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# Collar Rot Disease of Betelvine (*Piper betle* L.) under Coastal Saline Zone of West Bengal

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## ABSTRACT

Collar rot is an important disease of betelvine (*Piper betle* L.) caused by the soil borne fungus *Sclerotium rolfsii* Sacc. The pathogen was isolated from the infected vines cultivated in the coastal saline agro-climatic zone of West Bengal and characterized for its growth and survival under different pH, temperature and soil moisture. The pathogen was found to prefer acidic pH (optimum at pH 5.0 to 5.5) for its mycelial growth, formation of sclerotia and their germination. The saprophytic colonization ability of the fungus was drastically reduced at pH 7.7 and above. Germination of sclerotia was least at a soil moisture level of 20% of field capacity (FC). The increase in soil moisture increased the rate of sclerotial germination, resulting in quick loss of its viability. The saprophytic colonization ability of *S. rolfsii* was maximum at 60% of FC, which reduced rapidly above 70% of FC. The optimum temperature for maximum mycelial growth, sclerotial formation and its germination was 30±1°C. Mycelial growth, sclerotial formation, germination and saprophytic colonization ability of the fungus ceased completely at 40±1°C. These specific soil temperature and moisture requirements of the fungus *S. rolfsii* make the pathogen highly selective for its seasonal incidence and proliferation.

**Key Words:** Betelvine, collar rot, ***Sclerotium rolfsii*,** pH, temperature, soil moisture.



## INTRODUCTION

Betelvine (*Piper betle* L.) is an important cash crop in India, grown over 55,000 ha area. The heart shaped green leaves, commonly known as '*Paan'*, are widely consumed as mouth freshener. West Bengal accounts for more than half of the country's total production, securing the livelihood of nearly five lakh farm families (Anonymous, 2015). The coastal saline zone of this state is well known for producing the high quality *Mitha pata* cultivar of this crop that is known for its pleasant aroma. The perennial vines are grown inside a protected structure, called *boroj*, to create a moist and shaded microclimate, required for optimum crop growth. However, the same microclimate often becomes congenial for the growth of several pathogens inside the *boroj*. Among all such pathogens, it is very difficult to manage and eradicate the *Sclerotium rolfsii* Sacc, causing collar rot disease. The pathogen causes rapid wilting, resulting in 17-90% crop loss (Garain *et al*, 2020) in West Bengal condition.

The soil borne pathogen is reported to infect more than 600 plant species and can survive in soil upto 7 years, by producing its resting structure 'sclerotia' (Farr *et al*, 2006). However, the growth and survival of the fungus in a particular soil system are governed by many soil physicochemical factors like pH, temperature and moisture (Shim and Starr, 1997; Raghavendra *et al,* 2018; Maji *et al,* 2019). Such factors play an important role on pathogen biology and influence the development of diseases. In West Bengal, weather and soil conditions vary greatly from one agro-climatic zone to another. The present study was conducted to understand

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the influence of pH, temperature and soil moisture on the growth and development of *S. rolfsii*. Such information base will be helpful to create a non-conducive soil environment for growth and development of the pathogen by adopting simple cultural practices.

## MATERIALS AND METHODS

The study was carried out in Ramkrishna Ashram Krishi Vigyan Kendra at Nimpith of South 24 Parganas district. The symptoms of the collar rot disease was studied on above and below ground parts of the diseased vines.



### Isolation and identification of the pathogen

The collar region of infected betelvine was collected from field, cut into small bits, surface sterilized with 0.1% NaOCl solution and aseptically transferred to Petri plates containing sterilized PDA medium. The plates were incubated at 28±2°C for three days and the actively growing mycelium from the margin of the colony was transferred to PDA slants. The pure culture was maintained at 4°C and the fungal isolate was identified based on morphological and colony characteristics (Punja and Damini, 1996).

### Production of sclerotia

Sand-corn-meal medium, taken in 500 ml conical flask, was sterilized and inoculated with mycelial disks of *S. rolfsii*, aseptically and incubated at 28±2°C for 20 days. The sclerotia, formed in the medium were collected, air dried on sterile blotting paper inside laminar airflow hood and stored inside a polypropylene bag for further studies.

