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Gummy Stem Blight Disease Caused by *Didymella bryoniae* on *Luffa acutangula* and its Molecular Identification - A First Report in Karnataka, India

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A B S T R A C T

Luffa acutangula is an important vegetable crop in sub-tropical regions of the Asia. Gummy stem blight (GSB) is a major disease of Cucurbitaceae members. Field survey was conducted during 2018- 2019 in crop growing areas of Mysuru and Chamarajanagar districts. Based on morphological and microscopic observation, the fungus inciting GSB disease was identified as *Didymella bryoniae* which was further confirmed through molecular characterization techniques using ITS-rDNA primers. Based on the sequence data, phylogenetic tree was constructed using nBLAST, and the similarity sequence confirmed the pathogen as *D. bryoniae*. Sequence was submitted to GenBank with accession number MW990413. Pathogenicity test was performed on 25- days old plants and on detached healthy fruits with mycelial suspension of *D. bryoniae*. The pathogen was re-isolated from the infected experimental plants for confirmation. *In vitro* antifungal activity was conducted to evaluate the sensitivity of pathogen to different fungicides and botanical extracts by agar well diffusion test at different concentrations. Among the tested fungicides, Aliette and methanol leaf extracts of neem and *Ficus* was found to be most effective in inhibiting growth of the pathogen. This is the first report of GSB caused by *D. bryoniae* on ridge gourd from this region to our knowledge.

***Key words*:** BLAST, *Didymella bryoniae,* GenBank, GSB, ITS –rDNA, NCBI

Luffa acutangula (L) Roxb. (Cucurbitaceae) commonly known as ridge gourd and is an important vegetable crop, has attained greater significance due to its industrial and medicinal use [1]. From the nutritional point of view, it has low calorific value but rich in vitamins (Vit. B_2 and Vit. C) and more than 50 compounds have been isolated [2]. The bioactive compounds such as flavonoids, saponins, Cucurbitacin-B, anthraquinones, and gelatinous substance exhibit significant medicinal properties and are extensively used in the Indian Ayurvedic system of medicine. Leaf juice is used for treating a range of ailments including sores, inflamed spleen, ringworms, piles, leprosy, bites of insects and snakes, and dysentery [3]. Ridge gourd is being infected by many fungal, bacterial, and viral diseases such as downy mildew (*Psedoperonospora cubensis*), powdery mildew (*Erysiphe cichoracearum*/ *Sphaerotheca fuliginea*), gummy stem blight (*Didymella bryoniae*), *Fusarium* wilt (*Fusarium oxysporum*), angular leaf spot (*Pseudomonas syringae*), bacterial soft rot (*Erwinia carotovora*), mosaic disease, etc., which drastically reduce the yield and considerably limit their profitable production [4]. Generally, GSB disease is a devastating disease of Cucurbitaceae crops caused by *Didymella bryoniae*, which belongs to the division Ascomycota of class Dothideomycets. GSB disease distributed throughout the world, especially higher degree of disease incidence was reported from tropical and subtropical regions because the pathogen requires humid and warm condition for proper growth [5].

Didymella bryoniae exhibited a wide range of host specificity, no clear-cut definite number of hosts have been reported so far. It mainly known to affects cucurbits such as cucumber, sponge gourd, bitter gourd, ridge gourd, bottle gourd, watermelon, musk melon, etc., and in all these hosts the pathogen exhibits different phases of GSB symptoms like foliar blight, stem canker and fruit rots depending on the host susceptibility. *D. bryoniae* is a homothallic [6] and it produces sexual spores in culture plates as well as in the field which are referred as teleomorphic phase [7-8]. The first report of GSB disease in ridge gourd caused by *D. bryoniae* from India was from Jammu and Kashmir [9]. However, there is no report from the other parts of the country. Therefore, the present work was carried out to study the disease incidence in the selected areas of Karnataka region, isolation, identification, and molecular characterization of *D. bryoniae* and fungicide susceptibility.

MATERIALS AND METHODS

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Field survey and collection of diseased samples

A roving field survey was carried out in major ridge gourd growing fields of Chamarajanagar and Mysore districts (12° 30' 0.00" N and 76° 40' 12.00" E). A field visit was conducted during October 2017 – December 2018 and recorded the occurrence of GSB disease severity. Characteristic appearance of gummy exudates on infected stem and fruits, lesions at the margins of leaves, and water-soaked area at main veins of a leaf were collected. During the rainy season with high humidity severe symptoms such as rotting of fruits were observed. Selected fields were divided in five blocks and fifty randomly selected GSB diseased plants in each row were scored for disease severity [10].

