Antifungal potential of macro-algae against *Alternariasolani* causing early leaf blight in tomto(*Solanum lycopersicum* L.)

Comment [HS1]: Spelling Correction "Tomato"

ABSTRACT

This study aimed to investigate the occurrence and severity of early blight disease in major tomato growing areas of Tamil Nadu, India. The percent disease incidence ranged from 5.44%-25.05% across the surveyed villages, with the highest incidence recorded in KonguThirupathivillage of Coimbatore district (25.05%), followed by shoolagirivillage in Krishnagiridistrict (21.09%), and the lowest incidence in Sikkampatti village of Salem district (5.44%). The isolated pathogen exhibited characteristics such as branched septate mycelium, spores are elongated, muriform, beaked.The color variations, ranging from grey to ashy grey, whitish grey, and blackish gre. Pathogenicity tests were conducted on eight different isolates of *Alternaria solani viz.,* TA-1,TA-2,TA-3,TA-4,TA-5,TA-6,TA-7,TA-8. Among themTA-1 demonstrating the highest virulence (95.75% disease incidence) followed by TA-3 (90.01%). Furthermore, seaweed extracts were prepared using different solvents (methanol, ethyl acetate and hexane) and evaluated for their antifungal activity against the pathogen. *In vitro* screening of *Kappaphycus alvarezii* exhibited the highest inhibition of mycelial growth (76.00%) under aseptic conditions. This study provides valuable insights into the prevalence of early blight disease in tomato crops and highlights the potential of seaweed extracts as a natural antifungal agent.

Keywords:*Tomato, early blight disease, seaweeds, organic solvents, antifungal activity*

Comment [HS2]: Write in alphabetical order

1.INTRODUCTION

A member of the Solanaceae family, the tomato (*Lycopersicon esculentum* Mill) is one of the world's most widely grown and highly profitable vegetables. The vegetable has been consumed the second most after potatoes [1]. Because of their many colours, shapes, sizes, and tastes, tomatoes are enjoyed in many situations and can be eaten raw or cooked [2].It is cultivated because of its edible fruits, which may be eaten raw or cooked and are rich in minerals and vitamins A, B, and C. Plantdisease caused by bacteria, fungi, and viruses can severely affect tomato crops, which leads to reduced fruit yield and quality.

Early blight of tomato caused by *Alternaria solani* is one of the main diseases that affects tomatoes resulting in major yield losses. It infects many parts of the plant, like leaves, roots, seeds and destroyed up to 79% of the plants in the worldwide[3]. Fungal diseases are often controlled with chemical fungicides, but these chemicals are hazardous toenvironment, harm beneficial microorganism and make plants resistant to the fungicide.The development of alternative control

strategies, such as the application of bio-stimulants like seaweed extracts, is increasingly essential for the efficient management of diseases. Recent years have observed an increase in interest forcroalgae due to their antifungal, antibacterial, antiviral, antioxidant, anti-inflammatory, cytotoxic, and antimitotic properties [4]. According to reference [5], seaweeds are rich in antifungal compounds such phenolic compounds and terpenes, that promote plant growth and productivity by inhibiting the growth of diseases.Therefore, our present study the use of seaweed products as a natural and eco-friendly way of combating *A.solani* under *invitro* conditions.

2. MATERIALS AND METHODS

2.1 Survey on disease incidence of early blightof tomato

Early blight-infected leaf samples have been collected from major tomato-growing regions of TamilNadu Coimbatore, Dharmapuri, Krishnagiri, Theni, Salem, and Madurai. The purified cultures have been preserved on PDA slants and used for further study. The precent disease incidence was determined using Vidhyaksekaran's method [6].

$$\text{PDI \%} = \frac{\text{No of Plants Infected}}{\text{Total number Plants observed}} \text{x } 100$$

2.2 Symptoms

According to reference [7], the disease's initial manifestations were small, black necrotic lesions on the older leaves that eventually spread upward. As the lesions develop, they usually become larger and resemble target boards with concentric rings often surrounded by a yellow zone and the whole plant defoliates. Lesions on young tomato seedlings can entirely girdle the stem, resulting in "collar rot," which can cause reduced plant vigor or death[8] (Fig.2).