### Pathogenicity study

Five sclerotia of the isolated fungus were placed against the stem of a healthy betelvine plant inside a *boroj*, at the collar region and wrapped with a piece of sterile moist cotton. The cotton was re-moistened every day with sterile distilled water. The vine was studied for any disease development for the next 14 days. The associated pathogen was re-isolated from the infected collar region, cultured on PDA and characterized.

### Influence of pH on the growth and development of *S. rolfsii*

The pH of liquid PDA media was adjusted to a wide range (pH 4.5 to pH 8.5), by adding either 0.1N HCl or 0.1N NaOH. Culture of *S. rolfsii* was inoculated to the Petri plates containing PDA medium of different pH and incubated at 28±2$^0$C. The plates were observed for colony growth and sclerotia formation. On another set of PDA plates, 10 sclerotia were seeded, incubated at 28±2$^0$C and observed for their germination. Soil samples with a wide pH range (pH 5.2 to pH 8.8) were prepared by mixing different quantities of CaO to a previously collected soil sample (silty clay texture, pH 5.2). These samples were sterilized at 121°C and 15 p.s.i. pressure for 20 min, separately poured in plastic glasses and moistened with sterile water to 60% of their field capacity (FC). Fifty sclerotia were placed over the soil and incubated at 28±2°C and 80% relative humidity. Number of germinating sclerotia was counted up to 72 hr. In a separate experiment, culture of *S. rolfsii,* mass multiplied over sand-corn-meal medium, was mixed with the soil samples of different pH, at the rate of 2% inoculum. Pieces of betelvine stem (2-inch length) were cut from healthy vines and autoclaved at 15 p.s.i. pressure for 20 minutes. Ten pieces of betelvine stems were vertically inserted in the soil and the glasses were incubated at 28±2$^0$C and 80% RH. Soil moisture was maintained at 60% FC. The percentage of stem pieces colonized by white mycelial growth of *S. rolfsii* was recorded after 5 days and expressed as saprophytic colonization ability of the fungus.

### Influence of temperature on *S. rolfsii*

Mycelial discs of *S. rolfsii* were seeded in Petri plates containing PDA medium and incubated at a wide range of temperature (10±1$^0$C to 45±1$^0$C). The growth of the colonies was measured after 24, 48 and 72 hr of incubation and the radial growth rate (mm/day) was determined. The same plates were further

incubated at the respective temperatures for 21 days, to observe sclerotia formation. Sterile soil (silty clay texture, pH 6.1) was taken in plastic glasses and moistened with sterile water to 60% of FC. Fifty sclerotia were placed over the soil and incubated at different temperatures at 80% relative humidity. Number of germinating sclerotia was counted up to 72 hr. In a separate experiment, 100g of sterile soil was poured in 3 plastic glasses and moistened with sterile water (60% of FC) and inoculated with *S. rolfsii* (2% inoculum). Ten pieces of betelvine stem, autoclaved at 15 p.s.i. for 20 min, were placed in each glass and incubated at different temperature and 80% RH. Percentage of stem piece colonized by white mycelial growth was counted after 5 days and expressed as saprophytic colonization ability of the fungus at different temperature.

**Influence of soil moisture on *S. rolfsii***

Field capacity (FC) of the soil was calculated as FC = $[(W_w - W_D)*100/ W_D]$, where, $W_W$ and $W_D$ denotes weight of the saturated soil and dry soil, respectively. Plastic glasses were poured with 100 g sterilized soil and moistened with known quantity of sterile distilled water to obtain soil samples with different moisture content (ranging from 20% to 90% of FC). Ten sclerotia were placed over moist soil samples and incubated at 28±2°C and 80% RH. Soil moisture content were maintained to the respective values in each treatment by periodically weighing the glasses and adding sterile distilled water to the glasses, wherever required. The number of germinating sclerotia was counted upto 72 hours. The soil with different soil moisture content was mixed with *S. rolfsii* (2% inoculum). Ten pieces of sterilized betelvine stem were vertically placed on the soil (half inserted) and incubated at 28±2°C and 80% RH. Four replications were maintained for each treatment. Percentage of stem pieces colonized by white mycelial growth, was counted after 5 days and expressed as saprophytic colonization ability of the fungus at different temperature.