Pathogen isolation

Leaf and fruits showing GSB symptoms were collected and brought to the laboratory in sterile plastic cover. Each sample was cut into small pieces of about 0.5cm using a sterile blade and surface sterilized with 1% sodium hypochlorite (v/v) solution for 1-2 minutes followed by washing thrice with sterile distilled water and blotter dried. The samples were transferred on to the potato dextrose agar (PDA) medium amended with chloramphenicol (40mgl^{-1}) in petri plates and incubated at room temperature with 12 hours photoperiod for 7 days at 25°C. The colony diameter growth was recorded using colony diameter scale. The culture plates showing GSB morphological features were sub-cultured on quarter strength PDA (QPDA) for proper sporulation and were further subjected to morphological and microscopic identification and molecular analysis.

Morphological identification of isolated fungi

Observation of colony morphology on culture plates was compared with the characteristic's features resembled with a description of the pathogen. The mycelia and other reproductive structures were collected from agar plugs and tease onto the glass slide, stained with 1% cotton blue prepared in a lactophenol and gently tapped after placing coverslip, observed under a light microscope and photographs were capture with camera (Nikon Eclipse E400) and identified [11].

Koch's postulation

Pathogenicity test was conducted for the confirmation of isolated pathogen by spraying fungal suspension on detached healthy fruits and on 25 days old healthy plants raised poly-house. Petri plates of two weeks old fresh fungal culture grown on QPDA media was flooded with water and used as inoculum. Spores load was adjusted to 1x10^{6} conidia ml^{-1} and used immediately. The suspension was sprayed on plants with a hand atomizer twice at 24 hours interval. Healthy plants without inoculum suspension were maintained under the suitable condition as a control. For fruit detached test, 8mm disc of the pathogen was inoculated on healthy ridge gourd fruit. After 6-8 days of post-inoculation, primary infection, and symptoms were observed in the inoculated region. The data was recorded frequently at different time intervals and pathogen exhibit GSB symptoms on the experimental plant were re-isolated and its identity was confirmed.

DNA extraction and PCR amplification

Morphologically identified pathogenic fungi that exhibited GSB symptoms were used for further molecular characterization. About 100-120 mg of fungal mycelium was scarped from pure culture under aseptic conditions and the genomic DNA was extracted by the cetyltrimethylammonium bromide (CTAB) method [12]. The genomic DNA was washed in sterilized, nuclease-free water and stored at -20°C. Each isolate was subjected to PCR amplification through using Internal Transcribed Spacer primers sequences such as ITS1 (5′- TCCGTAGGTGAACCTGCG-3′) and ITS4 (5′- TCCTCCGCTTATTGATATG- 3′). The PCR analysis was carried out in a 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). Add1.0 µl of 2 mM dNTPs, 1.0 µl of 20 pM of each primer, and 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 Eppendorf mastercycler gradient with specific conditions 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 [13].

ITS sequencing and phylogenetic tree analysis

The end products of PCR were purified and eluted from spin columns with 30µl of nuclease-free water and DNA concentration was determined through a spectrophotometer. About 12µl of samples was prepared in a sequencing tube with 3µl of DNA template, 1µl of 10pM ITS1 or ITS4 primer and brought up to a final volume by adding 8µl of nuclease-free water. This sample mixture was sequenced and blasted (http://www.ncbi.nlm.nih.gov/blast) against known sequences deposited in NCBI (National Center for Biotechnology Information) database to make sure the available species identification. ClustalW software was used to align multiple sequences of the isolates and percent homology between aligned sequences was calculated [14]. 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 [15]. 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. The representative sequence from the present report was shown in the phylogenetic tree that shared a common clade with the GenBank sequence thus confirming the pathogen identity.