2.3 Isolation of Early blight pathogen

The infected plant samples taken from major tomato growing districts of Tamil Nadu, based on the symptoms caused by early blight. The affected specimens will be used to isolate the pathogen. A sterile scalpel will be used to cut the diseased tissue from the infected plant, along with some healthy tissue, into small pieces (5 mm diameter). The plant exhibiting typical symptoms will then be rinsed three times with sterilized water and disinfected with 1% sodium hypochlorite solution for a minute. The infected samples will be placed aseptically on potato dextrose agar (PDA) medium, and the plates will be incubated at room temperature in the invert position. After seven days of incubation, the fungal growth transferred aseptically to PDA slants and purified following Single Spore Technique [9].

2.4 Pathogenicity Test

Earthen pots of 30 cm diameter were filled with sterilizedpottingmixtureandplacedinglasshouse.Thepottingmixtureconsistsofredsoil+sand+FYM(2:1:1) wassterilized in an autoclave at 121 °C at 15 psi for 2 hrs for twoconsecutive days. For testing the

pathogenicity, the tomato cultivar PKM 1 was used. Three tomato seeds per pot were planted in the PKM1 variety. The pathogenicity of eight distinct *A.solani* isolates (TA-1, TA-2, TA-3, TA-4, TA-5, TA-6, TA-7, and TA-8) was examined under potculture condition. Thirty to forty-five days after seeding, the tomato plants were sprayed with a spore solution that contained $2.5x10^6$ spores/ml. The plants started to show symptoms of early blight. The inoculated plants were re-isolated if thesymptoms were similar to those from the infected field. The re-isolated cultures again inoculated to PKM-1 cultivar and observe the characteristics symptoms.Control pots are sprayed only with sterile distilled water [10].

2.5 Molecular Characterisation

2.5.1 PCRamplificationoftheInternalTranscribedSpacer(ITS) regionof the rDNA

CTABmethodwasusedtoextracttheDNA,afterthepathogenic isolates had been cultured in potato dextrose brothfor15days.AmplificationofITSregionsinisolateswascarried out by using a universal primer ITS1 and ITS4. PCRwasperformedwithareactionvolumeof10µlandthereactioncycleconsistedof60secondsat94°Cford enaturation, 45 seconds at 53 °C for annealing, and 90 sec at72°Cforextension [11]. ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3')ITS4(5'-TCCTCCGCTTATTGATATGC-3').

2.5.2 Agarosegel electrophoresis

Based on the procedure outlined by reference [12], agarose gel electrophoresis was carried out to assess theDNA's purity and to separate the products of the polymerasechain reaction. The gel was photographed and analysed usingan ultraviolet transilluminator. The PCR products sizes wereevaluatedbycomparingthemtoastandard1kbladder(BangaloreGenei Pvt. Ltd.,Bangalore, India).

2.6Collection of Seaweeds

Different kinds of seaweed were carefully chosen from the deep-water regions of Rameswaram, Tamil Nadu. To remove any debris or contaminants, the seaweed samples were carefully cleaned twice: once with fresh water and once with distilled water. The seaweed samples were then allowed to air dry for two or three weeks under shade after being carefully wiped to eliminate any remaining moisture. Following the process of drying, the seaweed samples were ground into a powder and kept in an roomtemperature with dry conditions and an average temperature between 28 and 37 degrees Celsius [13]. The seaweed extracts were made using a variety of solvents, including hexane, ethyl acetate, and methanol to assess their antifungal activity.