**Statistical analysis**

The experiments were set in completely randomized design (CRD) and the data were analysed using IBM SPSS Statistics 20. Mean separation was accomplished by Fisher's LSD test at $p \leq 0.05$. Angular transformation was used to normalise the data for the values in percentage.

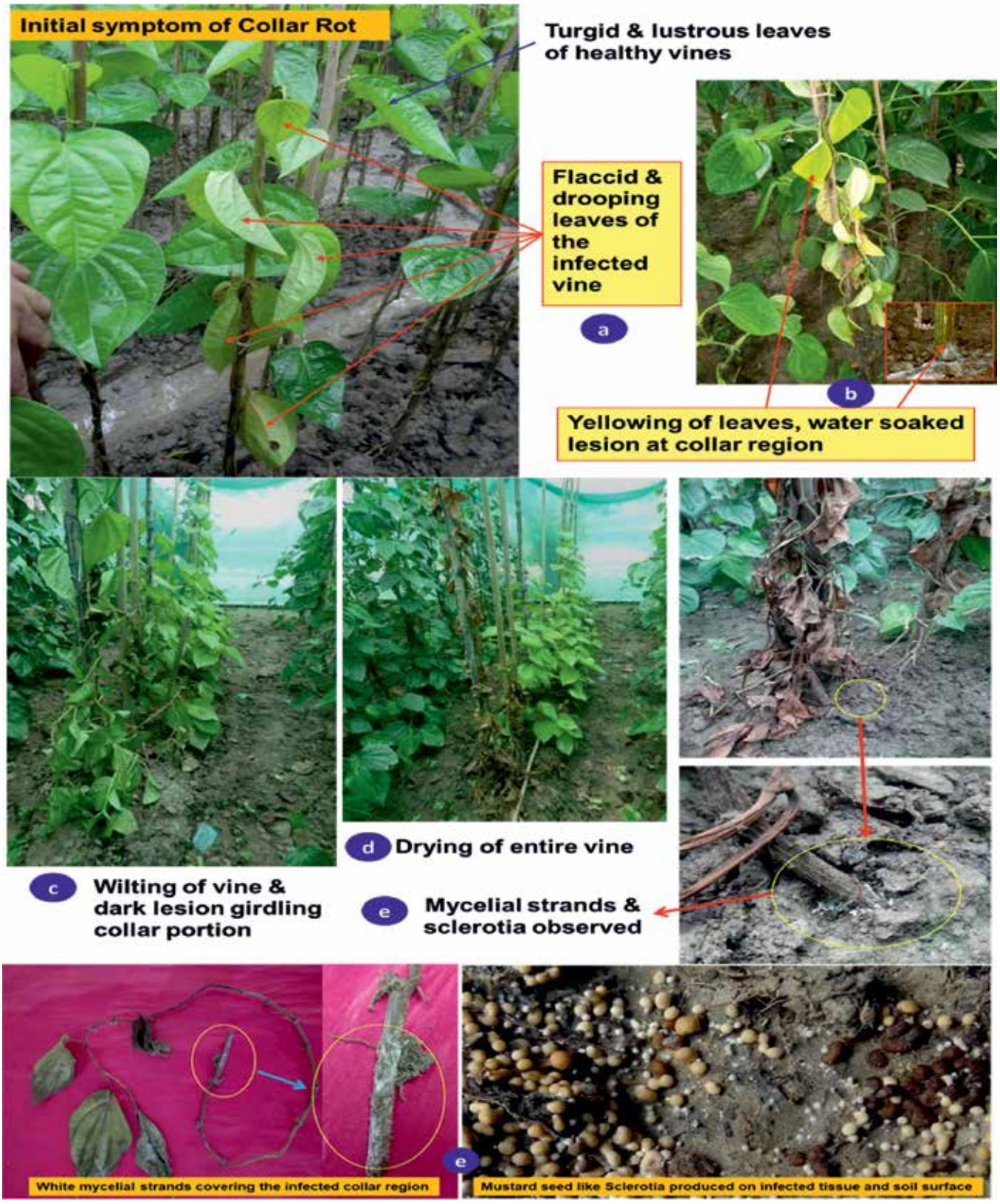
## RESULTS AND DISCUSSION

**Symptoms of collar rot disease**

At initial stage, the leaves of the infected vine showed loss of turgidity and lacked their characteristic luster, which gradually turned flaccid and started drooping (Pic. 1). Simultaneously, a water soaked depressed spot developed on the collar region of the infected vine, at soil level. The spot started to enlarge and soon turned into a shrunken, dark brown to black lesion, girdling the stem. After 3 to 4 d, the leaves became pale to yellow in colour and the infected vine showed wilting symptoms. After 7 to 10 d, thick, white, mycelial strands wrapped around the infected collar region of the vine and started radiating out horizontally, on the soil surface. By this time, the entire vine gets dried up. Light to dark brown and spherical to ellipsoidal sclerotial bodies started to develop on the surface of infected collar region and on the adjacent soil surface. The infected vines died within 3 to 7 d from the appearance of the symptoms.

**Characteristics and identification of the pathogen**

The fungus produced profuse white, fluffy, ropy mycelia, radiating in a fan shaped manner on PDA medium (Pic. 2). The hyphae were branched, septate and hyaline when young, later turning white in colour. Small white tufts of mycelium started to form on 5 to 10 d old cultures, first along the periphery of the plate and later on, all over the plate. These mycelial tufts turned into smooth, spherical, mustard seed like hard structures (sclerotia) that changed their colour from white to creamy to dark chocolate brown. Diameter of the sclerotia varied




Pic 1. Development of symptoms of Sclerotium rolfsii induced collar rot disease in betelvine (a – e)

from 0.8 to 1.2 mm with a mean of 0.97±0.05 mm. Average number of sclerotia produced per plate (90 mm diameter) was 211.5±2.9. Weight of the sclerotia ranged from 0.4 to 0.7 mg with a mean of 0.54±0.03 mg. The fungal pathogen was identified as *Sclerotium rolfsii* Sacc., based on its morphological and colony characteristics (Punja and Damiani, 1996; Watanabe, 2002).

