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RESEARCH ARTICLE

Occurrence of mycoparasitic fungi *Sphaerellopsis paraphysata* on pearl millet rust pathogen, *Puccinia substriata* in India.

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ABSTRACT

A mycoparasite was isolated on the rust infected pearl millet crop. It was isolated from an unusual pycnidia which was present along with the uredospores of rust. It reduced the uredial inoculum to a considerable extent. The morphology and molecular characters of the mycoparasite was studied. Its role as hyperparasite was established using Scanning Electron Micrographs, which clearly showed coiling and puncturing of uredospores of Puccinia substriata, by the mycelia of S. paraphysata. The confirmatory studies on the positive pathogenicity on rust fungi, showed a colourless septate mycelium, with an average conidial size of 9.27-12.92 ×2.55-5.87 µm and pycnidial size of 76.14-198.04×56.25-184.19 µm. The 540bp amplicon size of ITS sequence showed 96% similarity with S. paraphysata. The spore germination of Puccinia substriata on pearl millet leaves was reduced by 76%, 56%, 70% and the disease severity percentage was 13.3% on an average whereas in control it was 86.6%. The intimate association between S. paraphysata and Puccina substriata was proved by ultra microscopic structures in this investigation.

Keywords : *Pearl millet, Sphaerellopsis paraphysata, Mycoparasite, Rust, Characterization.*

INTRODUCTION

Pearl millet (*Pennisetum glaucum*), is a vital food crop in the semi arid tropics of the world, (Obilana, 2003). The rust disease caused by *Puccinia substriata* on pearl millet has been the most devastating disease of pearl millet that has been reported from many countries. It cause losses of upto 76% in grain yield and lessens the fodder quality (Aldir *et al.,* 2006). Nature contains a huge assortment of fungicolous fungi, either as parasites or amensols or saprobes (Hawksworth *et al.,*1995). Mycoparasitism, which is very basic in nature, has gotten extraordinary consideration because of the scan for biological control options for plant diseases (Hijwegen, 1988). *Sphaerellopsis* spp. is biocontol organism that is recently being exploited for rust control in many countries. It is an ubiquitous mycoparasite in rust that mostly occurs in its anamorph. The sexual stage of *Sphaerellopsis* is *Darluca filum* (Nischwitz *et al*., 2005; Yuan et al., 1998) and it can reduce rust spore production upto 98% (Yuan *et al.,* 1999). Since the fungicidal control of rust disease has hazardous impact on environment there exists a huge need for natural antagonist. Hence, the present study was focused on the isolation, morphological and molecular confirmation of the mycoparasite *S. paraphysata* so that it can be exploited against the rust fungi.

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MATERIAL AND METHODS

Isolation and identification of the mycoparasitic fungi

The rust infected pearl millet and other host plant leaves were collected from various districts of Tamil Nadu (Table. 1). The collected leaves were surface sterilized with 0.1% mercuric chloride. The rust pustules were scrapped and pycnidia were collected in 1 ml of water in eppendorf tubes. 500µl of the collected pycnidia were poured in a petri dish, 20ml of water agar was added in it and thoroughly mixed which was allowed to solidify completely. The dispersed pycnidia in petri plate were marked with the help of stereo zoom microscope and incubated at 25°C for 48hrs. The germinated pycnidiospores were identified and transferred to petri dish using a sterile cork borer containing V8 juice agar selective medium added with streptomycin to avoid bacterial contamination. Inoculated petri plates were incubated at 25°C for 20 days (Pei, 2003). Pure culture of the biocontrol agent was obtained by single hyphal tip method (Leyronas *et al.,* 2012). Stock cultures were subcultured at an interval of 30 days to maintain the viability of the culture.