2.7Preparation of Seaweed Extract through Soxhlet Apparatus

Twenty grams of partly mixed seaweed powder were put into a Soxhlet instrument to make the seaweed extract. This seaweed powder was encased in cellulose thimble paper and refluxed for 12 hours with 150 milliliters of the solvents *viz.,*ethyl acetate, methanol, acetone and hexane. After extraction, the solvent was filtered using whatsman No. 1 filter paper to get rid of any impurities. A rotary evaporator operating at 40 °C and 45 revolutions per minute was used to evaporate the

collected solution until all of the solvent had evaporated [14]. After being diluted with the appropriate solvents, the final extract was kept for future use at -4 °C[15].

2.8Antifungal Activity of Seaweeds against *A.solani*

(Poisson food Technique)

Poissoned food technique was used to test antifungal properties of seaweed extracts. Different seaweed extracts (1%, 3%, and 5%) were incorporated into potato dextrose agar medium, which was then autoclaved and transferred into sterile petriplates. Using a sterile cork borer, discs of 5 mm in diameter were cut from the periphery of an *A. solani* culture which was 5 days old. The discs were then aseptically placed onto PDA plates poisoned with seaweed extracts. As a control, the medium was used without adding the extract. Following 48, 72, and 96 hours of incubation, the colony diameter of the infected plates was measured and recorded at 25 °C. For every treatment, three plates per replication were maintained. The average colony diameter was measured after the experiment was conducted twice. The percentage inhibition (PI) of mycelial growth was calculated using the formula suggested by Pandey*et al*.[16].

$$PI = \frac{Dc - Dt}{Dc} \times 100$$

Where,

PI = Percent Inhibition

C= Average diameter of fungal growth (cm) in control,

T= Average diameter of fungal growth (cm) in treatment.

2.8.1 Different concentrations of promising seaweeds extract against the *A.solani*

The different concentrations of methanol extract of *S. wightii* and *K. alvarezii* were prepared from the stock solution (1%, 3% and 5%) by poisson food method.

2.9Statistical Analysis

To evaluate the mean differences among the treatments, an analysis of variance (ANOVA) was conducted and Duncan's Multiple Range Test at a significance level of 5% was employed [17].

3. RESULTS AND DISCUSSION

3.1 Survey

Roving method of survey conducted in major tomato growing areas of Tamil Nadu cultivating tomato.Kongu Thirupathi, Viraliyur, Kambainallur, Vadakarai,

Krishnagiri, Dharmapuri,Salem, Perambalur,Madurai and kinnathukadavu are the places surveyed. Samples were taken from plants having early blight symptoms. The results showed that the percentages of disease occurrence varied from 5.44% to 25.05%.KonguThirupathi village in Coimbatore showed the highest disease incidence of 25.05%, followed by shoolagiri village in Krishnagiri district, which showed21.09%. However, the lowest disease incidence percentage of 5.44% was recorded in Salem district (Table 1 and Fig.1).

3.2 Isolation of tomato early blight

The isolated pathogen cultures in the current investigation began initiallywhite but eventually developed to a greyish-white color with cottony growth on PDA medium. The single hyphal tip approach was used to purify the cultures in order to maintain pure cultures. The disease took around 10 to 12 days to cover the entire 9 cm plate. As shown in Fig. 3, the pure cultures were frequently sub-cultured and kept in the refrigerator to ensure the purity of the isolate.Mycelium are septate, branched and have dark color, whereas spores are elongated, muriform, beaked, and septate. The margin of colonies was either uneven or smooth. These results were in consistent with the initial observations of the genus Alternaria infecting crops published[18]. Colony traits of *Alternaria* exhibit color variations, ranging from grey to ashy grey, whitish grey,blackish grey and mycelial growth was observed flat or elevated, colony texture varied from velvety to rough, and colonies were seen to be smooth or uneven [19].

3.3 Pathogenicity

The pure culture of *A.solani* was obtained by single spore isolation method and sub culture was used for pathogenicity experiment by following Koch's postulate. The pathogenicity experiment was carried by pre-inoculation with spore suspension and homogenized mycelial bits of *A. solani* on foliage of 30 days old plants of PKM 1 cultivar of tomato. After inoculation, the symptoms appeared on inoculated leaves as brown,oval or circular necrotic spots with concentric rings and surrounded by a border of yellow host tissue shown in (Fig.4). The fungus was re-isolated and purified culture from these artificially infected leaves was similar to that of original culture. Thus, pathogenicity on tomato early blight was confirmed.

