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Research Article

IDENTIFICATION AND MOLECULAR CHARACTERIZATION OF *STAGONOSPOROPSIS CUCURBITACEARUM* CAUSES GUMMY STEM BLIGHT DISEASE ON *COCCINIA GRANDIS*- A FIRST REPORT IN KARNATAKA, INDIA

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ABSTRACT
Gummy stem blight caused by *Stagonosporopsis cucurbitacearum* (syn. *Didymella bryoniae*) is one of the major disease in cucurbits. The goal of the study is to identify and characterize *S. cucurbitacearum* causing gummy stem blight in *Coccinia grandis*. The infected samples collected from the *C. grandis* growing fields, surface sterilized and inoculated on PDA medium. The morphology of the inciting pathogen was thoroughly studied. For further confirmation, the molecular characterization was done by using PCR amplified DNA product with specific ITS1 and ITS4 - rDNA primers and sequenced for further analysis. Phylogenetic tree was constructed using nBLAST data, which confirmed that the pathogen as *S. cucurbitacearum*. The isolate was deposited in the GenBank and accession number was obtained (OL774656, OL774657, and OL774658). The pathogenicity test was carried out on detached fruit assay on ivy gourd. The characteristic symptoms on fruits and re-isolation also confirmed presence of *S. cucurbitacearum*. *In vitro* evaluation of fungicides and plants extract against pathogen was carried out, the results showed that, Kavach (34.66±0.33 at 1.0 mg L^{-1}), effectively inhibit the growth followed by Indofil, Aliette, Sectin, and Nativo. Likewise, the methanol extract of Neem and *Ficus* exhibits significant inhibitory activity. Therefore, this study revealed *S. cucurbitacearum* responsible for causing gummy stem blight disease in *C. grandis* host and this is the first report in Mysuru of Karnataka, India. Further the effective management is required to minimize the disease incidence and yield loss of this high nutritional vegetable crop.

Keywords: Cucurbits, GSB, *Stagonosporopsis cucurbitacearum,* ITS-rDNA, nBLAST, GenBank.

1. INTRODUCTION
Ivy gourd (*Coccinia grandis* L.) also known as little gourd or scarlet fruit gourd is a perennial, dioecious, tropical cucurbitaceous vegetable crop which gives continuous production for about 7-8 months of the year. Ivy gourd is believed to have originated in India [1]. It is one of the important minor cucurbit vegetable crop. This crop is extensively cultivated in Fiji, China, Africa, Central America, Malaysia, Australia and other tropical Asian countries. In India, generally cultivated in parts of various states which includes Uttar Pradesh, West Bengal, Assam, Chhattisgarh, Karnataka, Madhya Pradesh, Andhra Pradesh, Tamilnadu, Gujrat, Maharastra, Bihar and Odisha [2, 3]. Various parts of *C. grandis* have significant medicinal value as reported by many researchers. Ivy gourd leaf extract is active against *Shigella flexneri*, *Bacillus subtilis*, *Escherichia coli, Salmonella choleraesuis* [4]. It also exhibits other medicinal properties including antidiabetic, oxidant, larvicidal, cooling effect to the eye, gonorrhea, hypolipidemic skin diseases and urinary tract infection [5, 6].

Ivy gourd is the host for wide range of diseases including fungal diseases such as powdery mildew, seed-borne anthracnose, black rot, stem rot and viral diseases such as watermelon mosaic virus may leads to severe loss in the yield. Upon thorough review of existing literature and to the best of our knowledge, this is the first report of susceptibility of *C. grandis* to gummy stem blight disease from Karnataka. *Stagonosporopsis cucurbitacearum is* responsible for causing gummy stem blight (GSB) disease in majority of cucurbits. The pathogen exhibited wide range of host specificity and mainly affects cucurbits, no clear cut definite number of hosts has been reported so far. In the entire host, same pathogen exhibits different

phases of GSB symptoms like foliar blight, stem canker and fruit rots depending on the host susceptibility. *S. cucurbitacearum* is a homothallic [7] and it produces sexual spores in culture plates as well as in the field referred to as teleomorphic phase [8, 9]. However, there is no report on molecular characteri-zation of *S. cucurbitacearum* isolated from ivy gourd infected with GSB disease. Therefore, the present work was carried out for molecular characterization and *in vitro* screening of fungi toxicants and organic extracts against pathogen associated with GSB was systematically studied.

