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First report of scab of pomegranate (*Punica granatum* L.) caused by *Sphaceloma punicae* in India

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Abstract
Pomegranate is an important commercial fruit crop of India, it is affected by many diseases caused by various pathogens, resulted in poor quality and quantity of fruits. Survey was conducted in southern Karnataka and found that, the maximum PDI of scab was observed in Tipturu (15.56 PDI) followed by Kanakapura (13.10 PDI) and Arasikeri taluk (9.49 PDI). The fungus *S. punicae* exhibited maximum radial growth of 90.00 mm on Oat Meal Agar. At temperature of 30 ^{0}C with highest radial growth (79.33 mm) and dry mycelial weight (283.23 mg) was recorded and good sporulation at 25 ^{0}C was noticed. Optimum pH for the growth of pathogen is 6.5 to 7.

Keywords: Scab, pomegranate, *Sphaceloma punicae*

Introduction
Pomegranate is regarded as a fruit of paradise is one among the major fruit crops of arid zone. In India, it is regarded as a "vital cash crop", grown in an area of 1.93 lakh ha with the production of 17.73 lakh tons during 2014-15 (Anon., 2015) [1]. Among the different states growing pomegranate, Maharashtra is the largest producer occupying 2/3rd of total area in the country followed by Karnataka, Andhra Pradesh, Gujarat and Rajasthan. Karnataka accounts 19,040 ha area and 2.04 lakh tons production with an average productivity of 10.74 ha^{-1} in 2014-15 (Anon., 2015) [1].
Pomegranate has proned to many fungal and bacterial diseases *viz.*, aspergillus fruit rot, penicillium fruit rot, cercospora fruit rot, anthracnose, scab, wilt and bacterial blight. Among the fungal diseases, scab of pomegranate caused by *Sphaceloma punicae* which cause 10 to 15 per cent fruit loss during survey was conducted in southern Karnataka and also reduce the quality of fruits. Scab disease of pomegranate is one of the main factors for low productivity. Scab of pomegranate is spread at faster rate within the field of pomegranate during rainy season and reduce the quality of fruits which are unfit for marketing and also get lower prize.
The scab disease is spreading in pomegranate field and damage the crop. In recent years, there has been a major thrust on pesticide residue free organic pomegranate production. Taking the task into consideration, efficient botanicals and bio-agents need to be explored to fit into the management schedule and also there is a lack of information on management of scab of pomegranate through fungicides, but there is large number of chemicals available in the market as fungicides and their bio-efficacy and suitability was evaluated.

Material and Methods
A. Survey for occurrence and severity of pomegranate scab in southern Karnataka
The intensive survey was carried out during 2018-19 to know the incidence of scab disease of pomegranate in Kanakapura, Pavagada, Tipatur, Arasikere, Sira, Hassan, Kolar and Hosadurga talukas. In field, plants were selected in zigzag manner and the severity of scab disease of pomegranate was recorded by following 0 to 4 scale. Per cent Disease Index (PDI) was calculated by using following formula proposed by Wheeler (1969) [8].

$$\text{Percent disease index} = \frac{\text{Sum of the individual disease ratings}}{\text{Number of fruits}} \times \frac{100}{\text{Maximum disease grade}}$$

Disease scoring

Scale	Percent fruit infection
0	No infection
1	1-25% fruit area covered
2	26-50% fruit area covered
3	51-75% fruit area covered
4	>75% fruit area covered

B. Collection and isolation of the pathogen

The standard tissue isolation and single spore isolation technique was followed for isolation of scab pathogen from infected fruits collected during the time of survey which is described here.

The diseased fruits portions along with some healthy parts were cut into small pieces or bits and were washed with tap water. These bits of fruits were surface sterilized by using mercuric chloride (0.1%) solution for 15 seconds and then washed in sterilized distilled water for three times to remove the traces of mercuric chloride and later aseptically transferred to sterile petriplates with Potato Dextrose Agar (PDA). The plates were incubated at room temperature (27±1 ^{o}C) for about one week and observed for fungal growth and sporulation. Morphological identification of fungus was done with the aid of microscopic observation by considering specific keys *viz.*, septation of mycelium and spore character. The identified fungus was later transferred to sterile PDA slants and incubated at 27±1 ^{o}C for further use.