Evaluation of antifungal activity

Evaluation of the antifungal activity of five fungicides namely, Kavach, Aliette, Indofil, Nativo, and Sectin; and two plants extracts of *Azadirachta indica* and *Ficus glomerata* was carried out to test the susceptibility of pathogen. Organic solvents namely, petroleum ether, chloroform, ethyl acetate, and methanol were used for extraction and subjected for *in vitro* poison food and agar-well diffusion techniques at four different concentrations (0.025, 0.50, 0.75, and 1 mg ml^{-1}). For poison food technique, Erlenmeyer flasks containing 100 ml PDA medium with fungicides and botanicals at four different concentrations separately were mixed under aseptic conditions. After thorough mixing, 20 ml of poisoned food was poured into 90mm diameter Petri plates. From 10 days old pure culture, a 5mm diameter mycelial disc of test fungus was placed at the center of solidified PDA, three replications were maintained for each concentration. The Petri plates were incubated at 24±1°C and after seven days of incubation, colony diameter was recorded. 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 till 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. Each plate was incubated at 37°C for three days. The zone of inhibition around the well was recorded by measuring with colony diameter scale (NCCLS, 1993.

Statistics

All the *in vitro* experiments were conducted in triplicates and values were expressed utilizing± standard error (SE). All the experiments were subjected to Analysis of Variance (ANOVA) using SPSS v. 16.0 (SPSS Inc., Chicago, IL, USA). Significant effects of the F-value (p >0.05) treatment means were separated using Tukey's HSF (Honest Significant Differences) test.

RESULTS AND DISCUSSION

Disease status in the selected field

During field survey in ridge gourd cultivated areas of Mysore and Chamarajanagar districts of Karnataka state, India, the disease was confirmed as GSB based on symptomological characteristics (Fig 1A). Primary symptoms of GSB disease were observed on the leaves, which then gradually progressed on to stem during the pre-harvesting time. Initially, symptoms appeared on leaf margins, and gradually it acquired the entire leaf (Fig 1B-E). The appearance of blights followed by gummy exudates on stem and rotting of fruits were major symptoms, hence the disease named as GSB and black rot disease respectively. The late-stage disease symptom observed was oozing out of plant sap along with amber-colored gummy substances (Fig 1F-H).

Morphological characters of the isolated fungal pathogen

Morphological characters of the isolated fungal pathogen were studied both in the host as well as in pure culture under a light microscope. In host the mycelial hyphae were branched, septate, smooth, and hyaline, and measures 3.2 - 5.3 µm width. Black pycnidia of the anamorph (*P. cucurbitacearum*), 120.0 - 172.0 µm in diameter formed on the surface of infected leaves and fruits. Colony morphology of culture plates was observed, and the characteristics features were compared, and it resembled the descriptions of the causal agent of GSB disease described by Keinath *et al.* [11]. Culture plates were characterized by circular colonies with cottony growth, initially white to creamish color, later turned to grayish, and then finally black (Fig 2A-B*)*. The pathogen developed in culture plates has produced hyaline to light brown septate, submerged hyphae with sparse aerial mycelium measuring about 3.4 to 4.5 µm in width. The mycelium was irregularly branched, and hyphae slightly bulged at septa (Fig 2C). The pathogen produces chain like chlamydospores under stressful conditions (Fig 2D). Pycnidia were also formed after 10-12 days of incubation with globose to irregular shape and rarely solitary to confluent, glabrous, or with mycelial outgrowths and it measured 167.7-326.8 µm in diameter. In later stages, the oozed pycnidiospores from the pycnidia were variable in shape with sub-globose to ellipsoidal, aseptate, and rarely one septate with 6.5-9.5 x 2.2-4.2 µm in size (Fig 2E-F). The asci were hyaline, bitunicate, cylindrical to subclavate, short stipitate or sessile, biseriate, eight spored, and measured 50 x 12.5 µm in size (Fig 2G-H). Ascospores were 16 x 5 µm in size, hyaline, mono septate, constricted at the septum and rounded at ends with upper cell usually wider than the lower one (Fig 2I).

Pathogenicity test

Reconfirmation of the GSB disease causal fungus on ridge gourd was done by conducting pathogenicity test applying Koch postulates on 25 days old ridge gourd plants. The study proved that *D. bryoniae* was responsible for causing GSB in *L. acutangula* plants (Fig 3). A whitish mycelium formed on inoculated fruit followed by oozing out of gummy exudates (Fig 3A-B). Pycnidia were produced on the fruit surface after 10 days of inoculation (Fig 3C-D). A small spot with a concentric ring was observed on the margin of young leaf (Fig 3E-H) and progressively expanding the spot towards the center of the leaf. A creamish colored blight was produced on the main stem of the plant (Fig 3I). Gradually, these lesions became dry, rough, cracked at the internodal region of the stem, and produced brownish gummy exudates. GSB symptoms were not observed on untreated control plants. Tissue of inoculated plant parts exhibiting GSB symptoms was used to re-isolate the pathogen and identity was confirmed through cultural and microscopic characteristic features (Fig 3J).