2. MATERIAL AND METHODS

2.1. Field survey and Collection of diseased samples

A field survey carried out during the month of October - December 2019 in major ivy gourd growing regions of Ankasettypura village, Chamarajanagar district (11.92°N and 76.95°E), revealed the occurrence of GSB disease. The leaf, stem and fruits samples exhibiting GSB symptoms were collected and brought to the laboratory in sterile plastic cover for isolation and further pathological studies. Characteristic symptoms like blights appeared on stem and fruits followed by exudation of amber-colored gummy substances. The lesions at the margins of leaves and water-soaked area at main veins of leaf were found to be observed. During rainy season with high humidity severe symptoms were observed.

2.2. Pathogen Isolation

Collected infected leaf and fruit samples were cut into small pieces of about 0.5cm using sterile blade and surface sterilized with 2% sodium hypochloride (v/v) solution for 2-3 min. followed by washing thrice with sterile distilled water and blotter dried. The samples were inoculated on the potato dextrose agar (PDA) medium amended with chloramphenicol (40mg L^{-1}). Inoculated plates were incubated at room temperature with alternative of 12hr. photoperiod for 7 days at 25°C and colony growth rate was recorded by measuring colony diameter after specific time of incubation. The culture plates showing morphological characters were subcultured on quarter strength PDA (QPDA) for proper sporulation and were further subjected to morphological identification and molecular analysis.

2.3. Morphological identification of isolated fungi

It was carried out through the observation of colony morphology on culture plates and compared with the characteristics which resembled the descriptions of the pathogen given by Sutton (1980) [10]. The mycelia and other reproductive structures were removed from agar plugs and teased onto the glass slide using 1% cotton blue stain and placed a coverslip. The mounted samples were then observed under light microscope and photographs were taken, compared and identified based on the standard procedures.

2.4. Koch's postulation

Pathogenicity test was conducted through detached fruit assay. Healthy fruits were detached from the plant and brought to the laboratory; surface sterilized with 0.1% mercury chloride ($HgCl_2$) for 30 sec., followed by washing thrice in sterilized distilled water. About 10µl of spore suspension (1 X 10^6 conidia ml^{-1}) was sprayed on the tested fruit. The air-filled polythene bags were then placed in diffused sunlight at room temperature and observed daily for the appearance of symptoms. The results were recorded and photographed frequently for further studies.

2.5. DNA extraction and PCR amplification

The fungal DNA was extracted for further molecular characterization process using the modified protocol of White *et al.,* (1990) [11]. Under aseptic conditions, the mycelium (100-120 mg) was scarped from pure culture of a 7-day-old grown on PDA medium. Sterilized mortar and pestle was used to homogenize the mycelia in liquid nitrogen. Fine paste of ground tissues was later transferred to a 2.0 mL Eppendroff tube and used to obtain genomic DNA through DNeasy Plant Mini kit (BioSciences, Bangalore, India). The genomic DNA was washed out in sterilized, nuclease free water and stored at -20°C for further process. Each isolate was subjected to amplification through PCR using Internal Transcribed Spacer primers sequences such as ITS1 (5′-TCCGTAGGTGAACCTGCG- 3′) and ITS4 (5′-TCCTCCGCTTATTGATATG- 3′). The PCR analysis was carried out with Applied Biosystems Thermo cycler (GeneAmp®, USA), mixture of about 25µl containing 1µl of DNA sample with 2.5 µl of 10X PCR buffer (2.5 mM $MgCl_2$, 50mM Tris- HCl, 1.5 mM (NH_4) SO_4, and 0.1% Triton X-100), 1.0 µl of 2 mM dNTPs, 1.0 µl of 20 pM of each primer, 0.2 µl of Taq DNA polymerase and made up to 25µl with 18.3 µl of nuclease free water. Further amplification process was carried out in an Eppendroff master cycler gradient ES specific condition

including an initial denaturation at 90°C for 2 min., primer annealing at 55°C for 15 sec., followed by extension of primer for 30 sec., and final extension for 10 min. at 72°C.

2.6. ITS sequencing and Phylogenetic tree analysis

Phylogenetic analysis of the isolated pathogen was carried out using the ribosomal internal transcribed spacer (ITS) sequence for characterization of pathogen. The end products of PCR was purified and eluted from spin columns with 30µl of nuclease free water and DNA concentration was determined through spectro-photometer. About 12µl of samples were prepared in sequencing tube with 3µl of DNA template (300 ng), 1µl of 10pM ITS1 or ITS4 primer and brought up to a final volume by adding 8µl of nuclease free water. These sample mixture was sequenced and blasted (http://www.ncbi.nlm.nih.gov/blast/) against known sequences deposited in NCBI (National Center for Biotechnology Information) data base to make available species identification. Clustal W software was used to align multiple sequences of the isolates and percent homology between aligned sequences was calculated [12]. BIOEDIT tool was used to construct sequence identity matrix and further using these sequences phylogenetic tree was constructed based on Neighbour-Joining method through MEGA6 software [13]. All the characters were run unordered and of equal weight and gap were treated as missing data. For further analysis, 1000 bootstrap replicates were performed to assess the node support of the generated trees. Representative sequence from the present report was showed in phylogenetic tree shared common clade with the GenBank sequence thus confirming the pathogen identity.