C. Proving the pathogenicity

The healthy young fruits of pomegranate plants were selected and washed thoroughly with tap water, and wiped using moist cotton swab. The inoculum suspension from 15 days old culture was prepared in potato dextrose broth with 1×10^{6} spores mL^{-1} and used for spraying. Branches were covered with polythene bags for 48 hr. to ensure successful penetration of the pathogen into the tissue. Similarly control plant without inoculation with test organism was sprayed with sterile distilled water for comparison.

The polythene bags removed after 2 days and observation were taken regularly for the appearance and development of symptoms. After appearance of disease symptoms re-isolation was made from the diseased tissue of artificially infected plants. The obtained culture was compared with original culture to confirm the identity of the fungus and subsequent confirmation of Koch's postulates.

D. Cultural characters of *S. punicae* on different solid and liquid media

The cultural characters of *S. punicae* studied on seven non synthetic/semi-synthetic and two synthetic solid and liquid media

Twenty mL of each sterilized and cooled medium as listed above was poured aseptically into sterilized Petriplates. Five mm disc of the *S. punicae* was taken from actively growing culture with the aid of cork borer and a disc was placed at the center of Petridish and then incubated at 27 ± 1 ^{o}C for 15 days. Each of this experiment was replicated thrice and observations regarding cultural characters such as the colour, diameter and pigmentation of colony was recorded.

E. Growth studies of *S. punicae* on different liquid media

The liquid media composition and preparation were same as that of solid media except that the agar was not added. All the liquid media were sterilized, cooled and to this disc of test fungus was inoculated separately under aseptic condition and then incubated at 27 ± 1 °C for 15 days. The mycelial mat was harvested by filtering through whatman number 42 filter paper and then dried and weighed. The best non-synthetic/ semisynthetic and synthetic media was found out and used for further studies.

Dry mycelial weight (mg) = Total weight of filter paper along with mycelia – Initial weight of filter paper

Physiological studies

Effect of temperature on the growth of *S. punicae*

The fungal growth was tested at 20, 25, 30 and 35 ^{o}C. For each treatment level, three replications were maintained. Twenty five mL of potato dextrose broth (PDB) was added into each of 100 mL conical flask and sterilized at 1.1 $kgcm^{-2}$ pressure for 20 minutes at 121 °C. The flasks were allowed to cool after sterilization. Later, the flasks were inoculated with 5 mm disc of fungus which was collected from 15 days old culture and incubated at respective temperatures. The mycelial mat was harvested by filtering through Whatman No. 42 filter paper of 9 cm diameter and dried. The dry mycelial weight was recorded and results were analysed statistically.

Effect of pH on the growth of *S. punicae*

The liquid medium used in this study was potato dextrose broth. Hydrogen ion (p^{H}) concentration of the media was determined by using p^{H} meter. Adjustment of p^{H} was done using 0.1 N alkali (Sodium hydroxide) or 0.1 N acid (Hydrochloric acid). Reaction of liquid media was adjusted to required p^{H} *viz*., 5.0, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0. Twenty five mL of the medium was added to 100 mL conical flask and sterilized at 1.1 $kgcm^{-2}$ pressure for 20 minutes at 121 °C. Each treatment was replicated thrice. To each flask, 5mm fungal disc was inoculated aseptically and incubated at 27 ± 1 ^{o}C for 12 days. The ideal p^{H} for growth of the fungus was determined by harvesting mycelial mat that was filtered through Whatman filter paper and dry mycelial weight (mg) was recorded.

Results

Survey for occurrence and severity of pomegranate scab in southern Karnataka

Roving survey was conducted during 2018-19 to assess the severity of scab of pomegranate in major growing areas *viz.*, Hassan, Tumakuru, Chitradurga, Ramanagara and Kolar. Totally 25 fields of 9 talukas and 5 districts were surveyed.