rDNA sequence analysis

Nuclear encoded ITS regions of the rDNA sequence were analyzed, and the result showed 90% significant alignment for the isolated fungal pathogen. The amplified PCR products (700 bp) were bi-directionally sequenced, and the concord sequences were blasted. The consensus sequences obtained from BLAST were aligned with the ITS sequence procured from GenBank (Supplementary Fig 1). A similarity search was carried out using nBLAST and the results showed that the sequence obtained from the present work exhibited 98% similarity with the GenBank reference sequence of *D. bryoniae* (MG198901). The query sequence was deposited in the GenBank with the accession number MW990413. Further, phylogenetic analysis of selected ITS regions revealed that the isolated fungi in the present work shared a common clade of *D. bryoniae*. It was represented by the reference sequences obtained from the GenBank, thereby conforming to the isolated pathogen as *D. bryoniae* (Fig 4).

Evaluation of antifungal activity

The *in vitro* efficacy of systemic fungi-toxicants and two botanicals were tested at 0.25, 0.50, 0.75, and 1mg ml^{-1} concentrations to study the mycelial growth of *D. bryoniae* and the result were tabulated (Supplementary Table 1-6). The data revealed that all the fungi-toxicants and plant extracts significantly inhibited the mycelial growth of *D. bryoniae*. In both poison food and agar well techniques, 1mg/ml concentration of Aliette fungicide significantly inhibited the mycelial growth (34.00 ± 1.15/ 12.33 ± 0.33) followed by Nativo (30.00 ± 0.57/ 14.33 ± 0.33), Indofil (28.66 ± 0.66/ 16.00 ± 0.00), Kavach (27.33 ± 0.66/ 16.66 ± 0.66) and Sectin (25.33 ± 0.66/ 18.66 ± 0.33) respectively (Fig 5 A-B).Among the four solvent extracts of two botanicals tested, ethyl acetate and methanol extracts of *A. indica* (29.33 ± 0.66 / 11.33 ± 0.33) (Fig 5 C-D) and *F. glomerata* (26.66 ± 0.66 / 10.00 ± 0.00) (Fig 5 E-F) showed significant results in both poison food and agar well technique respectively (Supplementary Fig 2-6).

Most of the cultivated cucurbits are more susceptible to GSB, although the degree of susceptibility varies among the hosts. The important phases are a foliar blight, stem canker, and fruit rot depends on host susceptibility [16]. According to the previous reports, muskmelon, watermelon, cucumber, *Cucumis sativus*, *Cucurbita pepo*, butternut squash, and tropical pumpkins are more susceptible to both foliar blight and canker phases of GSB [16]. *Cucurbita pepo*, *Cucumis melo*, *Crocus sativus*, and *Cucurbita moschata* are more susceptible to fruit rot than foliar blight [17-19]. The present study corroborates

with previous studies confirming the GSB disease symptomatology associated with ridge gourd in the study area. Chiu and Walker [6] and Koike [20] have also observed stem canker along with the gummy exudates in watermelon. Pandey and Pandey [21] gave an account of foliar infection caused by *D. bryoniae* on ridge gourd but there is no report of fruit rot disease. During the field survey, ridge gourd fruits infected with GSB exhibited black rot symptoms and secretion of gummy substance was observed in the present study; however, foliar blight, stem canker, and black rot symptoms were observed only when GSB disease is severe. The study highlights the percentage of disease incidence and a maximum of 45% disease incidence was observed during the month of June through November, 2018.

