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Characterization and Etiolgy of Pathogen(S) Associated with Wilt Complex Disease of *Lagenaria siceraria* in Himachal Pradesh

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***Abstract*:** The microscopic examination of the diseased plant parts of bottle gourd revealed the association of three pathogens of the wilt complex namely, *Colletotrichum orbiculare*, *Fusarium oxysporum* and *Didymella bryoniae.* Pathogenicity test with each of the three isolated pathogens was conducted on the highly susceptible bottlegourd variety MHBG 8 and the Koch's postulates were proved by *Didymella bryoniae* thereby, confirming the pathogenicity of the test pathogen.*D. bryoniae*exhibited white cottony aerial mycelium and olive green to dark green or black substrate mycelium. Pycnidia were brown coloured and contained hyaline, cylindrical conidia having rounded ends with two guttules, non or mono septate, measuring 3-5 x 2-3 µm. Pseudothecia expressed as dark brown with hyaline pseudoparaphyses protruding from ostiole and contained numerous asci with 8 ascospores each. Ascospores were hyaline, oval, monoseptate and measured 5-8 x 3-5 µm in size. Blast results of the ITS sequences obtained through PCR amplification of the fungal DNA using ITS1 and ITS4 primers were 99 per cent coinciding with that of *Stagnosporopsis cucurbitcearum*. Thus, on the basis of, morpho-cultural characteristics and molecular characterization, the test pathogen is identified and confirmed as *Didymella bryoniae* (*Stagnosporopsis cucurbitcearum*) to be the cause of wilt disease of bottle gourd.

***Keywords*:** Wilt complex, Bottle gourd, *Didymella bryoniae*, Pycnidia, Molecular characterization

The occurrence of wilt complex disease in bottle gourd, grown in Himachal Pradesh, resulted in sudden drying or wilting of the vines leading to complete failure of the crop resulting in huge losses to the farmers. Many pathogens like *Erwinia* spp., *Xanthomonas* spp., *Rhizoctonia* spp., *Colletotrichum* spp., *Fusarium* spp., *Didymella* spp., and an insect pest squash bug (*Anasa tristis*) are associated with the disease, but the exact cause of disease is not known. The pathogen is a newly discovered threat to bottle gourd production in Himachal Pradesh, therefore its correct identification was necessary to establish an effective management strategy.*Didymella bryoniae* (Auersw.) Rehm [anamorph *Stagonosporopsis* cucurbitacearum (Fr.)Aveskamp, Gruyter & Verkley.] is a major fungal pathogen with a widehost range within the family Cucurbitaceae. The pathogen has been reported to occur naturally on *Lagenaria siceraria* in Southeast Asia (Thaung 2008).

Basim et al (2016) observed that *D. bryoniae* isolate under *in vitro* conditions showed white aerial and olivaceous mycelium in the initial stages of growth, whereas olive to dark green or black substrate mycelium in the latter part. The colony surface in the Petri dish was rough and undulated.PCR based technique has more recently been applied for the successful identification of *Didymella bryoniae* (Keinath and Dean 2001). The internal transcribed spacers (ITS) gene of the nuclear ribosomal DNA operon ITS region (Druzhinina et al 2005), has been proposed as standard loci for use in DNA barcoding in fungi. The use of ITS as fungal barcode locus is most popular (Seifert 2009) because ITS is more specific region for distinction at the species level (Singh et al 2005).

Rajkumar et al (2016) carried out the PCR reactions using the cycling conditions of an initial denaturation step at 94°C for 3 min followed by 35 cycles with a denaturation step at 94°C for 15 sec, annealing at 52°C for 40 sec, extension at 72°C for 1 min, followed by a final extension at 72°C for 5 min. Basım et al (2016) and Choi et al (2010) separated the amplified PCR products by gel electrophoresis in 2% agarose gel with 0.5X TAE buffer, stained by keeping the gel in the ethidium bromide (0.5 µg/ml) for 10 min, and then visualized under an ultraviolet (UV) light imaging system which produced fragment sizes of approximately 560 bp (Basım et al 2016). Tsai and Chen (2012) observed that PCR products amplified using primers ITS1 and ITS4 had 98 to 99% nucleotide sequence identity with *Didymella bryoniae*.Keeping in view the economic importance of the disease, a comprehensive study was designed to ascertain the causal agent of the sudden wilt malady of bottle gourd based on morphological and molecular identification.