2.7. Evaluation of antifungal activity

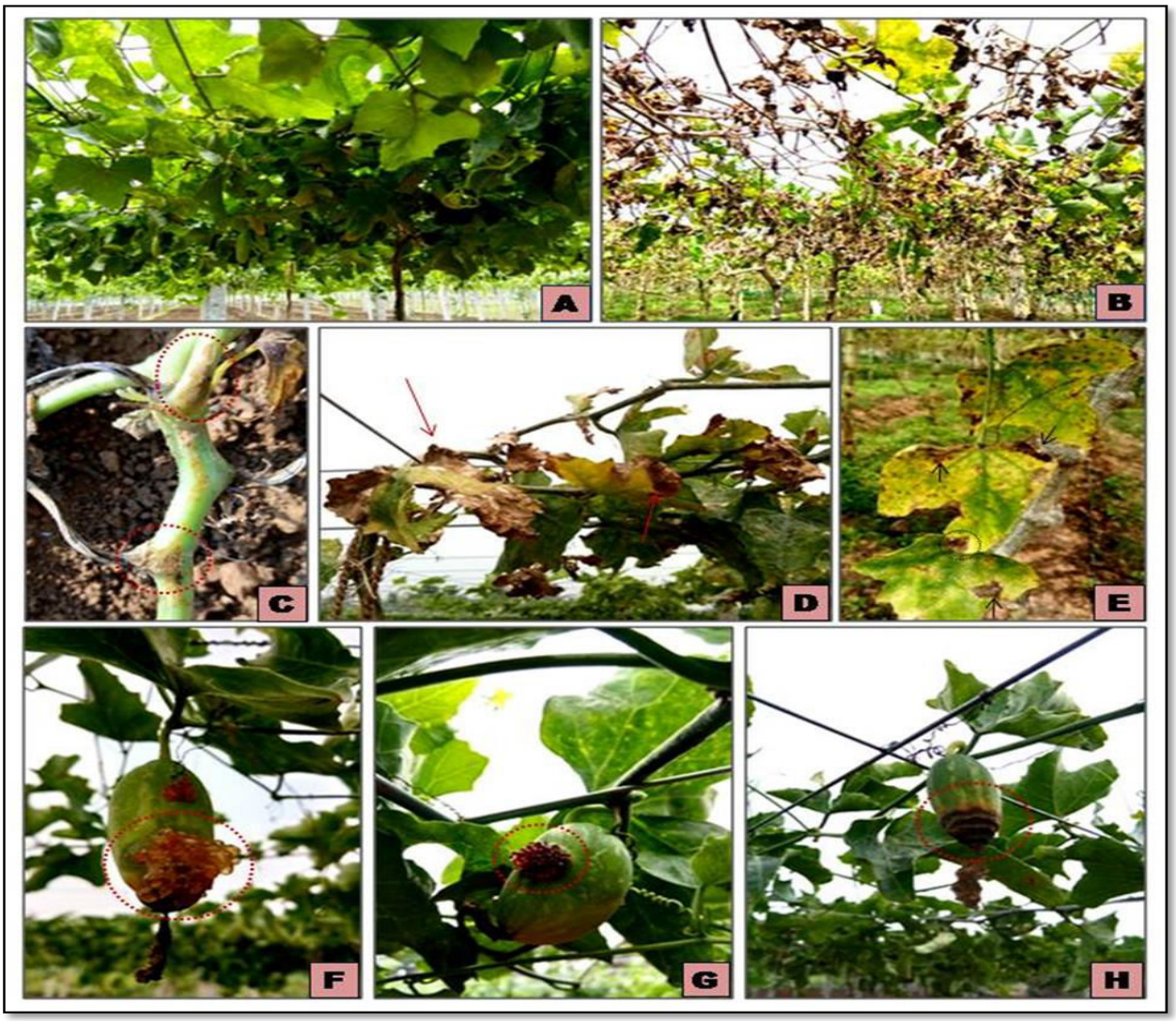
The antifungal activity of fungicides such as Kavach, Aliette, Indofil, Nativo, and Sectin and botanical extracts of neem and *Ficus* by using different solvents such as petroleum ether, chloroform, ethyl acetate and methanol was evaluated against *S. cucurbitacearum*. It was carried out through *in vitro* poison food and agar well technique at the concentrations of 0. 025, 0.050, 0.075, and 0.1mg mL^{-1}. Erlenmeyer flasks containing 100 ml PDA medium with fungicides and leaf extract was separately mixed under aseptic condition. After thorough mixing, 20 ml of poisoned food was poured in 40mm diameter petri plates. From 10 days old pure culture, 5mm diameter mycelial disc of test fungus was placed at the center of solidified PDA. The growth of the test fungus on non-poisoned PDA served as control. For the agar well diffusion method, the petri plates containing two weeks grown pathogen on QPDA medium was flooded with sterile distilled water and the suspension thus obtained was filtered through muslin cloth and stored at 4°C until further use. 100µl of the test organism was spread uniformly by using a sterile L- shaped glass rod onto the Petri plates containing solidified PDA medium, 8mm wells were made and loaded with 50µl plant extracts and /or fungicides at different concentration along with respective solvent and water which served as negative control. The Petri plates were incubated at 37°C for seven days. After seven days of incubation, observation on mycelia growth of test fungus was recorded by measuring with colony diameter scale [14]. Three replications were maintained for each concentration.

