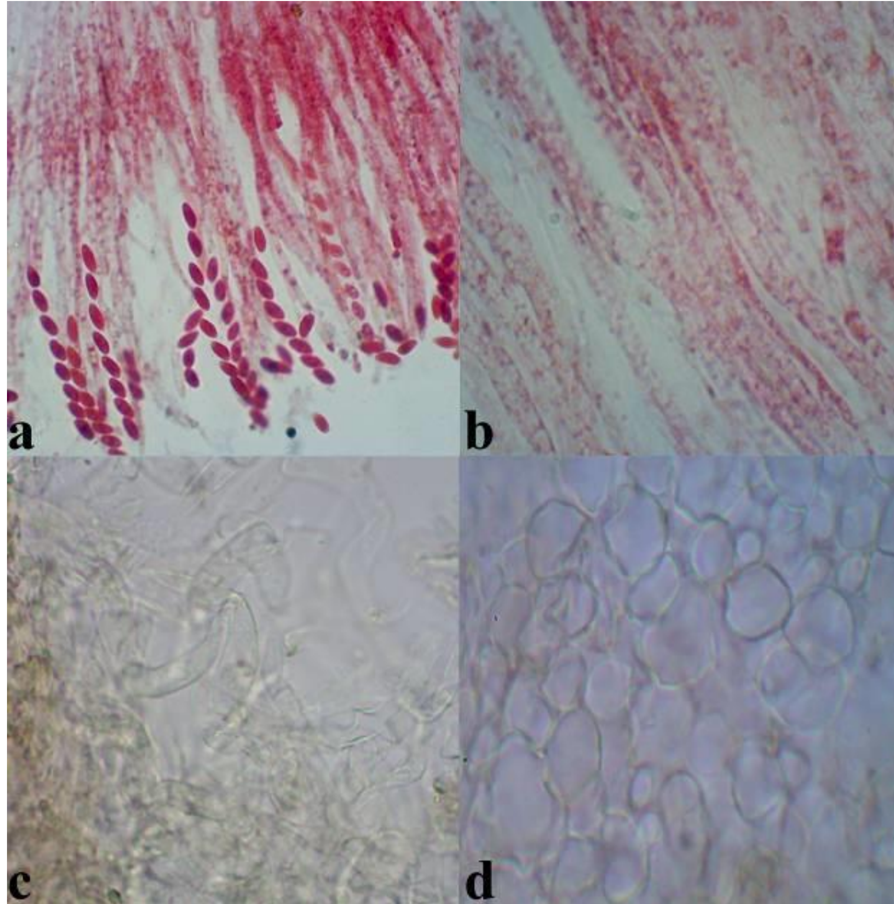

Figure 5a. Hymenial layer with ascus arranged in a row (450X magnification), **b.** Subhymenium (1000X magnification), **c.** Medullary excipulum (1000X magnification), **d.** Ectal excipulum (1000X magnification).

cells that fabricate the stroma (Figure 5d).

Ascus and ascospore

Ascospore morphology enables the separation between *S. sclerotiorum* and *S.trifoliorum*. *S. sclerotiorum* and *S. trifoliorum* are reported to produce monomorphic and dimorphic ascospores, respectively (Evans et al., 2008; Ekins et al., 2005). In the present study, asci were cylindrical, sac like, elongated, with truncated apex and measured 179.39 µm x 8.65 µm at 1000X magnification (Figure 6a). The lateral wall was thin (1.89 µm) and, apex region was thick (5.28 µm) when measured under 1000x magnification (Figure 6b). Each ascus contained eight, ellipsoidal, hyaline, monomorphic ascospores measuring 16.84 µm x 8.65 µm at 1000x magnification. Monomorphic ascospores confirmed the identity as *S. sclerotiorum* distinguishing from *S. trifoliorum*. These results confirmed the identity of the pathogen as *S. sclerotiorum.* Asci were intercepted with supporting sterile hyphae called paraphyses (Figure 6c). The paraphyses were hyaline, filliform with clavate apex. Ascospores were monomorphic and uniform as per the distinctive character of *S. sclerotiorum* (Figure 6a). Ascospores measured 16-19 µ in length and 8-9 µm in width. Ascospores that germinated at both ends were also observed (Figure 6d). Developmental stages of ascospores like empty ascus and immature ascus with under developed ascospores were also observed and recorded (Figures 6e and 6f).