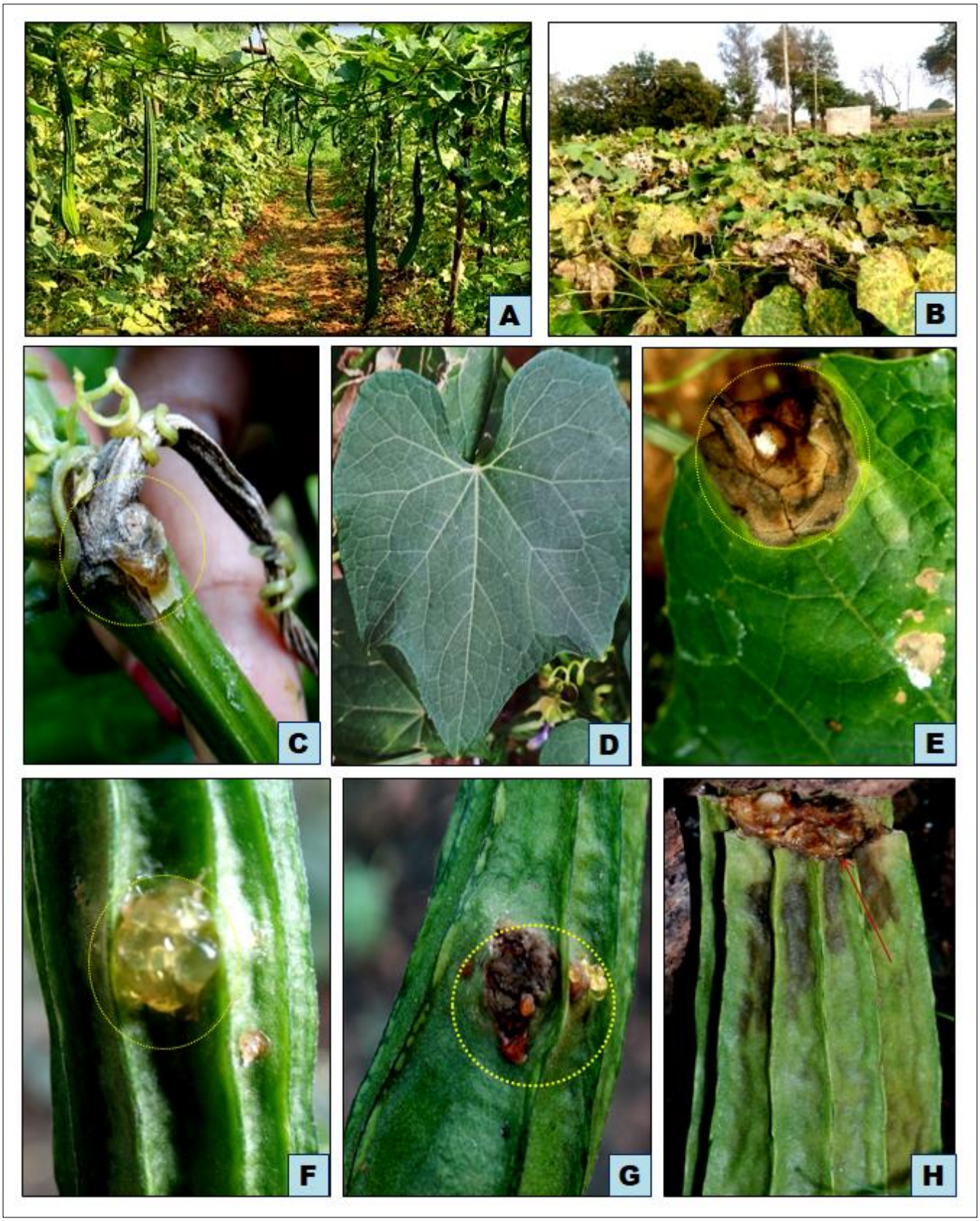

Fig 1 Gummy stem blight symptoms *in Luffa acutangula*: (A)- Healthy plants in a field; (B)- Symptoms associate with chlorosis and drying of the leaves; (C)- Oozing out of gummy substance from cracked stem; (D)- Healthy leaf; (E)- Lesions on infected leaves; (F & G) - Oozing of reddish brown gummy sticky substance from the fruits; (H) - Cut end of fruit exhibiting symptoms