3. RESULTS AND DISCUSSION

3.1. Disease status in selected field

During November - December 2019, *Coccinia grandis* plants were found to exhibit symptoms of gummy stem blight disease at major ivy gourd growing fields of Chamaraja Nagar district, Karnataka, India (Fig.1A). The primary characteristic symptoms of GSB disease was yellowing of leaf margins followed by appearance of minute brown spots on leaf lamina, gradually it turned to light yellow halo patches (Fig.1B, D, E). In advanced stages of infection, stem blight symptoms were noticed on the plants having an age of 35-45 days. Initially, small, light brown slightly oily spots of irregular shape and size were noticed on the nodes of vine. Gradually enlarged lesion and cracking was visible on the stem, accompanied by gummy ooze (Fig. 1C). Slowly the spots increased in size, turned dark brown to black and covered almost entire node. In addition, on internodes the spots were in the form of short to long strips of yellowish brown in colour. As the strips enlarged, they became broad and turned dark brown in colour. On fruits lesions, a gummy exudate was seen after rainy season, it is one of the characteristic symptoms of GSB. Such fluid gathered in the form of small to big droplets of light red to dark amber in colour and dried to form gum exudates. Based on the thorough review, we believe that the majority of cultivated cucurbits are highly susceptible to *S. cucurbitacearum*, which includes *Cucumis sativus*, *C. melo, Cucurbita pepo, C. moschata* etc., [7, 15-20]. Therefore,

the results of this investigation highlights the various nature of symptoms of GSB and which is agree with the previous report of Mahapatra *et al.,* (2020) [21], observed the same symptoms on infected watermelon.