Among the isolates,TA-1 demonstrated the highest virulence, resulting in a disease incidence of 96.14%. It was closely followed by TA-4, which exhibited a disease incidence of 90.12%. On the other hand, isolate TA-8 displayed the lowest disease incidence, with a rate of 35.65%. Based on these findings, it was determined that the TA-1 isolate of *A.solani* was the most virulent among the tested isolates, warranting further investigation (Fig.5).

3.4Molecular Characterization of pathogen

To identify *Alternaria* spp. by sequencing, Polymerase Chain Reaction was performed for eight isolates using universal primers such as ITS1 and ITS4. The amplified ITS-DNA sequence was run in Agarose gel and ITS-DNA sequence of 560 bp was viewed using gel documentation system shown in (Fig.6) .

Further, the amplified ITS PCR product was sequenced by Sanger sequencing. The ITS sequences of the pathogen were BLAST searched at NCBI database and the ITS sequences of pathogen showed 98% homology to that of *A. solani* and obtained sequences were entered into GenBank database to get accession number(PP724524).

3.5*Invitro* Screening of Seaweeds against the pathogen *A.solani*

The present study aimed to investigate the antifungal activity of seaweed extracts, specifically bio-stimulants, against the tomato early blight pathogen *A. solani*. Different solvent extracts, namely methanol, ethyl acetate, and hexane were obtained from five seaweed species, including *Sargassum wightii, Kappaphycus alvarezii, Gracilaria edulis, Caulerpa racemosa,* and *Ulva lactuca* (Fig.7). These extracts were then analysed for their antifungal potential. Notably, the results revealed a novel finding in the field of natural antifungals.

Among the tested solvents the methonalic extract showed the highest reduction percetange compared to others. Among the different seaweed extracts, *Kappaphycus alvarezii* exhibited an unprecedented and remarkable reduction in mycelial growth, with a size of only 2.61 cm under aseptic conditions. This dramatic inhibition translated to a maximum percent reduction over the control, reaching an impressive 76.00%. This significant antifungal activity of *Kappaphycus alvarezii*showcases its untapped potential as a potent source of natural compounds for combating early blight of tomato. Furthermore, *Sargassum wightii* demonstrated a noteworthy second-highest percent reduction in mycelial growth, with a size of 2.66 cm and a reduction of 71.10%. This finding highlights the promising antifungal properties of *Kappaphycus alvarezii*, which can potentially contribute to the development of alternative strategies to manage tomato early blight. The *Gracilariaedulis*, although exhibits lower percent reduction over the control at44%, still displayed some degree of antifungal activity (Table.2, Fig.8,Fig. 9 and Fig. 10). This suggests the presence of unique bioactive compounds within that may contribute to the overall antifungal potential of seaweed extracts. Overall, the novel findings of this study shed light on the previously unexplored antifungal activity of seaweed extracts against *A. solani*. The remarkable inhibitory effect of *Kappaphycus alvarezii* and the promising results obtained from *Sargassum wightii* emphasize the potential of these seaweed species as valuable sources of natural antifungal compounds. These findings open new avenues for further research and the development of eco-friendly alternatives for managing early blight of tomato.

Table 1: Disease incidence of early blight disease in major tomato growing areas of Tamil Nadu