MATERIAL AND METHODS

Sterilization of glass ware/plastic ware/soil: The glass ware was dry sterilized at 160-180^{0}C for 2 hr in a hot air oven. Growth media were sterilized in autoclave at 1.1 kg/cm^2 pressure for 20 min, whereas plastic ware was surface sterilized with 100 per cent alcohol.

Isolation and purification: Isolation of the fungal pathogen(s) was conducted from symptomatic fresh plant tissues observed microscopically. Small pieces of tissue (approximately 1 × 1 cm) were cut aseptically from the margin of the lesions. The pieces were disinfested in 1.0 per cent sodium hypochlorite for 15-20 sec, followed by rinsing three times with sterile distilled water, and finally blotted on sterilized filter papers to remove the excess moisture. The bits were then transferred to potato dextrose agar (PDA) and quarter strength potato dextrose agar (QPDA) medium slants under aseptic conditions and were incubated in BOD incubator at 25°C under 24 hr continuous photoperiod produced from cool white fluorescent bulbs. Precautions were taken to avoid contamination of culture from time to time. To obtain a pure culture from a single conidium, a small piece was cut from an area of the colony, taking care to avoid other microorganisms. The piece was put into 10 ml sterile water in a test tube, and was agitated for 20 to 30 sec to release conidia. The spore suspension was serial diluted and 1 ml spore suspension was poured in Petri plates containing 2 per cent water agar. Then after 1 day the Petri plates were examined under microscope, a single spore was marked and transferred to another petri plate containing potato dextrose agar or one quarter strength potato dextrose agar using fine tip needle. Fungal colony arising from single spore was multiplied on PDA or QPDA medium and used for further studies.

Pathogenicity Test

Preparation of inoculum: Inoculum was prepared by flooding a Petri dish of the 15 days old isolate growing on PDA or QPDA with sterilized distilled water and gently scraped to release conidia. The suspension was filtered through four layers of sterile cheese cloth to remove mycelial bits and media. Spore concentration/inoculum load was determined by counting the number of spores in a haemocytometer and adjusted to 1x10^5 conidia/ml.

Pathogen inoculation: To confirm the identity of isolated pathogen(s) obtained from bottle gourd plants, pathogenicity test was performed by inoculating the isolated pathogen on highly susceptible bottle gourd variety MHBG 8. Healthy plants of susceptible variety were raised in plastic pots (15 cm dia) filled with sterilized soil. Fully established 30 days old plants were inoculated by spray method using spore suspension of test pathogen. Sterile, distilled water was sprayed on corresponding control plants. The inoculated and control plants were placed in net-house at 25-30°C temperature and covered with plastic bags for 96 hr to maintain 100 per cent relative humidity and thereafter the pots were watered daily till the development of disease symptoms. The data on incubation and latent period was recorded. The pathogen was reisolated from the diseased plants to prove Koch's postulates.

Morpho-cultural studies: The pathogen was identified on the basis of morpho-cultural characteristics. Sterilized Petri plates containing equal quantity (20 ml) of PDA/QPDA medium were inoculated with 5 mm culture disc of 10-15 days old culture of pathogen and incubated at 25±1°C. Various morphological traits both, macroscopic (colony colour, colony type, colony growth, pigmentation) and microscopic (size, shape and colour of fruiting bodies and spores) were recorded. Fruiting bodies were placed on a droplet of sterile water on a microscopic slide and crushed to release the contents. The length × width of the spores from the pathogen was measured at 400X magnification and was examined for the presence of septa.

Molecular Characterization

Raising mycelium mass: Five mm bits of the pure culture of the fungus were incubated in liquid potato dextrose broth and kept at 25°C for 8-10 days in orbital shaking incubators. Mycelia (10-15 g) and liquid media were separated by pouring the culture through a funnel lined with filter paper. The mycelia was dried between paper towels and stored at 4°C for further use.

Protocol for DNA extraction: The dried mycelium was ground into fine powder using liquid nitrogen in sterilized pestle mortar. The 50 mg of this powder was taken in 1.5 ml eppendorf tubes and 750 µl of SDS buffer was added to it and incubated at room temperature for 15 min. After incubation 750 µl of Chloroform: Isoethyl alcohol (24:1) is added to it and centrifuged at 14000 rpm for 10 min. The aqueous phase was taken into a fresh eppendorf tube and 400 µl of isopropyl alcohol was added to it and incubated overnight at -20°C.Next morning the tubes were subjected to centrifuge action at 10000 rpm at 4 °C. The supernatant thus obtained was discarded and pellets formed were washed with 200 µl of 70 per cent ethanol and dried for two hr by inverting the tubes over paper towel/tissue paper. At the end, pellet was dissolved in 50 µl of TE buffer centrifuged for one min and stored at -80°C.temperature.