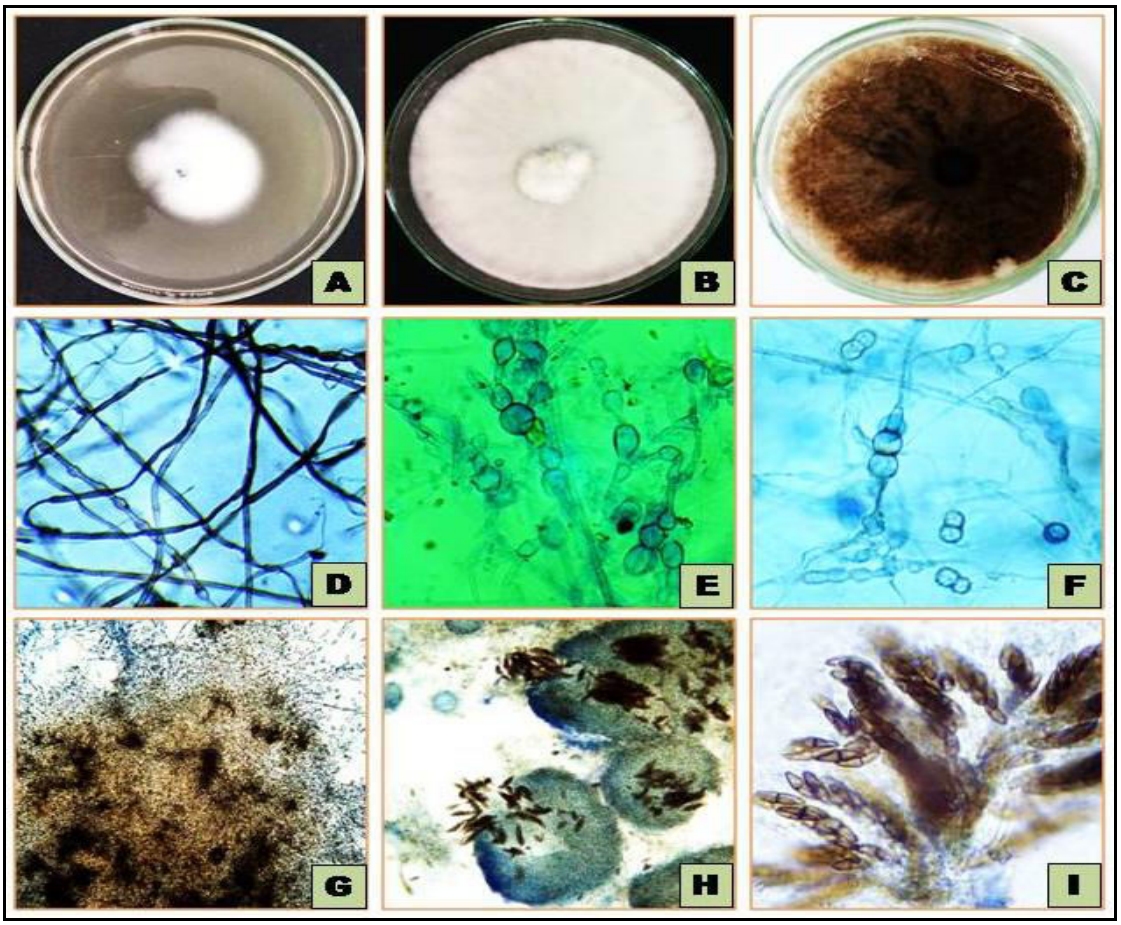
A- Healthy plants; B-Symptoms associated with chlorosis and drying of the leaves; C- Water soaked lesions on stems ; D- Tan lesions with necrotic areas on leaves; E-Lesions on infected leaves; F, G, &H -Oozing of amber-coloured gummy sticky substance from the fruits.

Fig. 1: Gummy stem blight symptoms *in Coccinia grandis*

3.2. Morphological characters of the isolated fungus

The pathogen was isolated on PDA medium and morphological characters of the isolated fungal pathogen were studied on culture plates as well as under microscope. Five days old mycelium of the pathogen on PDA was characterized by circular colonies with creamish white cottony growth turned to grayish and finally black (Fig. 2A-C).Colony morphology on culture plates was compared with the characteristics of the causal agent of GSB disease explained by Keinath *et al.,* (1995) [22]. The mycelium was hyaline, septate, smooth, and irregularly branched structure, measuring about 4.0-5.5 µm width (Fig.2D). Pycnidia and chlamydospores were formed after 2 weeks of incubation. Chlamydospores were spherical to ellipsoid, 7.7-13 × 6-12 µm, light brown, thick-walled, and were single or 3 - 8 in a chain (Fig. 2E & F). After 10 -12 days of incubation, the anamorph stage *Phoma cucurbitacearum* produced pycnidia and it is globose to irregular in shape, measured 170 - 325 µm (Av. 236.5 µm) in diameter (Fig. 2G & H). Large number of pycnidiospores was released from pycnidia and it is variable in shape, sub-

globose to ellipsoidal, aseptate rarely one septate and measuring about 6.5-9.5 x 2.2-4.2 µm in size (Fig. 2I). By considering these morpho-logical features compared with authentic description, the pathogen was identified as *S. cucurbitacearum* (Aureswarld) Rehm (Anamorph: *Phoma cucurbitacearum* (Foutrey) Sacardo). Similar description of the fungal pathogen *S. cucurbitacearum* responsible for causing GSB on muskmelon was given by Nuangmek *et al.,* (2018) [23]. Chiu and Walker (1949) [7]; Punithalingam and Holliday (1972) [24] collectively corroborate our identification of the anamorph, *Phoma cucurbitacearum*. Similar description of the teleomarph have been given by Corlett (1981) [9]; Kienath *et al.,* (1995) [22] and Pandey and Pandey (2003) [25].