Molecular confirmation

Genomic DNA Extraction

The fungal cultures were grown on V8 juice agar media, mycelium was scrapped from the culture plates gently. Fresh mycelia of 300g was taken for DNA extraction. 250 µl of C TAB buffer was added and the mycellia was ground with the help of pestile and mortar.The mixture was collected in two eppendorf tubes and incubated at 60 °C for 1 hour in waterbath. The extract was taken, equal volume of Phenol, Chloroform and Isoamyl alcohol (24:2:1) was added followed by gentle inversion. Centrifugation of this mixture was done at 13000 rpm for 10 minutes. The aqueous phase was taken with the help of micrpipette and added with equal volume of icecold isopropanol.The contents were incubated at -20° C for 30 minutes which precipitates the total DNA and pelletized with centrifuging at 12000 rpm for 15 min which was further purified by 70% ethanol wash followed by air drying the pellets and added with 200µl of double distilled water and The isolated DNA was quantified in nanodrop and adjusted to the concentration as it contained 100ng/µl and stored at -80° C.(Chakraborty *et al.,* 2010)

PCR Amplification

The primer pair ITS1 (5´-TCCGTAGGTG AACCTG CGG-3') and ITS4 (5´-TCCTCCGCTTATTGATATGC-3') (White *et al*.,1990) were used for the amplification of the ITS region. The PCR was performed in Eppendorf thermocycler using the profile 94°C of initial denaturation for 5 min, then 40 cycles of 94°C for 30sec, 52°C for 40 sec, and 72°C for 90 sec, followed by 72°C final extension for 10 min (Kajamuhan *et al*., 2015). Agarose gel Electrophorosis was used to observe the amplified products of ITS region using 1.2% agarose in 1% TAE buffer,5µl of PCR product along with 2µl of loading dye loaded on agarose gel visualized under UV transilluminator. 100 bp DNA ladder was used to estimate the size of the PCR product and the banding profiles were documented.

Pathogenecity

The pathogenicity confirmation was done by spraying the uredospores at the concentration of 10^6/ml on the plants that were raised in glass house at six leaf stage. After the uredospore establishment in the cumbu leaves, the conidia of *S. paraphysata* was sprayed at the concentration of 10^5/ml. The leaves were periodically observed for parasitization till the parasitization occurred. *S. paraphysata* was then reisolated from uredospores of the pearl millet plant.

Efficacy of mycoparasite against pearl millet rust pathogen

Three cultivars of cumbu (CO 7, CO 10, TNAU cumbu hybrid CO 9) was raised in pots in PL480 glass house in Department of Plant Pathology, TNAU, Coimbatore. Uredospore inocula were freshly collected from Millet Breeding Station, TNAU, Coimbatore by brushing the uredospores in an aluminium foil and were brought upto the concentration of 10^6 spores/ ml. The inocula of the mycoparasite *S. paraphysata* TNAU-Sp1 which showed better parasitization at *invitro* conditions (Cavity slide experiment) was prepared by homogenizing the cultures. Uredospores of the pathogen were sprayed on 20 days old plants and three days later the conidia of *S. paraphysata* were sprayed (Swendsrud and Calpouzos, 1970). Disease severity (%) was measured according to a modified scale of Cobb (Peterson *et al.,* 1948) and the number of rust spores germinated was calculated by scrapping the uredospores established , after inoculation with pathogen and the biocontrol organism, using haemocytometer. The rust spore germination percentage was calculated using (Eqn. 1), and the reduction in the rust spore germination % was calculated using (Eqn. 2).

$$Rust\ spore\ germination\ \% = \frac{\text{Number of rust spores germinated}}{\text{Number of rust spores observed}} \times 100 \quad \text{(Eqn. 1)}$$

$$\%\ inhibition\ of\ rust\ spore\ germination = \frac{\text{Control}-\text{Treated}}{\text{Control}} \times 100 \quad \text{(Eqn. 2)}$$

Scanning Electron Microscopic studies

The samples obtained from inoculation experiment was freshly picked and used for environmental scanning electron microscopic analysis to study the morphological characters and mode of parasitization of *S. paraphysata*. The leaf consisted of uredial sori with pycnidia and the rust pustules alone were selected and morphologically examined by ESEM (FEI-Quanta 250) using xT microscope control software available at the Department of Nano Science and Technology, Tamil Nadu Agricultural University, Coimbatore. The leaf specimen was cut into a size of 5 × 5 mm and directly fixed in carbon stubs coated with gold in a high vaccum evaporator (Sputter coating). ESEM analysis was carried out using an acceleration voltage of 8-10 kV (Carling *et al,* 1975). Image capture control was achieved using Auto-Montage v.5.0 (Synoptics), the images were captured as a series of focal planes and montage to produce a composite focused image.