Severity of fruit scab on pomegranate

Severity of scab of pomegranate was recorded as per cent disease index (PDI) and was ranged from 4.24 to 15.56. Highest PDI of scab was recorded in Kamasamudra (15.56%) followed by Kanakapura (13.65 PDI) and least PDI observed in Masanahalli (4.24) of hiriyur taluk (Table 1). Among the different district surveyed, mean highest PDI was recorded in Ramanagara (13.10) followed by Tumakuru (10.35 PDI) and least PDI was recorded in Chitradurga (5.49) (Table 1).

Table 1: Survey for the severity of pomegranate scab in southern Karnataka

Sl. No.	Districts	Village	Variety grown	Per cent disease index of scab
1	Tumakuru	Kamasamudra	Bhagwa	15.56
		Hosahalli	Ganesh	12.25
		Yogenahalli	Bhagwa	7.98
		Nandikras	Kesar	11.00
		Garudinagara	Bhagwa	7.78
		Huliyar	Bhagwa	12.67
		Gommayya thanda	Bhagwa	7.24
		C. K. Halli	Ganesh	8.32
2	Hassan	Ramanahalli	Bhagwa	9.80
		R.N.Koppal	Bhagwa	9.18
		Thumbapura	Bhagwa	9.50
		Nadakihalli	Kesar	4.12
3	Chitradurga	Ramanjanhalli	Bhagwa	7.12
		Masanahalli	Bhagwa	4.10
		Babbur	Bhagwa	5.26
4	Ramanagara	Kanakapura	Bhagwa	12.56
		Kanakapura	Bhagwa	13.65
5	Kolar	Harabikothanahalli	Bhagwa	9.78
		Thattali	Ganesh	6.56

Symptoms on pomegranate fruits

Symptoms are initially appears as a rusty spots on fruits, it was turns to pinkish lesion on fruits when it was fully formed, scab lesions are raised and buff to pink coloured. Typical symptoms include swollen circular, ellipsoid or irregular spots (0·2–5·5 × 0·2–3·0 cm) which often enlarge and merge on fruits (Plate 1) which results fruits become unfit for the market. The disease spread is more under humid and moist conditions.

Plate 1: Symptoms of scab on pomegranate fruits

Morphological identification and Pathogenicity proving for pomegrante pathogen

Isolation of scab pathogen is difficult but it could be isolated on Host extract dextrose agar medium from pomegranate fruits which was showing typical scab symptoms by using standard tissue isolation method. And pure culture obtained on PDA medium by single spore isolation method.

Identification of the fungus was carried out based on the morphological characters. The fungus produced septate mycelium and conidiophores were single celled hyaline with smooth cylindrical branches. It produced conidia were 2.9–4.8 × 10.2–12.0 µm in size, ovoid to sub-cylindrical, unicellular and hyaline in nature (Plate 2).

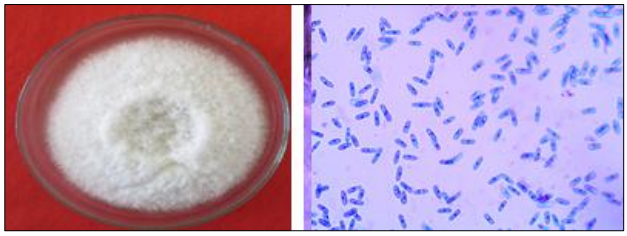


Plate 2: Microscopic observation septate mycelium and conidiophore bearing conidia

Pathogenicity

Fungus was isolated from infected pomegranate fruits and pure culture was obtained by single spore isolation method and used for pathogenecity test.

The pure culture of *S. punicae* was artificially inoculated to healthy pomegranate fruits by spraying of conidial suspension (10^6 conidia ml^{-1}). Initially, rusty spots on fruits was appeared seven days after inoculation and later on tenth day it was turned to pinkish lesion on fruits and on fifteenth day scab lesions are raised and buff to pink coloured. To prove the Koch's postulates, the pathogen was re-isolated from the infected fruits on PDA medium and the reisolated culture was found similar to the original culture (Plate 3).

Plate 3: Pathogenicity test of scab pathogen *S. punicae* on pomegranate fruit

Cultural characteristics of *S. punicae* on different solid media

Diversity in cultural characteristics of *S. punicae* was studied in seven non synthetic / semi synthetic and two synthetic media at room temperature 27 ± 1 °C (Table 2 and Plate 4).