Preparation of agarose gel electrophoresis: The DNA extracted was checked for its quality and quantity using agrose gel electrophoresis. 1.2 per cent agrose gel was prepared by adding 1.2 gms of agrose in 100 ml of TAE buffer (0.5 X) and heated gently till the agrose gets completely

dissolved and the mixture becomes transparent. Ethidium di bromide (3 µl/100 ml TAE buffer) was added to the mixture and cooled down before pouring in gel electrophoresis plate on which combs were fixed to make wells. The gel was left undisturbed for 30 min for solidification.3 µl of extracted DNA was mixed with 1 µl of gel loading dye and loaded in the wells of electrophoresis plate that were submerged in 0.5 X TAE buffer.Gel was run at 180 V for one hour and then visualized in ultraviolet (UV) transminator.

PCR amplification and DNA sequencing: Amplification and sequencing of the pathogen DNA was performed using a pair of universal primers ITS1 (5'TCCGTAGGTGAACCTGCGG3') and ITS4 (5'TCCTCCGCTTATTGATATGC3'). PCR reactions were carried out in a total volume of 50 µl. Reaction components included 5 µl of 10X PCR buffer, 2 µl dNTPs, 2 µl of 10 µM forward and reverse primers (ITS1/ITS4), 0.8 µl ExTaq, 3 µl of $MgCl_2$, 2 µl DNA template and 33.2 µl of water. Cycling conditions included an initial denaturation step at 94°C for 3 min followed by 35 cycles with a denaturation step at 94°C for 15 sec, annealing at 52°C for 40 sec, extension at 72°C for 1 min, followed by a final extension at 72°C for 5 min**.**The amplified PCR products were separated by gel electrophoresis in 1.2 per cent agarose gel with 0.5X TAE buffer as described above plus 3 µl of 1 kb plus ladder and water as negative control were also loaded in the adjacent empty wells for comparison and then visualized in ultraviolet (UV) transminator. The amplified PCR products were sent for custom sequencing.

RESULTS AND DISCUSSION

Isolation of the pathogen: Microscopic examination of the fresh samples of diseasedbottle gourd plants collected from different locations of the districts surveyed during cropping season 2020, exhibited presence of *Colletotrichum orbiculare, Fusarium oxysporum* and *Didymella bryoniae*. *Colletotrichum orbiculare* and *Fusarium oxysporum* were isolated on PDA and *Didymella bryoniae* on QPDA. The typical colony of *Didymella* developed on QPDA expressed as white aerial mycelium and olive to dark green or black subsrate mycelium. The results on the description of fungal growth culture of *Didymella bryoniae* are similar to those described by Jensen et al (2011), Keinath (2013) and Rajkumar et al (2016).

Pathogenicity: Data on pathogenicity test with each of the three isolated pathogens *viz., Colletotrichum orbiculare, Fusarium oxysporum* and *Didymella bryoniae* on the highly susceptible bottlegourd variety MHBG 8 reveals the expression of variable disease symptoms (Table 1, Fig. 1). *Colletotrichum orbiculare* produced water-soaked lesions on leaves that later turn into dark brown or black spots with an incubation period of 3 days and latent period of 5 days. *Fusarium oxysporum* caused vascular browning, root rot, main stem browning and wilting with 6 days of incubation period and 11 days of latent period. *Didymella bryoniae* caused water-soaked lesions on leaf margins, stem canker, gummy exudation from vines and leaf wilting with an incubation period of 3 days and a latent period of 10 days.The symptoms produced by *Didymella bryoniae* on inoculated bottlegourd plants were similar to those of the wilt disease under study and the pathogen was reisolated from diseased plants thereby proving Koch's postulates. Hence, confirming the pathogenicity of the test pathogen. Symptoms produced by *Didymella* on bottlegourd plants possessed marked appearance of the characteristic symptoms following lesion formation, canker production, gummy exudation, stem cracking and finally wilting of the vines. Similar observations have been described by Keinath (2013) giving an account of variation in symptomatology on the basis of their prevailing weather conditions.