RESULTS AND DISCUSSION

This study was conducted with an objective to confirm the morphological, molecular characteristics of *S. paraphysata* and to prove its mycoparasitic nature against pearl millet leaf rust caused by *Puccinia substriata*, further the potential to be used as a biocontrol organism was explored. The hyperparasite *Sphaerellopsis* spp. was collected from different crops infected with rust pathogen and they were designated as TNAU Sp1 to TNAU Sp12 (Table 1,). Out of these twelve samples, *S. paraphysata* was isolated from two samples (TNAU Sp1 and TNAU Sp2) and used for further studies.

Table 1. Collection and isolation of *Sphaerellopsis* spp from various hosts

S. No	Isolate Name	Location and District	District	GPS Data	Host	Average Conidial Size (m)		Average Pycnidial Size (m)	
						Length	Breadth	Length	Breadth
1	TNAU Sp1	Millet Breeding Station-TNAU	Coimbatore	11.0205 N, 76.9269 E	Cumbu	11.026^{j}	4.978^{d}	154.84^{e}	123.754^{e}
2	TNAU Sp2	Central Farm Unit-TNAU	Coimbatore	11.0104 N, 76.9499 E	Cumbu	11.65^{g}	2.55^{k}	114.52^{i}	110.31^{f}
3	TNAU Sp3	Onampalayam	Coimbatore	11.0102 N, 76.8671 E	Cumbu	12.35^{c}	4.64^{e}	176.85^{c}	160.43^{c}
4	TNAU Sp4	Aandipatti	Theni	10.0015 N, 77.6164 E	Cumbu	12.19^{d}	5.87^{a}	167.89^{d}	159.08^{c}
5	TNAU Sp5	Usilampatti	Theni	9.9651 N, 77.7885 E	Cumbu	11.32^{h}	5.41^{b}	134.29^{g}	110.98^{g}
6	TNAU Sp6	Devadanampatti	Theni	10.1466 N, 77.6436 E	Cumbu	12.09^{e}	5.1^{c}	145.678^{f}	137.90^{d}
7	TNAU Sp7	Omalur	Salem	11.7428 N, 78.0473 E	Cumbu	12.54^{b}	4.66^{e}	187.90^{b}	176.41^{b}
8	TNAU Sp8	Poosaripalayam	Coimbatore	11.0062 N, 76.9333 E	*Commilina officinalis*	9.27^{l}	3.96^{i}	98.54^{j}	90.67^{j}
9	TNAU Sp9	Poosaripalayam	Coimbatore	11.0062 N, 76.9333 E	*Cyanodon dactyon*	10.82^{k}	4.14^{g}	76.94^{k}	56.25^{k}
10	TNAU Sp10	Millet Breeding Station –TNAU	Coimbatore	11.0205 N, 76.9269 E	Sorghum	12.92^{a}	4.03^{h}	198.04^{a}	184.19^{a}
11	TNAU Sp11	Sugarcane Research Centre	Cuddalore	11.7480 N, 79.7714 E	Sugarcane	11.16^{i}	3.34^{j}	132.87^{g}	98.76^{i}
12	TNAU Sp12	Vazhavachanur	Thiruvannamalai	12.0724 N, 78.9882 E	Groundnut	11.87^{f}	4.56^{f}	128.90^{h}	100.12^{h}

*Values are average of 50 pycnidia and pycnidiospore. *Means followed by a common letter are not significantly different at 5% level by DMRT.*Length of Conidia, CD=0.674, SEd = 0.2394 *Breadth of Conidia CD= 0.4323, SEd = 0.1527 *Length of the Pycnidia, CD= 1.942, SEd= 0.686 *Breadth of the Pycnidia, CD= 2.0965, SEd= 0.7438