**Pathogenicity of the isolated *S. rolfsii***

Collar rot symptoms appeared 5 days after inoculating the isolated *S. rolfsii* in the healthy betelvine. The symptoms and signs were similar to those observed under field conditions. The non-inoculated vines remained healthy. The re-isolated culture of the fungus, obtained from the infected tissue of the inoculated vine, corroborated with the same morphological characteristics as of originally isolated culture. These results proved the Koch's postulate principles in establishing the pathogenicity of the isolated fungus.

**Influence of pH on growth and development of *S. rolfsii* on PDA medium**

The effect of pH of PDA medium was studied with nine different pH levels, ranging from 4.5 to 8.5, in respect of mycelial growth, sclerotia formation and sclerotia germination (Fig 1). The data indicated that pH 5.5 was most favourable for mycelial growth of the fungus (8.03±0.18 cm), which is statistically at par with pH 5 (7.65±0.09 cm). However, none of the pH values, under the study, was completely inhibitory for the growth of the pathogen. Thus, from the present findings it may be concluded that *S. rolfsii* is able to grow at wide level of pH with a pH optima at 5.0-6.0. Similar result was reported by Chaurasia *et al* (2013) while studying the mycelial growth of *S. rolfsii*. Formation of sclerotia was observed throughout the range of pH (4.5 to 8.5) on PDA medium (Fig 2). Sclerotia production was marginally reduced at pH 8.0 and above. Chaurasia *et al* (2013) also found pH 4.0 to 7.0 to be optimum for the production of sclerotia by *S. rolfsii* causing foot rot in brinjal. Acidic pH was ideal for germination of sclerotia. Cent percent sclerotial germination was found at pH 4.5 to 6.0. Sclerotial germination reduced drastically with increase in pH, beyond neutral (Fig 2). Lowest germination (46.50±3.30%) was observed at pH 8.0, which was statistically at par with pH 8.5 (48.50±9.91%). Punja and Grogan (1982) also reported that the sclerotial germination was inhibited above pH 7. Similarly, Raghavendra *et al* (2018) found least sclerotial germination of *S. rolfsii*, causing stem rot in groundnut, at basic pH (9.0).