Morphological identification of the pathogen: The test pathogen exhibited white cottony aerial mycelium and olive green to dark green or black substrate mycelium (Table 3, Fig. 3). Pycnidia was brown coloured and contained hyaline, cylindrical conidia having rounded ends with two guttules, non or mono septate, measuring 3-5 x 2-3 µm. Pseudothecia was of dark brown colour having hyaline pseudoparaphyses protruding from ostiole. Pseudothecia measured 100-120 x 40-80 µm and contained numerous asci with 8 ascospores each. Ascospores were hyaline, oval, monoseptate and

Table 1. Pathogenicity of pathogen(s) associated with wilt complex disease of bottlegourd

Pathogen	Symptom development	Incubation period (days)	Latent period (days)
Colletotrichum orbiculare	Water-soaked lesions on leaves that later turn into dark brown or black spots	3	5
Fusarium oxysporum	Vascular browning, root rot, main stem browning and wilting	6	11
Didymella bryoniae (Stagnosporopsis cucurbitacearum)	Water-soaked lesions on leaf margins, stem canker, gummy exudation from vines, leaf wilting	3	10

appear as two unequally sized triangles joined at the bases, pointing in opposite directions. They measured 5-8 x 3-5 µm in size. Basim et al (2016) observed that *Didymella bryoniae* isolate *in vitro* possess white aerial and olivaceous mycelium in the initial stages of growth and olive to dark green or black substrate mycelium in the latter part.Rajkumar et al (2016) observed hyaline and cylindrical conidia with rounded ends, non or mono septate and measuring 6-13x3-4 µm in size and hyaline ascospores measuring 15-21x5-8 µm in size that are monoseptate with two cells of differing sizes. Keinath (2013) mentioned dark brown to black coloured pseudothecia containing hyaline pseudoparaphyses protruding from the ostiole which are in accordance to the studies with respect to the test pathogen. Thus, the characteristic features of the test pathogen related, revealed its identity as *Didymella bryoniae*.