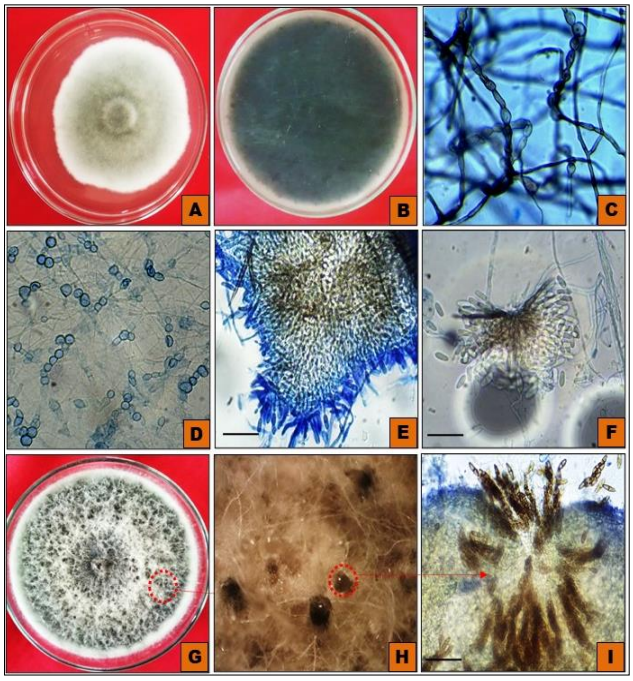

Fig 2 Culture plates and morphological characters of *Didymella bryoniae*: (A)- Colonies of *D. bryoniae* on PDA plates dorsal view; (B)- Ventral view; (C)- Mycelia on PDA after exposure to UV light; (D) - Chlamydospores in chain in hyphae; (E-F)- Conidia released from pycnidia; (G)- Culture plate with ascospores; (H)- Stereomicroscopic view of pathogen with ascospore; (I)- Microscopic view of Asci with ascospores

Fig 3 Pathogenicity test: Detached fruit test: (A-B)- White mycelial growth on fruit; (C)- Pycnidia formation on fruit: (D)- Stereomicroscopic view of pycnidia; (E-J)- Pot experiment: (E)- Before inoculation; (F)- After inoculation; (G)- Brown colour lesion on the cotyledonary leaf; Early symptom of GSB disease; (H)- Lesions on mature leaf; (I)- Cracking at the internodal region; (J)- Culture of pathogen re-isolated from infected plant