S.No	Place of collection	District	Isolates	Stageofthecrop	Geocoordinates		Early blight incidence(%)
					Latitude	Longitude	
1.	Kongu Thirupathi	Coimbatore	TA1	Flowering and early fruit formation	11.0017262º	76.8140771º	25.05[a]
2.	Viraliyur		TA2	Flowering and early fruit formation	11.0102502º	76.8021622º	14.06[c]
3.	Shoolagiri	Krishnagiri	TA3	Flowering stage	10.8172022º	76.9843839º	21.09[b]
4.	Kambainaallur	Dharmapuri	TA4	Vegetative stage	12.218872º	78.320145º	10.07[e]
5.	Puliyur		TA5	Flowering stage	12.219437º	78.322255º	8.09[f]
6.	Aviyur	Madurai	TA6	Early fruit formation stage	9.91311º	77.98699º	12.70[d]
7.	Sikkampatti	Salem	TA7	Flowering and early fruit formation stage	11.563942º	78.148933º	5.44[g]
8.	Koneripalayam	Perambalur	TA8	Vegetative stage	11.26138º	78.858461º	7.08[h]
						SEd =	**0.0857**
						CD (0.05) =	**0.1817**

Table2: *Invitro*screeningofdifferentsolventsofdifferentseaweedextractagainst*A.solani*(TA-1)

Different seaweeds	1%		3%		5%	
	Radial growth*(cm)	PIOC (%)	Radial growth*(cm)	PIOC (%)	Radial growth*(cm)	PIOC (%)
Sargassumwightii	7.13	20.00[b]	5.10	43.33[b]	2.66	71.10[b]
Kappaphycusalvarezii	6.76	25.50[a]	4.20	53.33[a]	2.16	76.00[a]
Ulva lactuca	7.40	17.70[c]	5.46	39.33[c]	3.16	65.5[c]
Caulerpa racemosa	7.50	16.66[d]	5.50	38.88[d]	3.43	62.22[d]
Gracilariaedulis	8.03	10.70[e]	6.23	30.77[e]	5.05	44.4[e]
Control	9.00	0	9.00	0	9.00	0
CD(P=0.05%)	**0.269**	**0.433**	**0.289**	**0.417**	**0.257**	**0.269**