Molecular characterization of the pathogen: DNA was

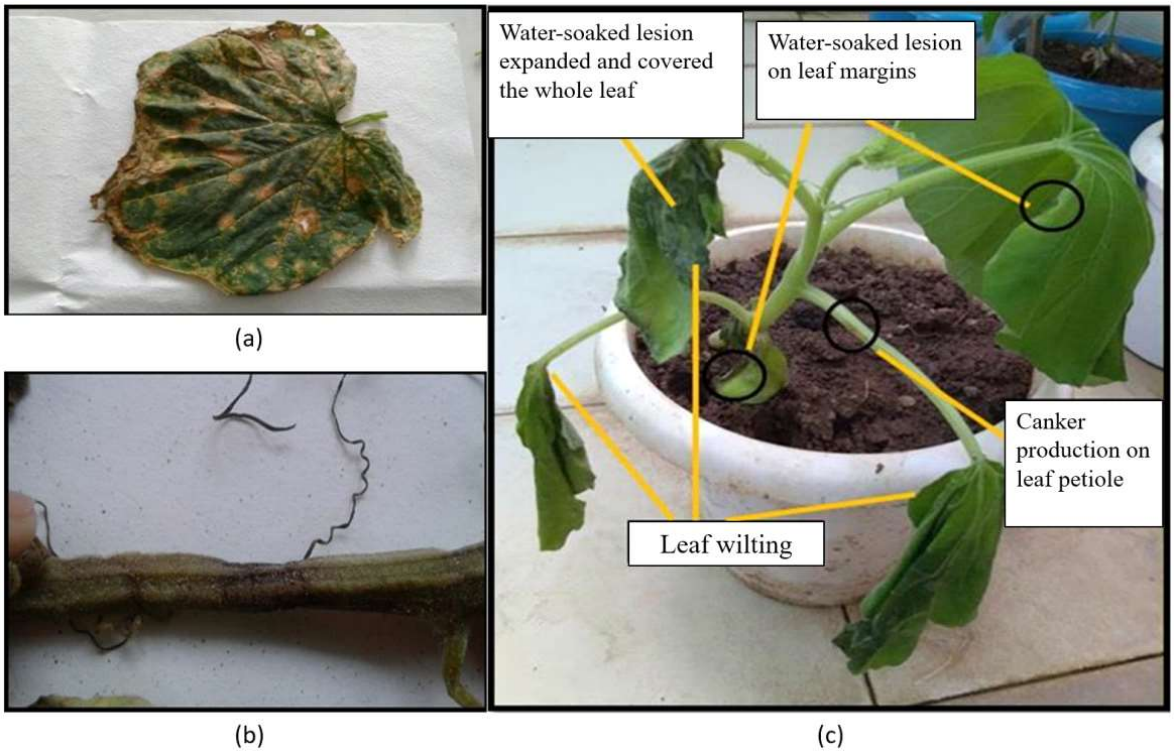

Fig. 1. Pathogenicity test of the isolated pathogen(s); (a) *Colletotrichum orbiculare* (b) *Fusarium oxysporum (*c) *Didymella bryoniae*

Table 2. Pathogen (s) associated with wilt complex disease of bottle gourd in different districts of Himachal Pradesh

District	Plant parts observed	Microscopic observations	Pathogen associated	Frequency of occurrence (%)
Kangra	Leaves	Acervuli and conidia	*Colletotrichum orbiculare*	30.0
	Leaves and roots	Macro and micro conidia	*Fusarium oxysporum*	10.0
	Leaves and stem	Conidia	*Didymella bryoniae (Stagnosporopsis cucurbitacearum)*	60.0
Hamirpur	Fruits	Acervuli and conidia	*Colletotrichum orbiculare*	10.0
	Stem	Macro and micro conidia	*Fusarium oxysporum*	25.0
	Stem and fruits	Pseudothecia and ascospores	*Didymella bryoniae*	65.0
Bilaspur	Fruits and leaves	Conidia	*Colletotrichum orbiculare*	25.0
	Stem and leaves	Macro and micro conidia	*Fusarium oxysporum*	15.0
	Fruit and stem	Conidia and ascospores	*Didymella bryoniae*	60.0
Una	Stem and leaves	Macro and micro conidia	*Fusarium oxysporum*	20.0
	Leaves, stem and fruit	*Pycnidia and conidia, pseudothecia and ascus*	*Didymella bryoniae*	80.0

extracted using SDS extraction buffer and checked for its quality in 1.2% agarose gel (Fig. 4). The amplified product using ITS1 and ITS4 primers were electrophoresed in 1.2% agarose gel and visualized in UV transamination (Plate 4.6). Amplification of 550 bp was obtained. Then the amplified product was sent for custom sequencing. The sequences obtained were as:

CAATACAATCCTTGGTATTCCATGGGGCATGCCTGT
TCGAGCGTCATTTGTACCTTCAAGCTTTGCTTGGTGTT
GGGTGTTTGTCTCGCCTCTGCGCGCAGACTCGCCTCA
AAACGATTGGCAGCCGGCGTATTGATTTCGGAGCGCA
GTACATCTCGCGCTTTGCACTCACAACGACGACGTCC
AAAAAGTACATTTTTTACACTCTGACCTCGATCATGATG
TGCCGCGTCCAGCCTCAAACCG

The sequence analysis using NCBI blast program showed 99% homology with the reference sequences of

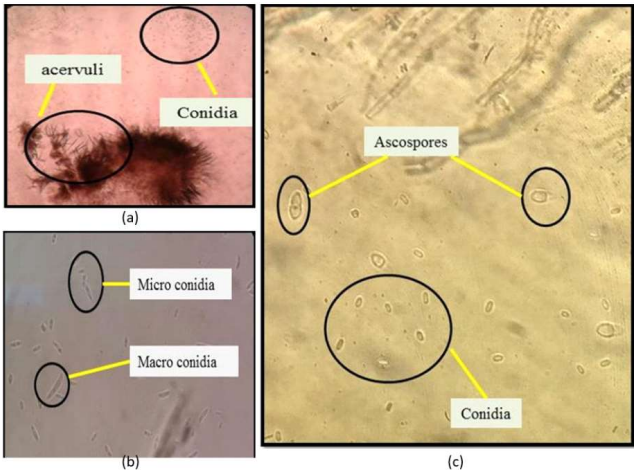

Fig. 2. Microscopic examination of the diseased plant samples; (a) *Colletotrichu orbiculare* (b) *Fusarium oxysporum* (c) *Didymella bryoniae*