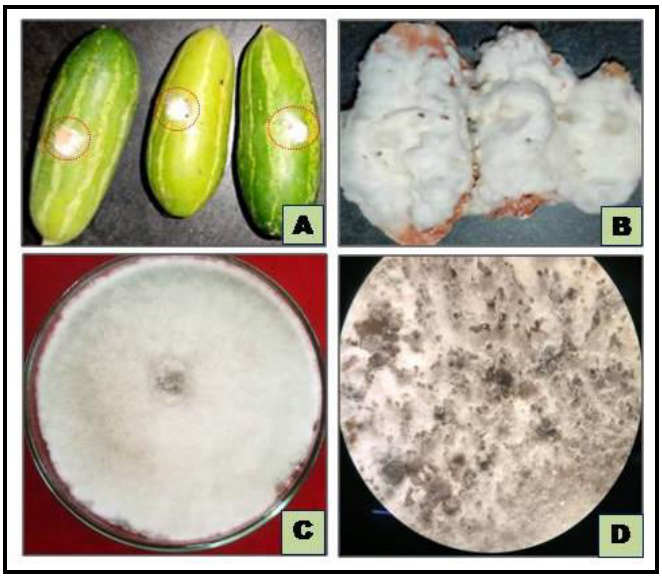


A- Colony formed on PDA after 3 days of incubation; B- Dorsal view of Colony; C- Ventral view; D- Mycelia of S. cucurbitacearum after exposure to UV light; E &F- Chlamydospores in chain in hyphae; G &H- Conidia released from pycnidia; I- Microscopic view of asci with ascospores.

Fig. 2: Culture and morphological characteristics of the pathogenic fungi *Stagonosporopsis cucurbitacearum*

3.3. Pathogenicity test

The pathogenicity of the causal fungus was established by confirming postulates of Koch on healthy, detached fresh fruit. The study proved that *S. cucurbitacearum* was responsible for causing GSB on *C. grandis*. A creamish coloured blight was produced on the inoculated region of fruits (Fig. 3A). This indicated the initiation of the disease, followed by expansion of the white cottony growth throughout the inoculated fruits (Fig. 3B). Inoculated fruits exhibited GSB symptoms, was used to re isolate the pathogen responsible for causing GSB symptoms and identity was confirmed through cultural and microscopic characters (Fig. 3C & D). Van Steekelenburg (1981) [15] obtained symptoms 3 to 4 days after inoculation of young cucumber plants and found wounding essential for fruit rot. Svedelius and Unestam (1978) [26] have observed more severe symptoms in three days after inoculation on detached injured leaves of cucumber than on uninjured leaves.

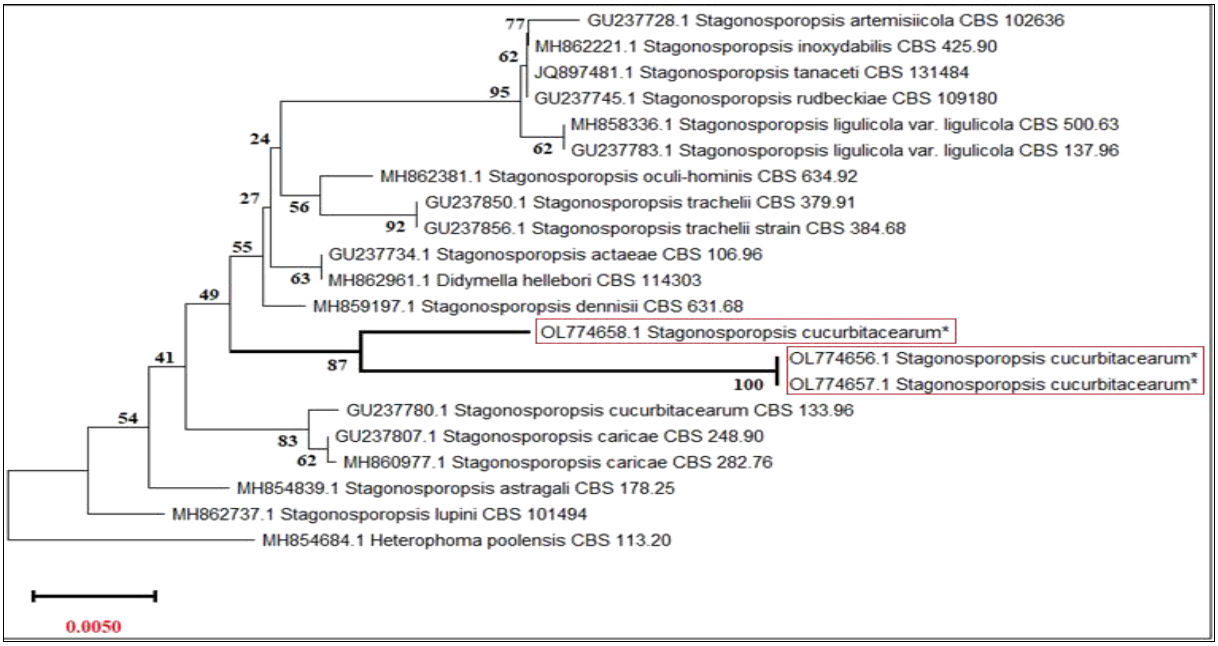


A -Healthy and fresh fruit inoculated with pathogen; B- After four to five days of inoculation white mycelial growth on fruit; C- Re-isolation of pathogen from fruit: D- Stereomicroscopic view of pycnidia.