*Mean of three replications

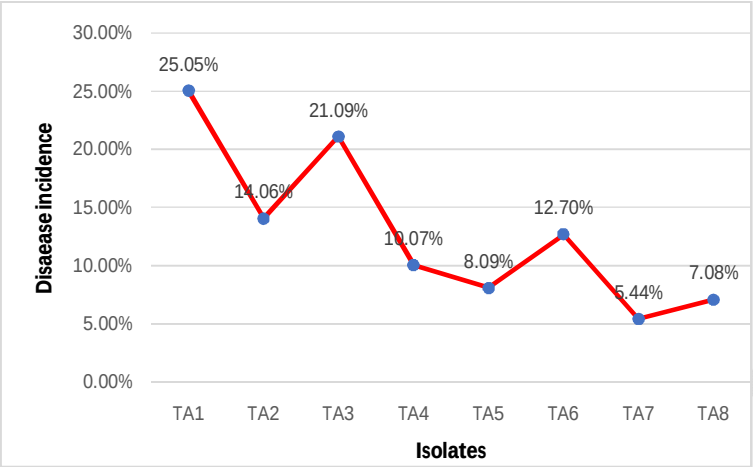

Fig. 1.Occurence of early blight from TamilNadu

Fig.2. Symptomsof Early blight

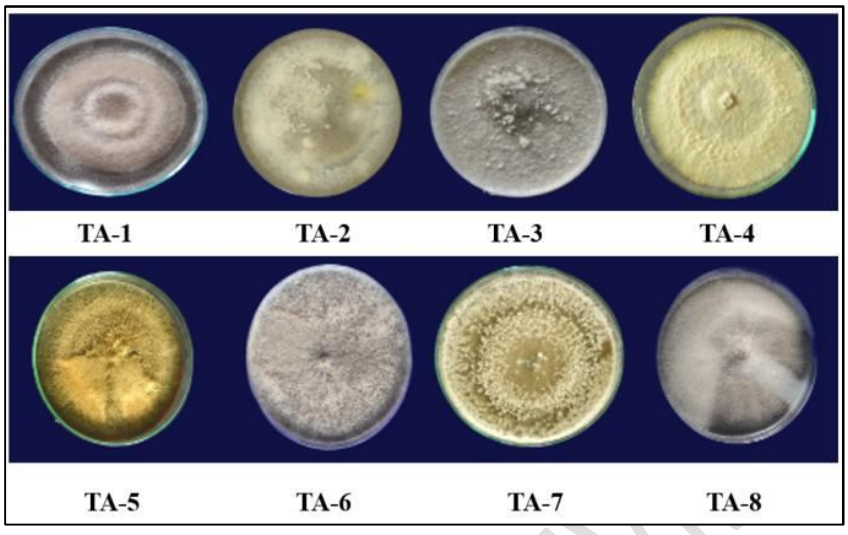

Fig.3. Differentisolatesof *A. solani*

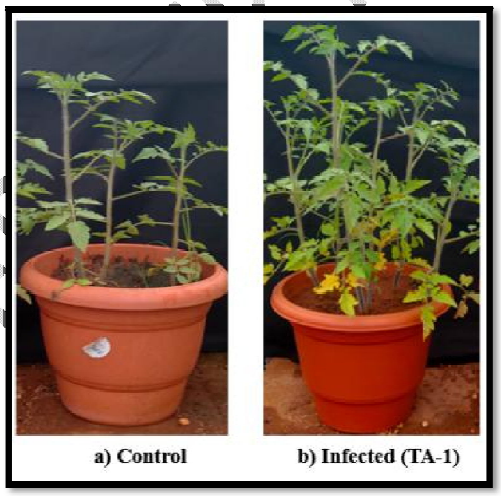

Fig.4. Pathogenicity test under glass house experiment

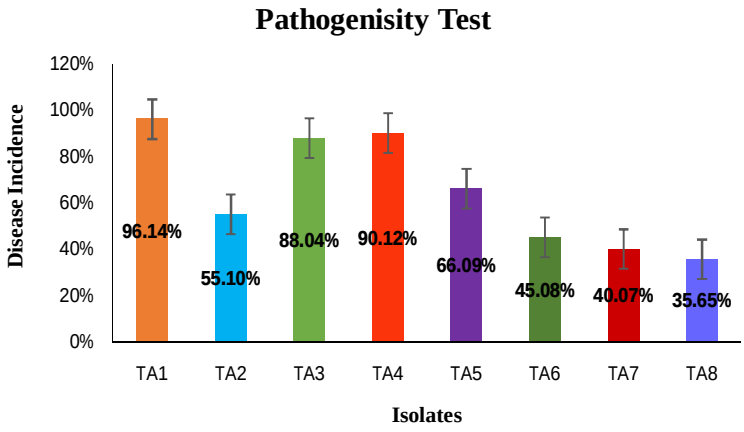