The radial growth, colony characters and sporulation of the fungus were recorded when the maximum growth was attained on any one of the tested media. The effect of different culture media on the growth of fungi differed significantly. Maximum radial growth of *S. punicae* was recorded on

oatmeal agar (90.00 mm), followed by potato dextrose agar (86.43 mm), richards's agar (81.93 mm) and the least radial growth was recorded in rose bengal agar (43.36 mm).

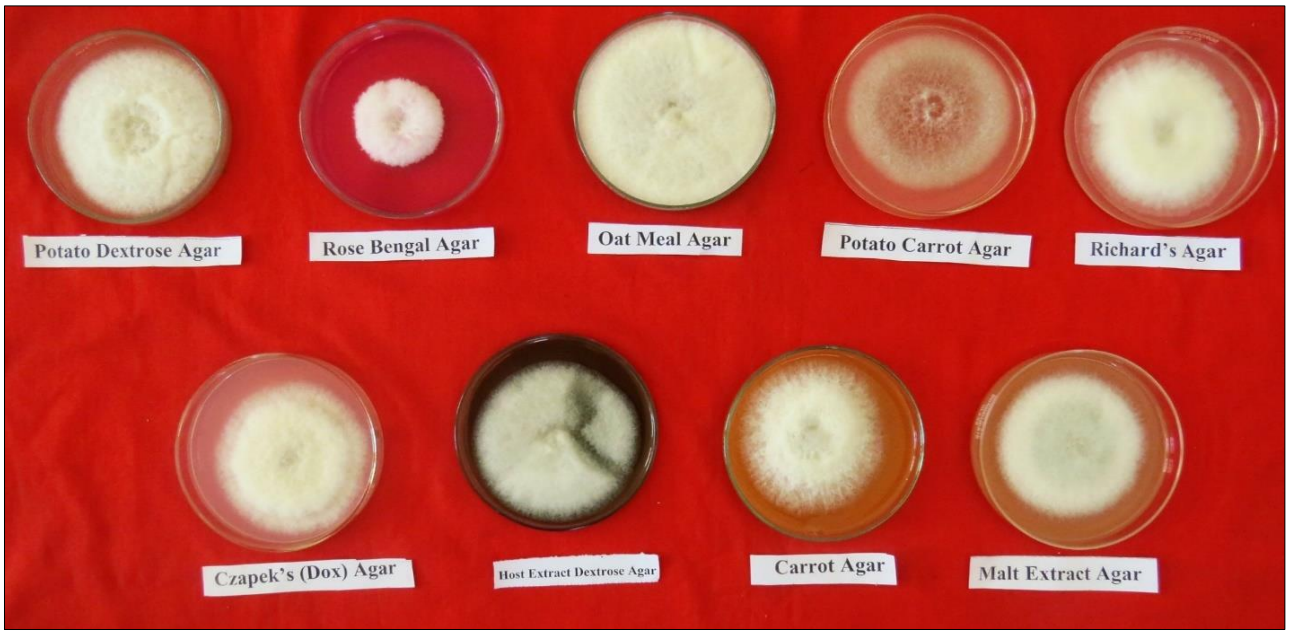


Plate 4: Growth of *S. punicae* on different solid media

Mycelial colour varied from greyish white to light brown on different media. The growth varied from flat, raised fluffy circular to sparse irregular. Pigmentation in the media varied from white, light grey to dark grey and light brown. Sporulation also showed greater variation in different media, ranging from good to poor. Good sporulation was recorded on host extract dextrose agar (6.62×10^3 ml^{-1}) followed by malt extract agar (5.83×10^3 ml^{-1}) and poor sporulation was recorded on Czapek Dox agar (1.36×10^3 ml^{-1})