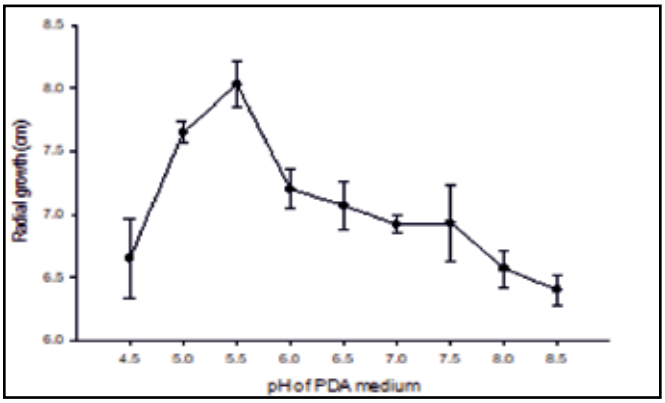



Fig 1. Mycelial growth of *S. rolfsii* on PDA medium at different pH. Error bars represent standard error of data.

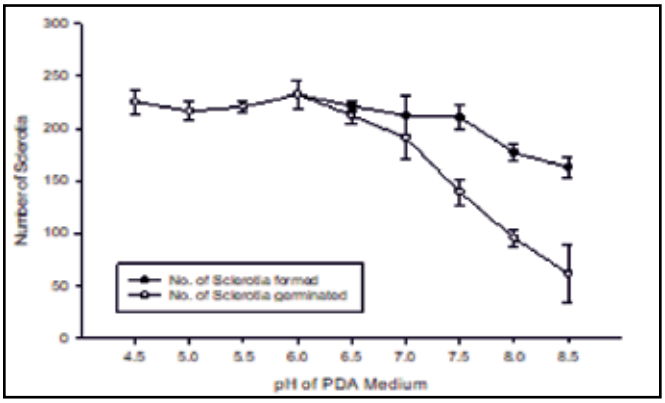



Fig 2. Formation of sclerotia of *S. rolfsii* and their germination on PDA medium at different pH. Error bars represent standard error of data.

**Influence of soil pH on sclerotia germination and saprophytic colonization**

Among the seven levels of soil pH studied, maximum sclerotial germination (87.33±2.40%) was found at pH 5.2, which was statistically at par

with pH 6.1 and 6.7 (Fig 3). Sclerotial germination was greatly reduced at alkaline pH. Lowest sclerotial germination (37.33±0.67%) was recorded at pH 8.8, which was statistically at par with pH 8.3. Shim and Starr (1997) also found greater sclerotial germination in acidic soil than alkaline soil while working with *S. rolfsii*, causing stem rot in peanut.

The saprophytic colonization ability of *S. rolfsii* was expressed as percent of betelvine stem colonized by the fungus in soil at seven levels of pH (Fig 3). The fungus showed a consistently high level of saprophytic colonization (67.50 to 72.50%), over a pH range of 5.2 to 7.2. But the saprophytic growth reduced drastically beyond soil pH of 7.7. The fungal pathogen prefers acidic to slightly alkaline soils for its saprophytic growth and proliferation as indicated from higher level of colonization of betelvine stems at pH 5.2 to 7.7. The comparatively higher sclerotial germination and saprophytic colonization were noticed at slightly acidic pH as compared to neutral and alkaline pH. This may be due to the fact that lower carbon loss takes place in sclerotia at acidic pH as compared to alkaline pH and carbon loss by sclerotia during incubation in soil at different pH is inversely correlated with its ability to infect plants (Hyakumachi *et al*, 2014).