Fig.5. Evaluating the virulence of different isolates of *A.solani*

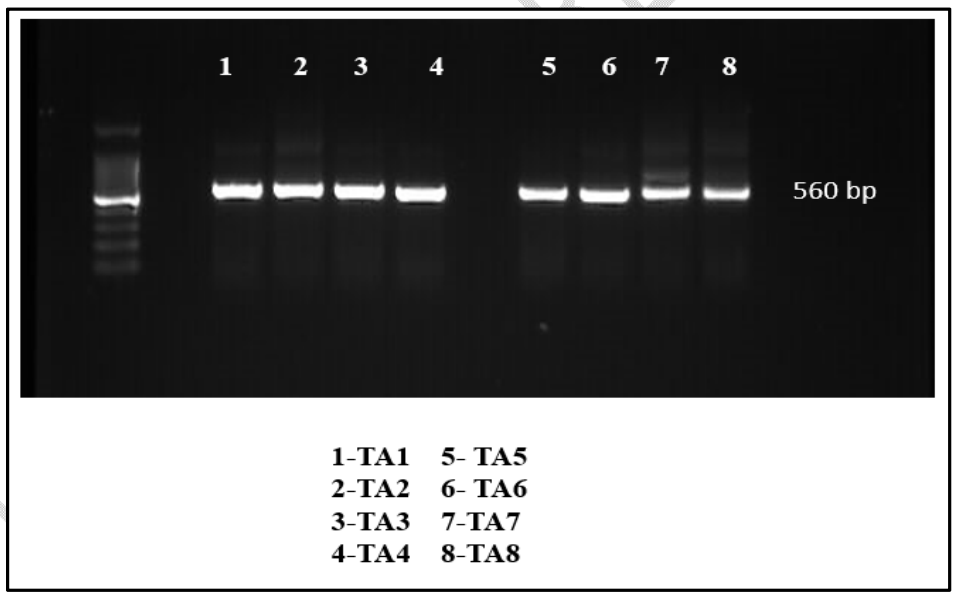

Fig.6. Gel Documentation of various isolates of *A.solani*

Fig.7. Collectionof seaweeds

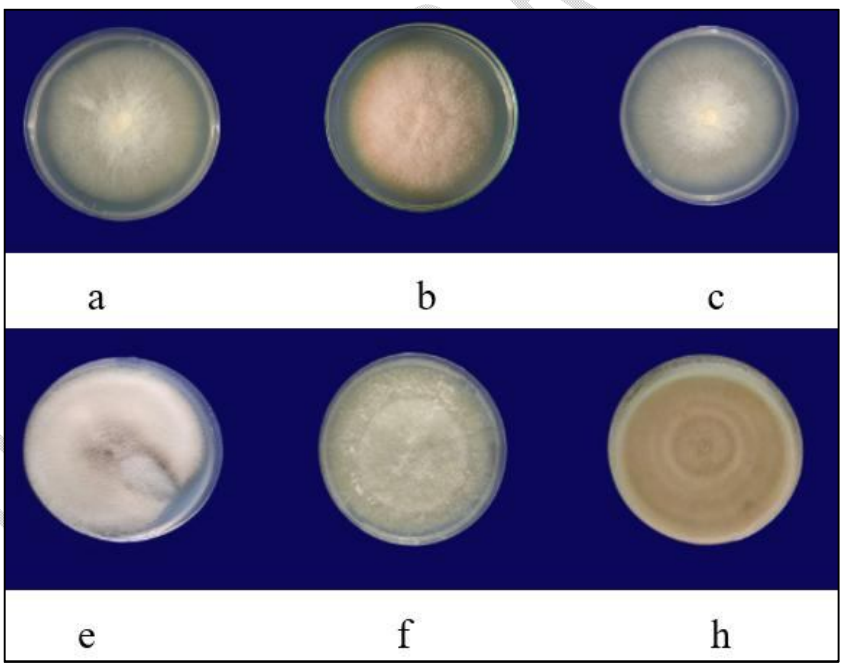

Fig.8. Methanolextract ofdifferent seaweeds @ 1%

(a.*Sargassum wightii,* b. *Kappaphycus alvarezii,* c. *Ulva lactuca,* d. *Caulerpa racemosa,* e. *Gracilaria edulis,* f. Control)