Table 2: Effect of different solid media on the growth of *S. punicae*

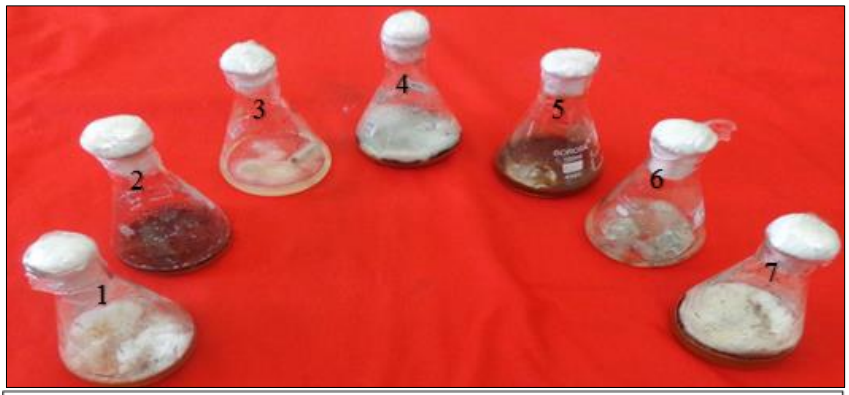
Sl. No.	Different Media	Radial growth (mm)	Type of growth	Colour	Pigmentation	Average no. of conidia ml^{-1}	Rating
	Non synthetic / Semi synthetic media						
1.	Potato Carrot Agar	79.17	Flat and circular	White with light brown	Light brown	3.90×10^3	+++
2.	Potato dextrose agar	86.43	Fluffy raised and circular	Greyish white	Dirty whitish to grey	5.73×10^3	++
3.	Rose Bengal Agar	43.36	Slightly raised and circular	White to grey	No pigmentation	3.50×10^3	++
4.	Oat Meal Agar	90.00	Sparsely raised and circular	Light grey	Light grey	5.48×10^3	++++
5.	Host Extract Dextrose Agar	78.26	Flat and circular	Greyish	Dark grey	6.62×10^3	+++
6.	Carrot Agar	71.13	Fluffy and irregular	Light grey	Reddish grey	5.10×10^3	+++
7.	Malt Extract Agar	79.20	Fluffy and irregular	Creamy grey	Light brown	5.83×10^3	++
	Synthetic media						
8.	Czapek Dox Agar	72.86	Fluffy raised and irregular	Creamy white	White	1.36×10^3	+
9.	Richards's Agar	81.93	Fluffy raised and irregular	Creamy white	White	3.73×10^3	++
	S. Em. ±	1.82					
	C.D. at 1%	3.50					

Mean of three replications
Sporulation: ++++ = Excellent > 9.0×10^3 +++ = Good > 4.0×10^3 to 9.0×10^3
++ = Moderate > 1.5×10^3 to 4.0×10^3 + = Poor < 1.5×10^3

Effect of different liquid media on growth of *S. punicae*

In present investigation, two synthetic and five non synthetic / semi synthetic broth were tested as described in "Material and Methods".

There was a significant difference among the different liquid media for growth of *S. punicae*. The maximum dry mycelial weight of *S. punicae* was observed in potato dextrose broth (362.00 mg) and found to be significantly superior over other nutrient broths, followed by potato carrot broth (323.67 mg) and least dry mycelial weight of *S. punicae* was recorded in Richards's broth (214.00 mg). Between synthetic and non / semi synthetic media, maximum growth was recorded in non / semi synthetic media (Table 3 and Plate 5).



1) Potato dextrose broth 2) Malt extract broth 3) Richard's broth
4) Carrot broth 5) Host extract dextrose broth 6) Czapek Dox broth
7) Potato carrot broth

Plate 5: Growth of *S. punicae* on different liquid media

Table 3: Effect of different liquid media on growth of *S. punicae*

Sl. No	Broth	Dry mycelial weight (mg)#
Non synthetic / Semi synthetic media		
1	Potato dextrose broth	362.00
2	Malt extract broth	274.33
3	Carrot broth	295.00
4	Host extract dextrose broth	243.00
5	Potato carrot broth	323.67
Synthetic media		
6	Czapek Dox broth	279.00
7	Richards's broth	214.00
S. Em. ±1%		1.64
CD at 1%		6.91