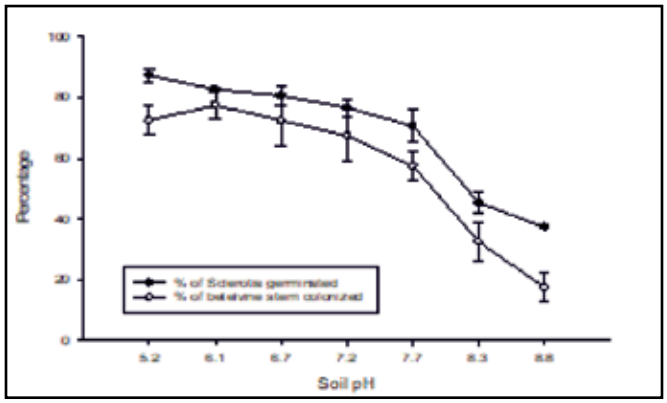



Fig 3. Effect of soil pH on *S. rolfsii* sclerotial germination and saprophytic colonization of betelvine stem. Error bars represent standard errors of data.

## Influence of soil moisture on sclerotia germination

The increase in soil moisture resulted in increase in sclerotial germination (Fig. 4). Least sclerotial germination (22.50±4.79%) was observed at 20% FC. The results indicate that availability of moisture is necessary for germination of the sclerotia in soil. In dry soil, the sclerotia did not germinate readily and kept its viability intact for longer time. In wet soil, most of the sclerotia germinated readily. Then the fate of the germinating mycelia depends either upon the presence of soil organic matter for saprophytic colonization or upon the presence of host tissue for infection or it succumbs to lyses and death. Similar observation was also reported by Palakshappa *et al* (1989), who found that *S. rolfsii* survived best at 20 - 40% soil moisture level.

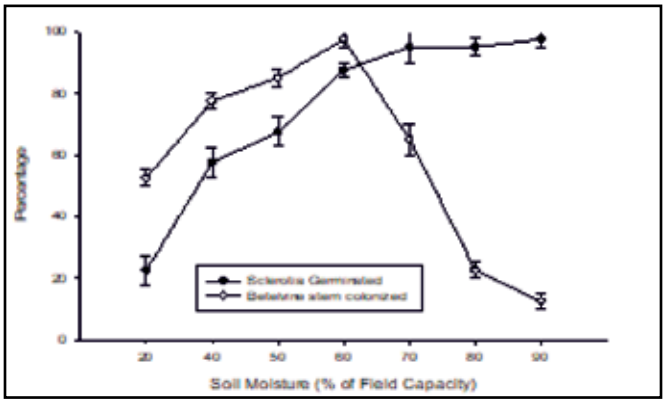



Fig 4. Effect of soil moisture on *S. rolfsii* sclerotia germination and saprophytic colonization of betelvine stem. Error bars represent standard errors of data.

## Influence of soil moisture on saprophytic colonization ability of *S. rolfsii*

The results (Fig. 4) indicated that the fungus sharply loses its saprophytic colonization ability at soil moisture above 70% of FC. The maximum saprophytic colonization (97.50±2.50%) was recorded at 60% FC. The near saturated to saturated soil moisture level may be detrimental for the mycelia by limiting the availability of oxygen to them. Beute and Rodriguez-Kabana (1981) reported that mycelia of *S. rolfsii* died rapidly in moist soil, irrespective of soil temperature. The present study indicates that though the soil moisture levels beyond 70% FC increase the germination rate of the sclerotia, it decreases the saprophytic colonization ability. Hence, the soil moisture level is one of the prime factors for survival, saprophytic and pathogenic colonization of *S. rolfsii* in the soil.