Fig. 3: Pathogenicity test by detached fruit method

3.4. rDNA Sequence Analysis

Genomic DNA was extracted and PCR reactions were carried out in a total volume of 25µL. The amplified PCR products (550 bp) were bi-directionally sequenced and the concord sequences were blasted. Similarity search was carried out using nBLAST and the results showed that the sequence obtained from the present work exhibited 100% similarity with GenBank reference sequence of *S. cucurbitacearum* (GU237780). The query sequences was deposited in the GenBank and got the accession numbers (OL774656, OL774657, OL774658). Further, phylogenetic analysis of selected ITS regions revealed that the isolated fungi shared a common clade of *S. cucurbitacearum* represented by the reference sequences obtained from the GenBank (Fig. 4), thereby conforming that the isolated pathogen is *S. cucurbitacearum*. Present work is strongly in agreement with earlier reports of Garampalli *et al.* (2015) [27] wherein, the molecular characterization analysis of new isolates of *D. bryoniae* from gherkin collected in Karnataka. Likewise, Mahapatra *et al.,* (2020) [21] characterized the rDNA- ITS sequencing of the pathogen causing GSB on watermelon in India. Babu *et al.,* (2015) [28] carried out genetic characterization of 35 isolates of *S. cucurbitacearum* obtained from cucurbits revealed that the isolates varied in pathogenicity and aggressiveness to different hosts. To the best of our knowledge, this molecular analysis of the isolated pathogen is the first confirmation of susceptibility of *S. cucurbitacearum* causes GSB disease on *C. grandis* from Karnataka, India.