Spermatia and croziers

Besides ascospores, spermatia or microconidia were also observed. Small, hyaline, globose spermatia were singly attached to phialides borne laterally on the hyphae (Figure 6g). Even though spermatia were observed, their exact role in reproduction remains unclear. Small hook like projections with bulged apex resembling croziers were observed in the apothecium (Figure 6h). The croziers later developed into ascus through meiosis and mitosis.

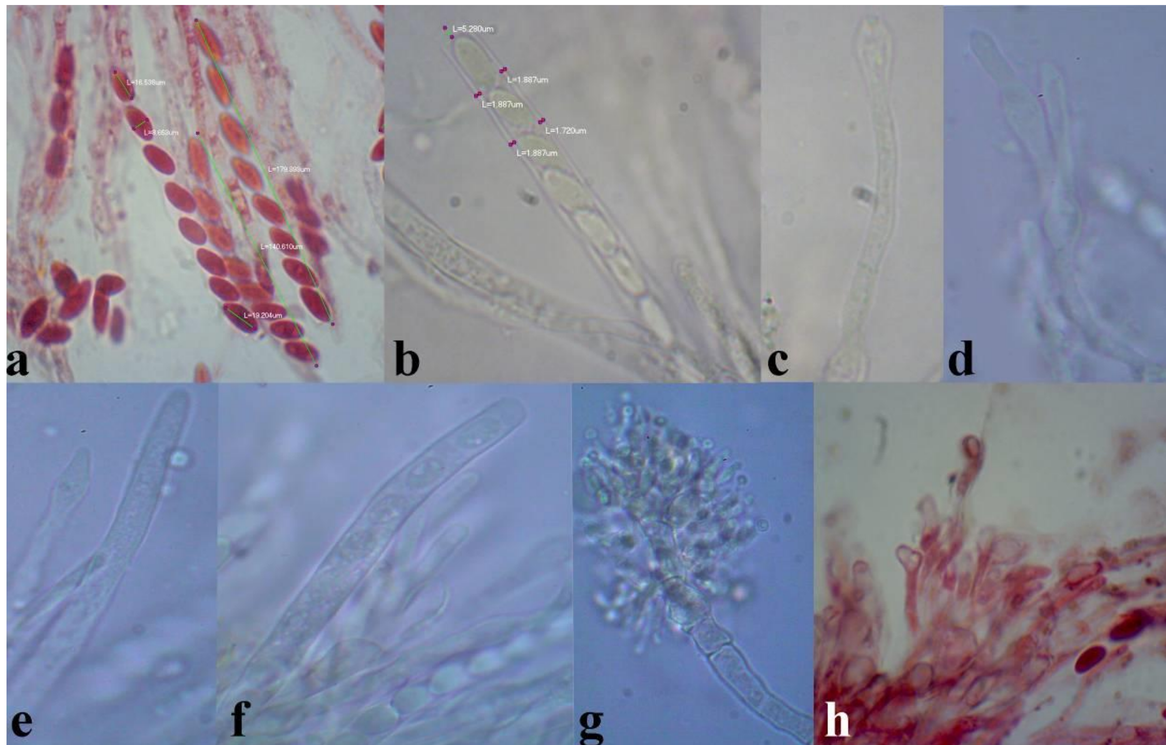

Figure 6. (a) Ascus with ascospore (1000X magnification), **(b)** Ascus wall (1000X magnification), (**c)** Paraphyses with bulged apex, **(d)**Germinating ascospore (1000X magnification), (**e)** Immature empty ascus, **(f)** Underdevloped ascospores inside ascus, **(g)** Spermatia (1000X magnification), **(h)** Croziers (1000X magnification).