Maiti and Sen (1982) also found that the sclerotia could survive 225 days in soil below 50% moisture level whereas, at 100% water holding capacity, the sclerotial population declined rapidly.

### Influence of temperature on growth and development of *S. rolfsii* in PDA medium

Among the various temperature levels studied (Fig 5), the optimum mycelial growth was found at 25±1°C to 30±1°C range, with the maximum mycelial growth rate (23.78±0.29 mm day$^{-1}$) been recorded at 30±1°C under *in vitro* condition. No mycelial growth was observed at and beyond 40±1°C. The optimum temperature for maximum sclerotia formation (246.33±4.33 per petri plate) was 35±1°C. Sclerotia formation reduced drastically below 25±1°C. No sclerotia formed below 20±1°C and above 35±1°C. Earlier published reports also indicated that the temperature range of 25 to 30°C was optimum for the *in vitro* growth of *S. rolfsii* (Zape *et al*, 2013).

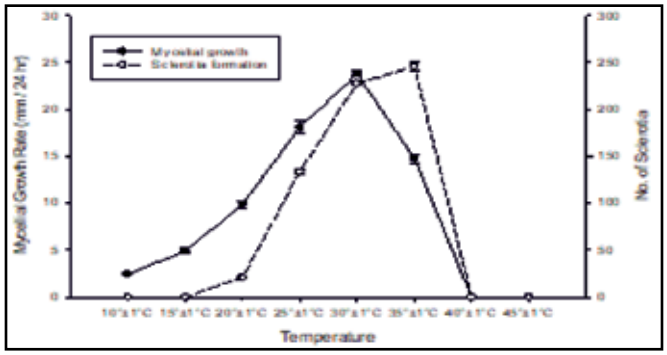



Fig 5. Effect of temperature on mycelial growth and sclerotia formation of *S. rolfsii* in PDA medium. Error bars represent standard errors of data.

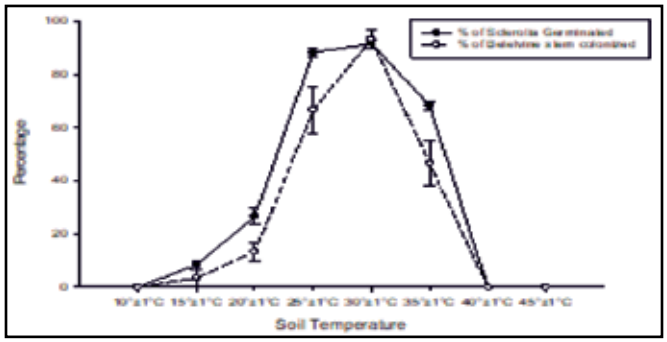



Fig 6. Effect of soil temperature on sclerotial germination and betelvine stem colonization by *S. rolfsii*. Error bars represent standard errors of data.

### Influence of soil temperature on sclerotial germination and saprophytic colonization

Sclerotial germination and saprophytic colonization were found to be highly dependent upon soil temperature (Fig 6). Maximum sclerotial germination (91.67±1.67 %) was recorded at 30±1°C, which was statistically at par with 25±1°C (88.33±1.67%). None of the sclerotia germinated at temperatures of 10±1°C and above 40±1°C. The peak saprophytic colonization ability (93.33±3.33%) was recorded at 30±1°C (Fig 6). However, no saprophytic colonization was recorded at below 15±1°C and above 40±1°C. The results corroborate with the findings of Palakshappa *et al* (1989) in betelvine and Maji *et al* (2019) in sunflower.

## CONCLUSION

From the present study it may be concluded that the fungus *S. rolfsii,* isolated from collar rot infected betelvine, could survive at wide range of pH and temperature but preferred acidic pH and temperature of 25-30°C for its optimum growth and development. The rapid decline of the saprophytic colonization ability of the fungus beyond 70% FC indicates that the soil borne pathogen loses its viability in moist soil. The findings opened up the scope for further research on cultural management of collar rot disease in betelvine in the coastal saline zone by managing the soil pH, temperature and moisture level.

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