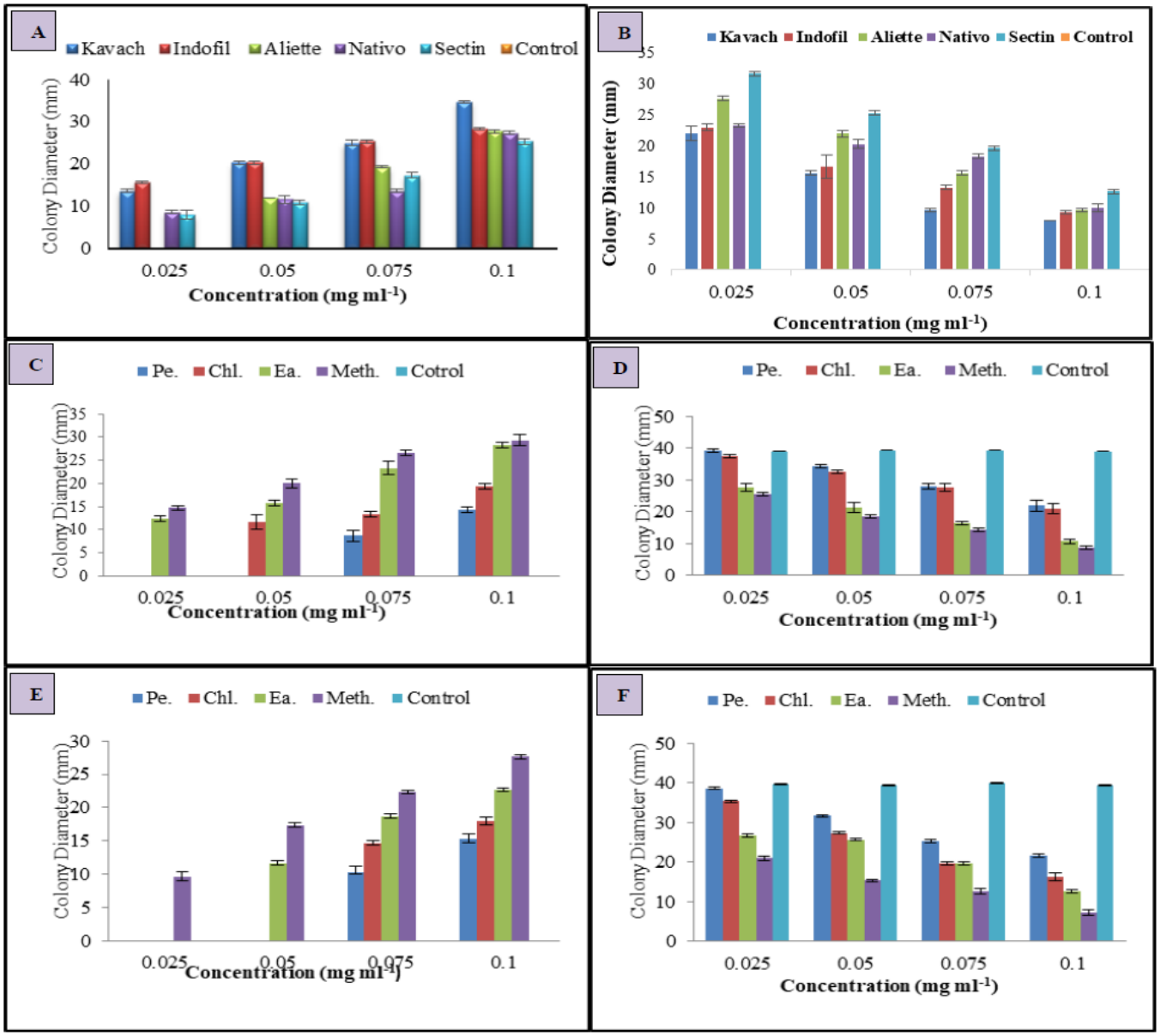


(Tamura-Nei Substitution model and nearest neighbor interchange search options with 1000 bootstrap replicates were used; Bar shows that the estimated nucleotide substitution per site is 0.0050).

Fig. 4: Phylogram constructed by Neighbor-joining method using reference sequence retrieved from NCBI GenBank

3.5. Evaluation of antifungal activity

The data on *in vitro* efficacy of systemic fungi-toxicants tested at 0.025, 0.050, 0.075, and 0.10 mg ml^{-1} concentration against *S. cucurbitacearum* is represented in the fig. 5. The results revealed that all the fungi-toxicants significantly inhibited mycelial growth of *S. cucurbitacearum* at all test concentrations. Kavach proved significantly superior to all other fungi-toxicants exhibiting (34.66±0.33) mycelial growth inhibition followed by Aliette, Indofil and Nativo in agar well method. Sectin proved least effective fungi-toxicant with mycelial growth inhibition (Fig. 5A). In poison food technique also Kavach (12.66±0.33) exhibited significantly superior antifungal activity against tested pathogen followed by Aliette, Indofil, Nativo, and Sectin (Fig. 5B). Among the four solvent extracts of two botanicals tested, ethyl acetate and methanol extracts of *A. indica* (29.33±1.15/ 8.66±0.57) (Fig. 5 C & D) and *F. glomerata* (27.66±0.33/ 7.33±0.66) (Fig. 5 E & F) showed significant results in both poison food and agar well technique respectively.



A & B - Five different fungicides; C & D - Azadirachta indica leaves extracts; E & F- Ficus glomerata leaves extracts.
Note: Error bars indicate significant differences ($p < 0.05$) in the antifungal activity among the groups.

Fig. 5: Inhibition of *S. cucurbitacearum* mycelia growth at four different concentrations of selected fungicides and botanicals by Agar well technique and Poisoned food technique

The overall result of antifungal activity reveals that the extent of mycelial growth inhibition of the test fungi-toxicant increased with increase in the concentration. Similar results were reported by Egel (2010) [29] thereby using different fungicide such as chlorothalonil, mancozeb, azoxystrobin, pyraclostrobin, and cyprodinil against *S. cucurbitacearum* and provide best control of GSB. Moumni *et al.,* (2021) [30] also studied the antifungal activity of the seven essential oils of various plant species against fungi of cucurbits including *S. cucurbitacearum* and reported inhibitory effect of extracts on the mycelial growth of the pathogen. Our findings are in strong agreement with the results reported by Utkhede (2002) [31] wherein, the reduction in GSB infection in greenhouse cucumbers and a great increase in the yield of fruits due to the application of bio agents.

4. CONCLUSION

In the present work first report of gummy stem blight disease in ivy gourd was investigated. Characteristic symptoms were observed on the host plant is gummy exudates on stem and fruits, circular lesion on leaves at the margins gradually expanded towards the midrib leads to death of the plant. *Stagonosporopsis cucurbit-acearum* responsible for causing GSB on *Coccinia grandis* an important vegetable crop with high medicinal value.

5. ACKNOWLEDGEMENTS

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Conflicts of interest

The authors declared that they have no conflict of interest.

Authors Contributions

Both the authors read and approved the final submitted manuscript.

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