The phenotypic characterization of apothecium revealed the components of apothecium identical to previous reports *viz*., ascospore (Saharan and Mehta, 2008), germinating ascospores (Saharan and Mehta, 2008), paraphyses (Saharan and Mehta, 2008), spermatia (Saharan and Mehta, 2008; Rollins, 2007) and crozier formation (Pellegrini et al., 1989). Thus developmental biology of the pathogen was comparatively studied.

Molecular characterization

The ITS region encompassing 18S rRNA, ITS1, 5.8S rRNA, ITS 2 and 28S rRNA can be regarded as an environmental barcodes for the identification of fungi (Bellemain, 2010). The 18S-28S rRNA gene has been reported to have approximately 600 bp (Wang et al., 2008; Jeon et al., 2006). In this study, the PCR product produced an amplicon length of ~600 bp (Figure 7) and the same was sequenced by Sanger dideoxy sequencing method at Excelris genomics, Ahmedabad. Sequencing was carried out using ABI 3730xl Genetic Analyzer (Applied Biosystems, USA). After sequencing the sequence was analysed with BLAST in NCBI (KP676452). The nucleotide sequence of 18S-28S rRNA gene acquired from the study fungus had 99% match with all the three species *viz*., *S. sclerotiorum* (KM272350), *S. trifoliorum* (JQ743329) and *S. minor* (KC836493). Under this predicament, morphological characterization gave a strong distinctive identification of the pathogen. This confirmed the identity of the pathogen as *S. sclerotiorum*.