Mean of three replications

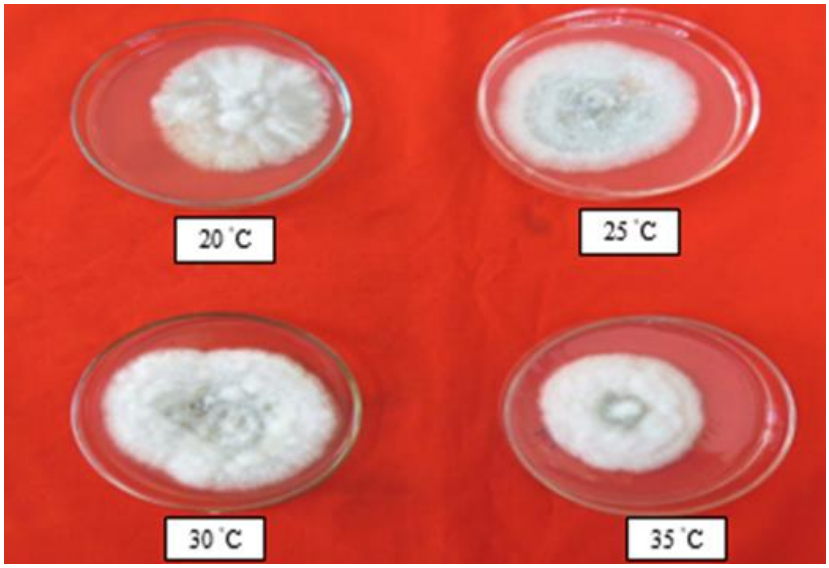


Plate 6a: Effect of different temperature on the growth of *S. punicae* on PDA

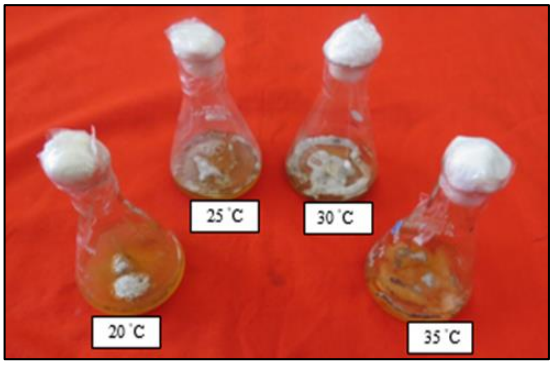


Plate 6b: Effect of different temperature on the growth of *S. punicae* on PDB

Effect of temperature on *S. punicae* on solid media

The fungus was grown on potato dextrose agar at four temperature levels *viz*., 20, 25, 30, and 35 ^{0}C to known the optimum temperature required for maximum radial growth and sporulation. As temperature increased from 20 to 30 ^{0}C the growth of *S. punicae* on PDA gradually increased and later declined with further increase in temperature. The growth differences at all the temperatures were statistically significant from each other. The maximum mycelial growth was recorded at 30 °C (79.33 mm) which was significantly superior over other temperatures tested and the least radial growth was recorded at 35 °C temperature (59.36 mm). Good sporulation was observed at 20 °C and 25 ºC and moderate sporulation was observed at 30 ˚C and 35 ˚C. Greyish white mycelial colour was observed at 20 °C, grey mycelial colour was observed at 25 ˚C and white mycelial was observed at 30 ˚C and 35 ˚C and type of mycelial growth was flat, fluffy raised, slightly raised and raised observed at 20, 25, 30, and 35 ˚C respectively (Table 4 and Plate 6a).

Effect of temperature on growth of *S. punicae* on liquid media

The pathogen *S. punicae* was grown on potato dextrose broth at four different temperature levels *viz*., 20 ^{0}C, 25 ^{0}C, 30 ^{0}C and 35 ^{0}C to know the optimum temperature required for maximum dry mycelial weight. The results obtained are presented in (Table 4, Fig. 5 and Plate 6b). The temperature of 30 ^{0}C was significantly superior to other temperature levels by recording the maximum dry mycelial weight (283.23 mg) followed by 25 ^{0}C (212.00 mg) and least dry mycelial weight was recorded at 35 ^{0}C (112.03 mg) (Table 4 and Plate 6b).

Effect of pH on the growth of *S. punicae* on solid media

The study was carried out to know optimum pH required for the radial growth and sporulation of *S. punicae* on solid media. The fungus was grown on potato dextrose agar at seven different pH levels *viz*., 5.0, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0.