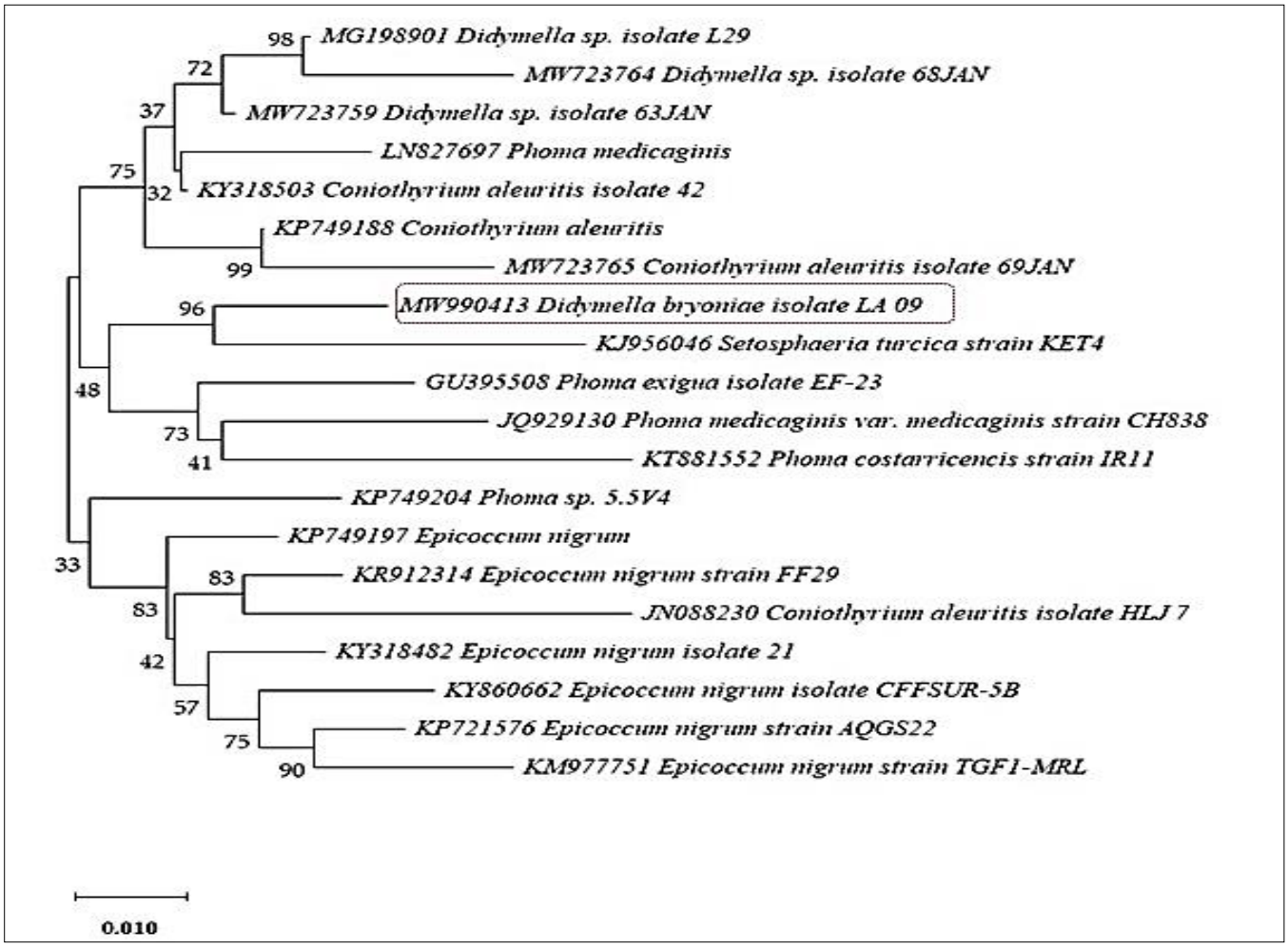

Fig 4 Phylogram constructed by Neighbour-joining method using reference sequence retrieved from NCBI GenBank (Tamura-Nei Substitution model and nearest neighbor interchange search options with 1000 bootstrap replicates were used; Bar shows that the estimated nucleotide substitution per site is 0.010)