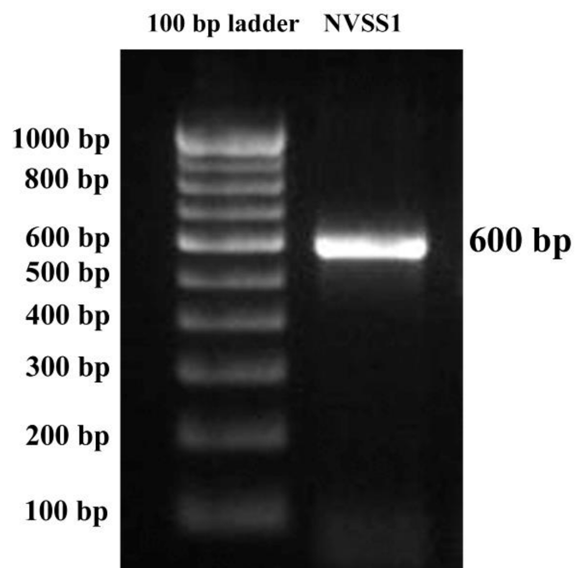

Figure 7. PCR amplification of 18S - 28S rRNA gene of *S. sclerotiorum.*

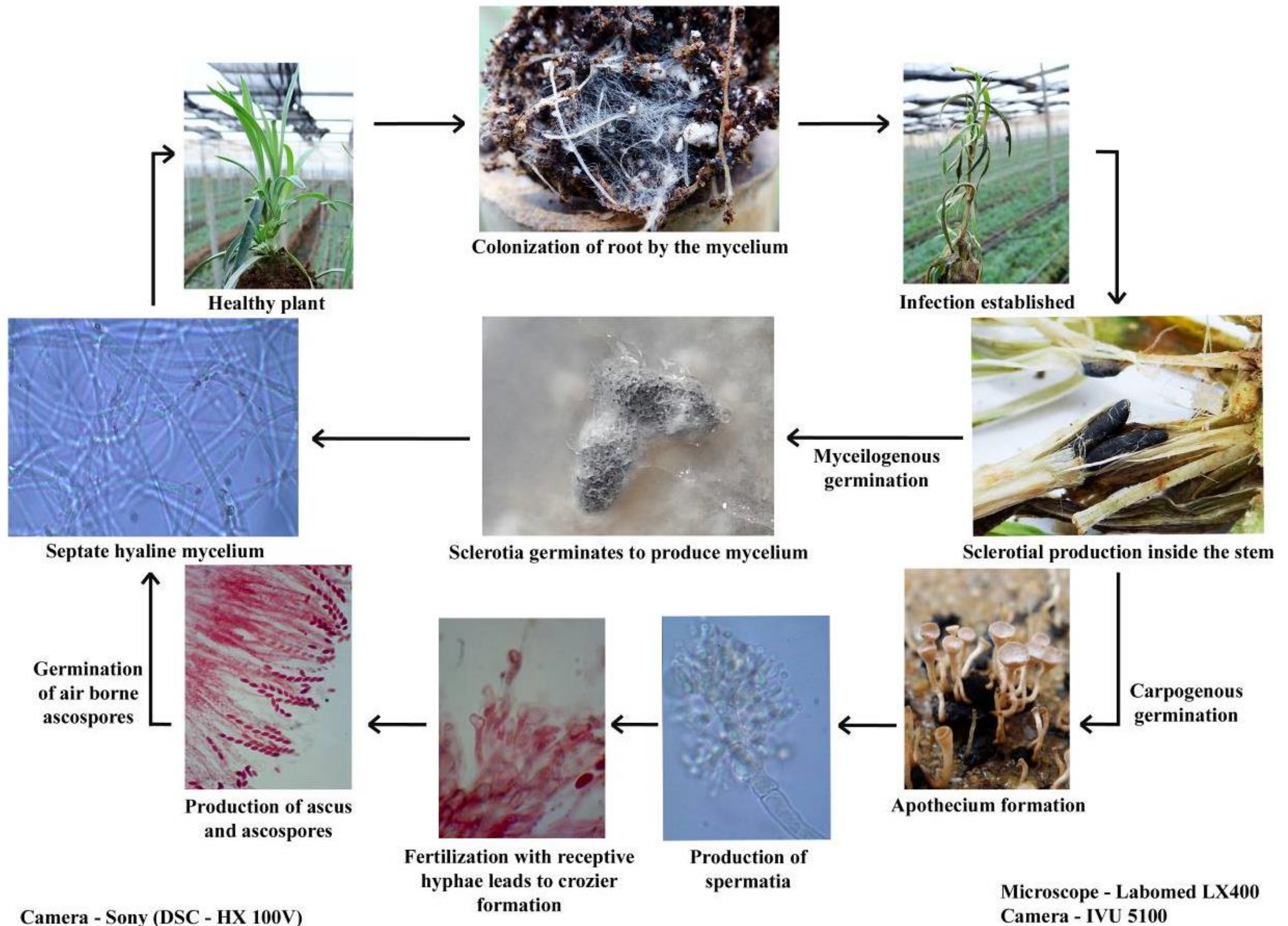

Figure 8. Life cycle of *S. sclerotiorum* in carnation.

Life cycle assessment

The life cycle and different stages of the pathogen were studied. The soil was rich in inoculum due to off season cultivation of leguminous plants. The pathogen resides in soil in the form of sclerotial bodies. The sclerotial bodies are hard and resistant to adverse environmental conditions. Sclerotia play a major role in the disease cycle, since they resist adverse environmental condition and provide required inoculum potential for disease establishment (Willets and Wong, 1980). Conducive environment favours stem rot in carnation, superseding myceliogenic/carpogenic germination of sclerotia (Bolton et al., 2006).

In the present study sclerotial bodies served as primary source of inoculum. Carpogenic germination of sclerotia and airborne dissemination of ascospores have been previously well documented (Heffer Link and Johnson, 2007; Saharan and Mehta, 2008; Purdy, 1979). Under carpogenic germination, apothecia were produced, inside which fertilization occurs and leads to the production of ascospores. The airborne ascospores gets drifted in wind and fall over the healthy flowers. Germinated ascospores gain access to colonize the root zone when the flowers fall off to ground following senescence. Carpogenic germination of sclerotia into apothecium was not observed till now in the field. However, an assumption was made and a lifecycle was designed based on previous studies (Figure 8).

Conclusion

Survey on the distribution of the pathogen *S. sclerotiorum* revealed that, stem rot disease is highly prevalent in Kothagri, Tamil Nadu. Developmental biology of the pathogen was studied in detail. Life cycle assessment shows the mode of infection of the pathogen as mycelium

colonizing root zone and advancing collar region. This provides basic information for future studies to control the disease by breaking developmental biology and infection cycle of the fungus.

Conflict of Interests

The authors have not declared any conflict of interests.

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