The significant difference was observed between different pH levels for radial growth of fungus on PDA media. The highest radial growth (87.23 mm) was observed at pH 6.5, followed by pH 6.0 (75.47 mm). The least radial growth (55.79 mm) was observed at pH 5.0 (Table 5 and Plate 7a).

Table 4: Effect of temperature on growth and sporulation of *S. punicae* in liquid and on solid media

Sl. No	Temperature (ºC)	On Potato dextrose broth	On Potato dextrose agar				
		Dry mycelia weight (mg)#	Radial growth (mm)#	Mycelial colour	Type of growth	Average no. of conidia ml^{-1}	Rating
1.	20	120.60	65.33	Greyish white	Flat	5×10^3	+++
2.	25	212.00	72.03	Grey	Fluffy raised	6.5×10^3	+++
3.	30	283.23	79.33	White	Slightly raised	3×10^3	++
4.	35	112.03	59.36	white	Raised	3.5×10^3	++
S.Em ±		1.33	5.43				
CD at 1%		3.49	7.058				

Mean of three replications

Sporulation

++++ = Excellent > 9.0×10^3
+++ = Good > 4.0×10^3 to 9.0×10^3
++ = Moderate > 1.5×10^3 to 4.0×10^3
+ = Poor < 1.5×10^3

Effect of pH on the growth of *S. punicae* on liquid media

The experiment was conducted to know the optimum pH requirement for the growth of *S. punicae* at different pH range *viz.*, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0 on liquid media.

The significant difference was observed between different pH levels for growth of fungus on liquid media. The maximum dry mycelial weight (359.67 mg) was observed at pH 7.0 followed by pH 6.5 (289.00 mg). The least dry mycelial weight (201.00 mg) was recorded at pH 5.0 (Table 5 and Plate 7b).

Table 5: Effect of hydrogen ion concentration (pH) on growth of *S. punicae* in liquid and solid media

Sl. No.	pH level	On Potato dextrose broth	On Potato dextrose agar
		Dry mycelial weight (mg)#	Radial growth (mm)#
1	5.0	201.00	55.79
2	5.5	231.00	70.43
3	6.0	249.69	75.47
4	6.5	289.00	87.23
5	7.0	359.67	72.17
6	7.5	241.00	71.59
7	8.0	210.33	70.87
	S.Em ±	0.72	0.64
	CD at 1%	3.04	2.73

Mean of three replications

Plate 7a: Effect of different hydrogen ion concentration (pH) on growth of *S. punicae* on solid media

Plate 7b: Effect of different hydrogen ion concentration (pH) on growth of *S. punicae* on liquid media

Discussion

Roving survey was conducted during 2018-19 to assess the severity of scab of pomegranate in major growing areas and Survey was conducted in southern Karnataka and found that, the maximum PDI of scab was observed in Tipturu (15.56) followed by Kanakapura (13.10) and Arasikeri taluk (9.49 PDI). sympotms obsereved as appears as a rusty spots on fruits, it was turns to pinkish lesion on fruits when it was fully formed, scab lesions are raised and buff to pink coloured. Typical symptoms include swollen circular, ellipsoid or irregular spots, resulted in poor quality and quantity of fruit (Xiao-Hui *et al.* (2004) [8] and reported that *S. punicae* was identified from scab-like lesions from fruit in China and also Jamadar *et al.*, (2011) [5] observed rusty spots on fruit and leaves in India. Jadhav and sharma (2011) [4] reported that symptoms of the pomegranate scab on leaves, fruits and stems of pomegranate).

The fungus *S. punicae* exhibited maximum radial growth of 90.00 mm on Oat Meal Agar (Results are in accordance with Hui-xiang *et al*. (2005) [3]. At temperature of 30 ˚C with highest radial growth (79.33 mm) and dry mycelial weight (283.23 mg) (Results are in accordance with Nechet *et al*, (2004) [6] was recorded and good sporulation at 25 ˚C was noticed. Optimum pH for the growth of pathogen is 6.5 to 7(Similar results were observed by Fothergill and Ashcroft (1955) [2].

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