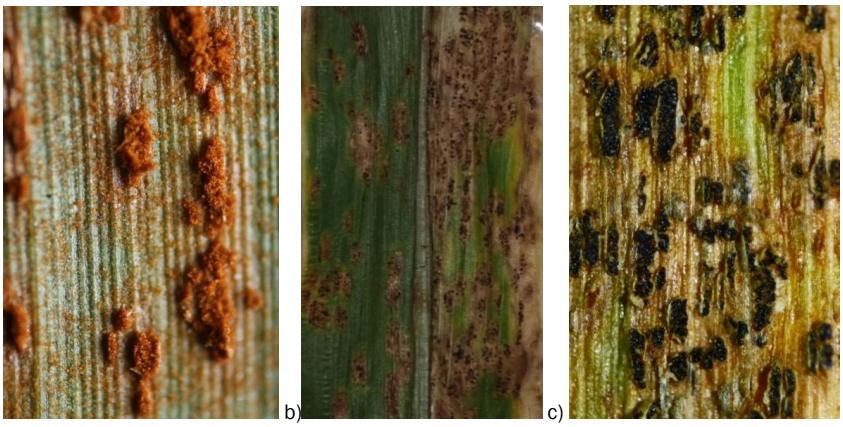
The pycnidia present in the leaf samples were black, spherical/globose with an average size range of 76.14-198.04 × 56.25-184.19 µm. The conidia was hyaline, double celled, single septate, with or without a gelatinous matrix at both the ends with an average size range of 9.27-12.92 × 2.55-5.87 µm (Table1).

Table 2. Studies on effect of *S.paraphysata* inoculation on rust spore germination and disease severity

S.No	Cultivar	Per cent uredospore germination(%)		Per cent Disease severity (%)	
		Control	Treatment with *S. paraphysata*	Control	Treatment with *S. paraphysata*
1	CO 7	99	23	80	10
2	CO 10	96	40	80	20
3	TNAU Cumbu hybrid CO 9	96	26	80	10

* Values are means of five replications T_{cal} = 11.214, T_{tab} = 4.302, Since $T_{cal} > T_{tab,}$ there is a siginificant difference between the treatments. *Control – Uredospores alone without *S. paraphysata* spray *Uredospores were sprayed three days before inoculation with *S. paraphysata*. *Average percent disease severity After treatment with *S. paraphysata* = 13.3 % *Average percent disease severity in control = 86.6%

Based on the morphological and conidial characters it was identified as *Sphaerellopsis* sp. (Kranz and Brandenburger,1981) who defined the conidia of *S. filum* as hyaline, double celled, single septate and pynidia as dark, globose with an ostiole at the top. The culture grown on V8 juice agar was initially



a) b) c)

Figure 1. Leaf rust symptoms on pearl millet parasitized by *S. paraphysata* a) Rust infected pearl millet leaves, b) Parasitized rust infection with *S. paraphysata* c) Pycnidia of *S. paraphysata* on cumbu rust infected leaves.

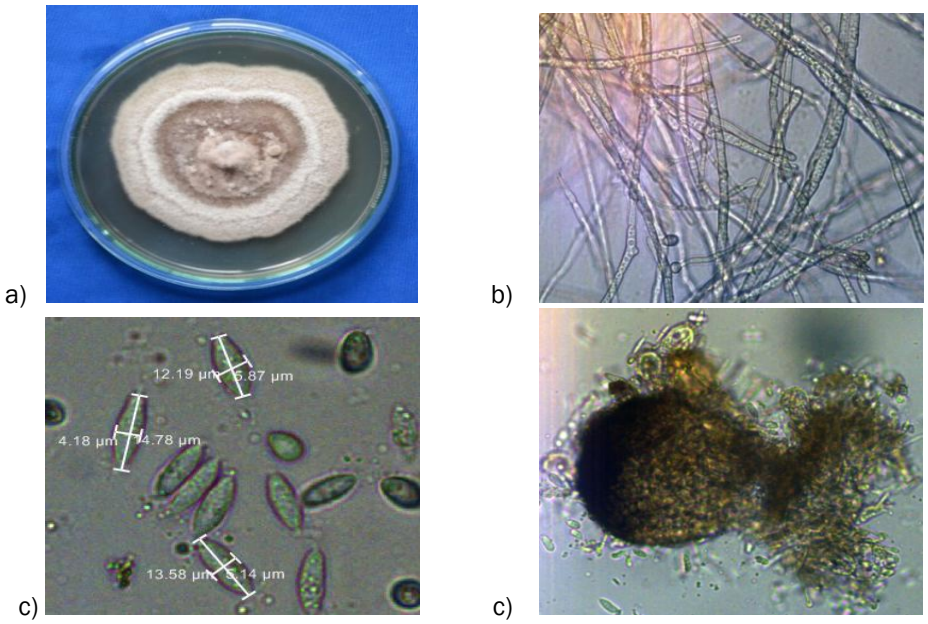


Figure 2. Cultural and spore characters of *S. paraphysata in vitro*
a) Colony of *S.paraphysata* on V8 juice agar b) Hyaline septate mycelia of *S.paraphysata* c) Conidia of *S. paraphysata* d) Pycndia of *S. paraphysata*

cottony, fluffy and white in colour but centre turns greyish on sporulation. The culture has a definite margin sometimes submerges in media, sporulation occurred on the third week of incubation. The mycelium was hyaline and septate. There was no morphological difference between TNAU Sp1 and