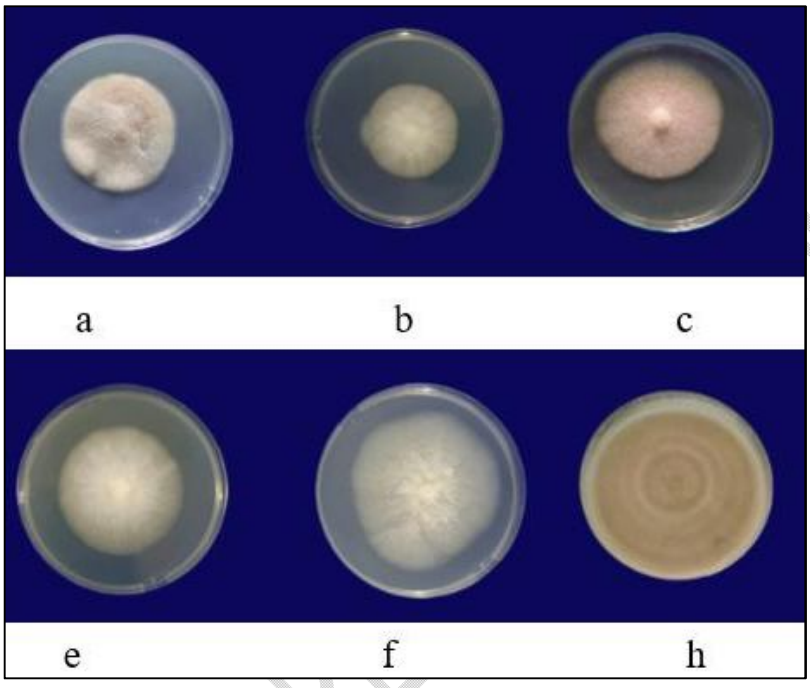

Fig.9. Methanolextract ofdifferent seaweeds @ 3%

(a.*Sargassum wightii,* b. *Kappaphycus alvarezii,* c. *Ulva lactuca,* d. *Caulerpa racemosa,* e. *Gracilaria edulis,* f. Control)

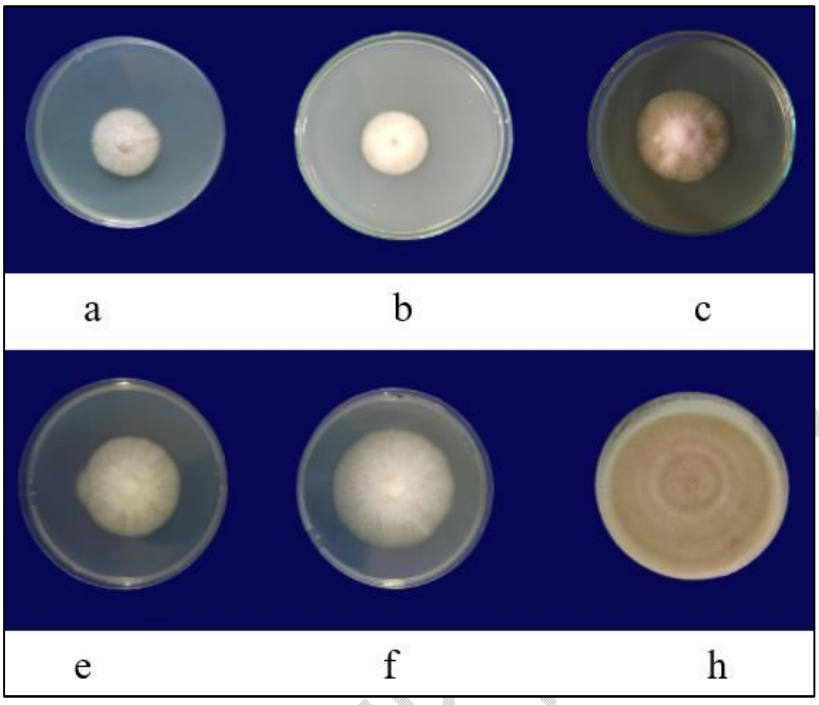

Fig.10. Methanolextract ofdifferent seaweeds @ 5%

(a.*Sargassum wightii,* b. *Kappaphycus alvarezii,* c. *Ulva lactuca,* d. *Caulerpa racemosa,* e. *Gracilaria edulis,* f. Control)

Conclusion

In conclusion, the survey were conducted in major tomato-growing areas of Tamil Nadu revealed varying levels of early blight disease incidence, with Kongu Thirupathi village in Coimbatore district having the highest disease incidence percentage. The pathogen *A.solani*was successfully isolated and characterized from infected plants. Among the tested isolates, TA-1 demonstrated the highest virulence. Additionally, seaweed extracts, particularly *K.alvarezii*exhibitedhighest promisingantifungalactivityagainst*A.solani*. These findings provide important understandings for the management of early blight disease in tomato and highlight the potential of seaweed extracts as alternative control measures. Further research is needed to explore the active compounds responsible for the antifungal activity and their practical application in disease management strategies.

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