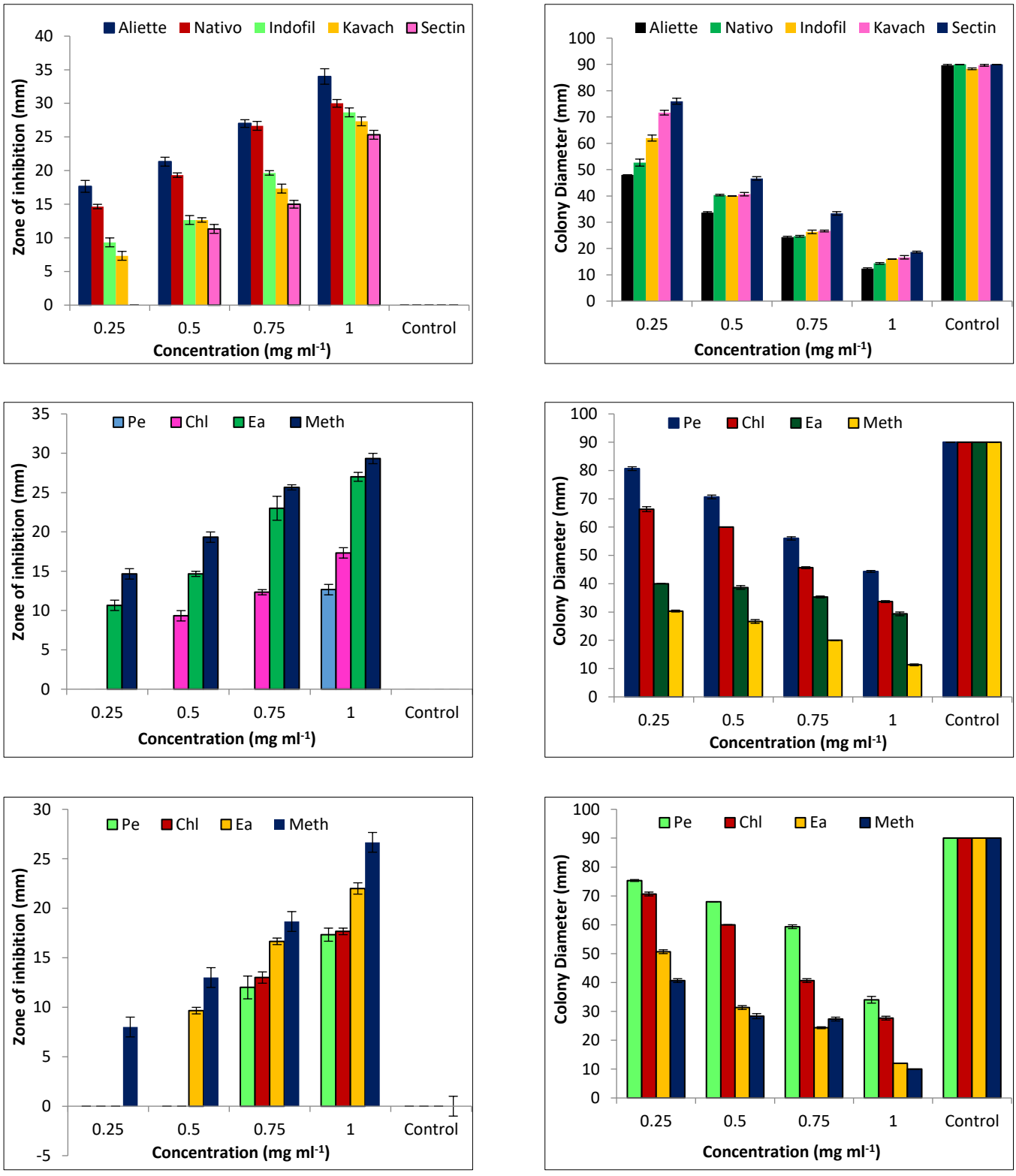

Fig 5 Inhibition of *D. bryoniae* mycelia growth at four different concentrations of selected fungicides and botanicals by Agar well technique and Poisoned food technique. (A & B): five different fungicides. (C & D): *Ficus glomerata* leaves extracts; (E & F): *Azadirachta indica*

Note: Error bars indicate significant differences ($p< 0.05$) in the antifungal activity among the groups

The morphological features on the culture plate and its microscopic study the pathogen was confirmed as *D. bryoniae* (Aureswarld) Rehm (Anamorph: *Phoma cucurbitacearum* (Foutrey) Sacardo). The work of Chiu and Walker [6] and Punithalingam and Holliday [5] corroborate our study to confirm the pathogen as *D. bryoniae* causing GSB on ridge gourd. Pathogenicity test was carried out for confirmation of pathogen. In earlier reports, Van Steekelenburg [22] observed symptoms in 3-4 days after inoculation on young cucumber plants. Bala and Hosein [23] have also reported that after 48 hrs of incubation with the spore concentration of $1x10^7$ *D. bryoniae* suspensions on the seedling of different cucurbitaceous crops, the plants started developing the typical disease symptoms in all the test plants where they have maintained in polythene tents at 26-28°C with high relative humidity. Detached injured leaves showed severe symptoms after three days of inoculation than in uninjured which corroborates the earlier report of Svdelius and Unestam [24].

Our results are strongly in agreement with earlier reports where Garampalli *et al.* [25] characterized the rDNA- ITS

sequencing of the pathogen causing GSB on gherkin from the Karnataka region and Santos *et al.* [26] carried out the analysis of new isolates of *D. bryoniae* from cucurbits collected in Brazil revealed that the isolates varied in pathogenicity and aggressiveness to different hosts. Babu *et al.* [27] reported that genetics characterization of 35 isolates of *D. bryoniae* from cucurbits revealed genetic variability. The previous work on ITS- rDNA has suggested that the sequence similarity creates a significant tool to identify the unknown organisms. With the molecular analysis of the isolated pathogen in the present work, the identity of the fungal pathogen was confirmed as *D. bryoniae* (Sexual stage: *Phoma cucurbitacearum*) which according to our knowledge, is a first report on ridge gourd.

Overall, the extent of mycelial growth inhibition of the tested fungicides increased with an increase in their concentration. The results are in accordance with the findings of Paret *et al.* [28]. Egel [29] examined different fungicide and suggested that chlorothalonil, mancozeb, azoxystrobin, pyraclostrobin, and cyprodinil provided the best control of GSB. Utkhede [30] reported a significant reduction in GSB infection in greenhouse cucumbers and a great increase in the yield of fruits due to the application of bioagents. The results of the present work revealed that, the isolated pathogen is susceptible to the all the fungicides used in *in vitro* study and can be used in the management of GSB disease. Further, the results of plants extract also proved to be effective and can be used as alternative fungicides if the grower practicing organic farming.

CONCLUSION

Gummy stem blight is a major disease of Cucurbitaceae members which causes maximum yield loss in tropical and sub-tropical countries. The present work reports GSB disease on ridge gourd for the first time from this region. Systematic investigation on the nature of symptoms and isolation, identification and molecular characterization of pathogen was carried out and confirmed as *Didymella bryoniae* responsible for causing GSB on *Luffa acutangula* with disease incidence of 30- 50%. Further, the work also enlightened on the disease management strategies by *in vitro* studies using five fungicides and two botanical extracts against the pathogen and revealed that the isolated pathogen was highly susceptible to all the fungicides and also to both the plant extracts. In conclusion, results of present investigation can be used in GSB disease management strategies.

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Conflicts of interest

The authors declared that they have no conflict of interest in the present work.

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