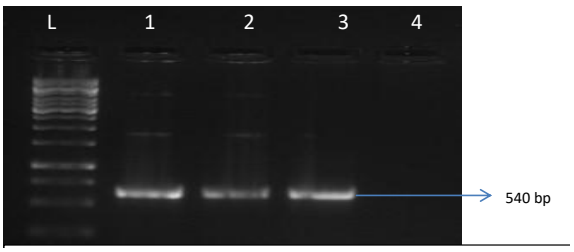


Figure 3. - PCR amplification of ITS region of *S. paraphysata*
L - Ladder 1Kb, 1 – TNAU-Sp1, 2- TNAU-Sp2, 3-Positive Control, 4-Negative control.
TNAU Sp2

The cultural characters were in accordance with Driessen *et al*. (2004) who reported that the developing colonies of *S. filum* in V8 juice Agar was white,cottony, with branched septate mycelium which grows both on the surface and submerged within the agar.

The ITS regions of *S. paraphysata* (TNAU Sp1 and TNAU Sp2) were amplified with the primer pair ITS1 and ITS4 for the initial confirmation and identification of the clear taxonomic position. The amplicon length of ITS1 and ITS4 region of *S. paraphysata* was 540bp (Fig.3.). The NCBI BLAST analysis showed that the isolated organism was *S. paraphysata* with 96% similarity and both isolates were deposited in NCBI databases with accession numbers *viz.*, MK863554 for TNAU-Sp1 and MK918510 for TNAU-Sp2. This was in accordance with Liesbach *et al.,* (2004) who reported that all the isolates of *Sphaerellopsis* spp. was amplified at 494 – 537bp.

Efficacy of mycoparasite against *Puccinia substriata* showed that S. *parphysata* reduced the spore germination by 76%, 56%, 70% under *in vitro* from the uredospores collected from the three cultivars CO 7, CO 10, TNAU Cumbu hybrid CO 9 respectively. The uredospores were sprayed three days before the application of conidia of *S. paraphysata*. The uredospores established after twelve days of inoculation and parasitization occurred after one week of application of *S. paraphysata* conidia. The disease severity of rust on the three cultivars ranged from 10 % to 20% with an average of 13.3%. In control, the severity was 80 % (Table 2,). This was in accordance with Yuan *et al,* (1999) who reported that *S. filum* reduced the spore production of Willow rust (*Melamspora epitea*) by 98%.

Sutton, (1980) has said that the efficacy of the hyperparasite depends upon its ability to degrade the rust sori and thus to stop the germination of uredospores. The morphological characters of *S. paraphysata* did not deviate much from that of *S. filum* on observation with SEM. The pycnidia was black, globose and 70-150 µm in diameter which was partially immersed on the rust spores (Fig.2). An ostiole was seen to be present at the top of the pycnidia through which the conidia gets exuded.The ultrastuctural examination using Scanning Electron Microscope proved hyperparasitic potential of *S.*

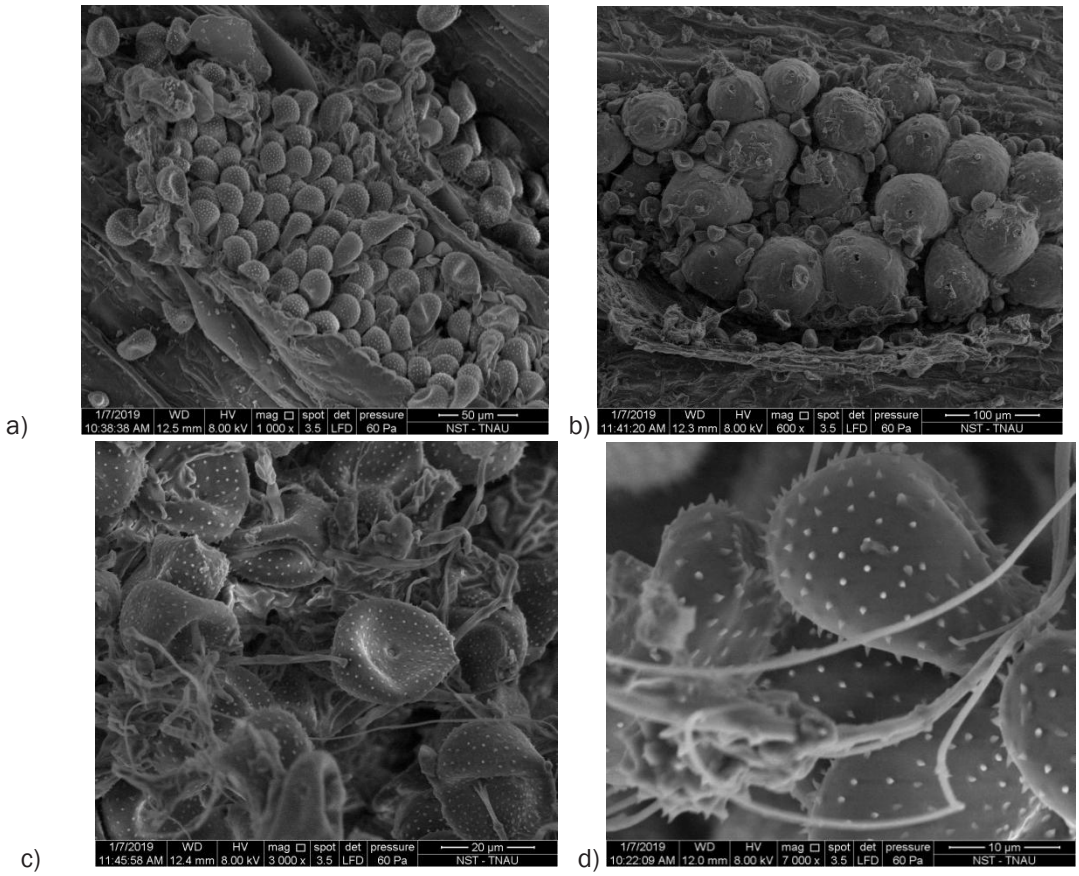


Figure 4. Scanning Electron Microscopic images showing parasitization of rust spores
a) Uredospores extruding from rust inoculated leaves b) Pycnidia partially immersed in rust uredospores c) Mycellia of *S. paraphysata* coiling around the uredospores d) Penetration of mycelia into the uredospores

paraphysata as most of the rust spores colonized by the mycelia were deformed and shrunken. In this study, the mycelium of *S. paraphysata* was found to coil and penetrate the uredospores of *P. substriata.* Special penetration structures such as appersoria was not observed (Fig. 4). The morphological observations indicated in the present study were similar to that of the prior description elaborated by Carling *et al.,* 1975, Plachecka, 2005, who observed that the hyphae of *S. filum* penetrating the *P. recondita* with its appressoria and those uredospores in contact with *S. filum* was seen disintegrated.It clearly indicated that *S. paraphysata* may be an efficient mycoparasite for rust fungi. Further, it could be grown on artificial media. This is an added advantage for this mycoparasite for mass production and commercial exploitation, as a biocontrol organism against rust fungi (Pei, 2003).

CONCLUSION

Morphological and molecular characters revealed that the mycoparasite is *S. paraphysata*. In glass house studies also there was a decrease in rust disease severity and uredospore germination on inoculation with the mycoparasite. Further SEM micrographs has revealed the intimate mycoparasitic association of *S. paraphysata* against *P. substriata*. Since *S. paraphysata* expressed effective mycoparasitism on uredospores of *Puccinia substriata* which enabled reduction in rust incidence, the organism can be mass multiplied and used as a component in IDM for rust disease management.

Abbreviations

ESEM – Environmental Scanning Electron Microscope
ITS – Internal Transcribed Spacer
IDM – Integrated Disease Management.
SEM – Scanning Electron Microscope
TNAU-Sp1 – *Sphaerellopsis paraphysata1*
TNAU-Sp2 – *Sphaerellopsis paraphysata2*
PCR- Polymerase Chain Reaction
SEM – Scanning Electron Microscope

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