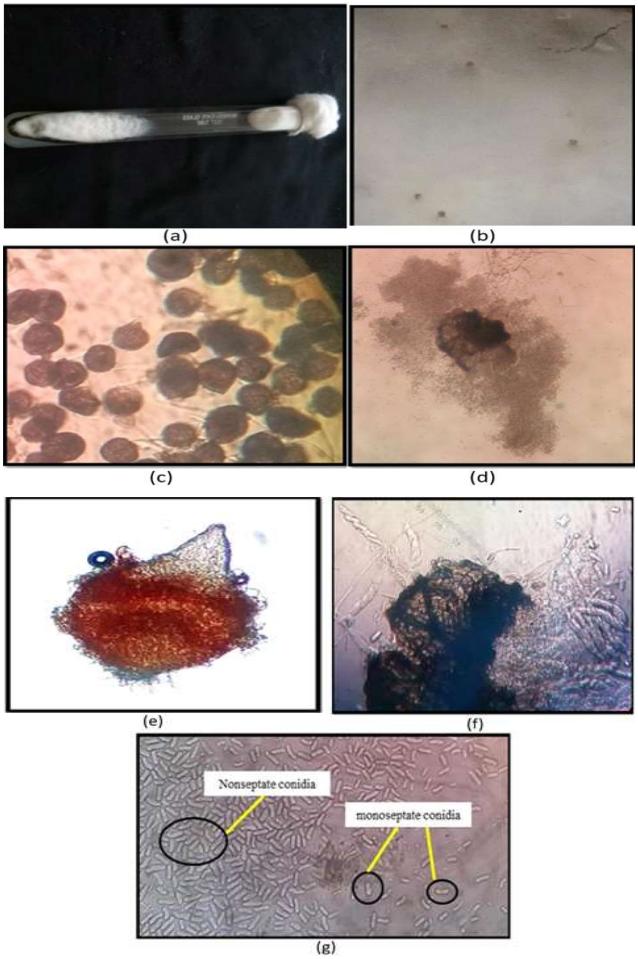

Fig. 3. Morpho-cultural features of the test pathogen causing wilt disease of bottle gourd; (a) pure culture of test pathogen, (b) fruiting bodies on culture, (c) brown coloured pycnidia, (d) pycnidia liberating conidia (e) pseudothecia with hyaline pseudoparaphyses, (f) pseudothecia liberating asci, (g) mono septate and non septate conidia

Table 3. Morpho-cultural features of the test pathogen causing wilt disease of bottle gourd

Character	Isolated test pathogen	*Didymella bryoniae**
Aerial mycelium	White coloured aerial mycelium	White aerial mycelium
Substrate mycelium	Olive to dark green or black coloured substrate mycelium	Olive to dark green or black substrate mycelium
Pycnidia	Brown coloured	Tan or brown coloured with a dark ring of cells around the ostiole
Conidia	Hyaline, cylindrical, rounded ends with two guttules, non or monoseptate, 3-5 x 2-3 µm	Hyaline, oblong to cylindrical with rounded ends,mainly with two guttules, and non or monoseptate, 6-13 x 3-5 µm
Pseudothecia	Dark brown, hyaline pseudoparaphyses protruding from ostiole, asci with eight ascospores, 100-120 x 40-80 µm	Dark brown to black, slightly immersed in the host tissue, hyaline pseudoparaphyses are present, protruding from the ostiole. Contain numerous asci with eight ascospores each, pseudothecia ranged from 195-205 µm in diameter
Ascospores	Hyaline, monoseptate, oval, appear as two unequally sized triangles joined at the bases, pointing in opposite directions, 5-8 x 3-5 µm	Hyaline and monoseptate, 13-21 µm in length and 5-8 µm in width, they are oval and appear as two unequally sized triangles joined at the bases, pointing in opposite directions

*Keinath et al 1995

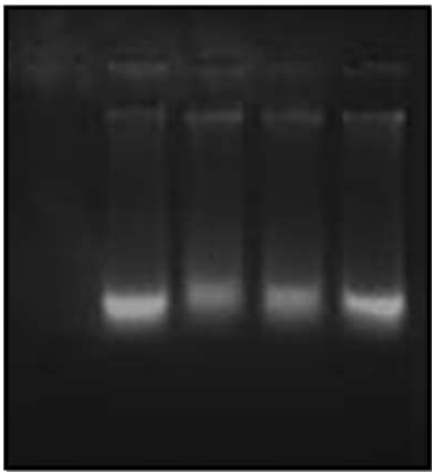

Fig. 4. DNA check of *Didymella bryoniae*

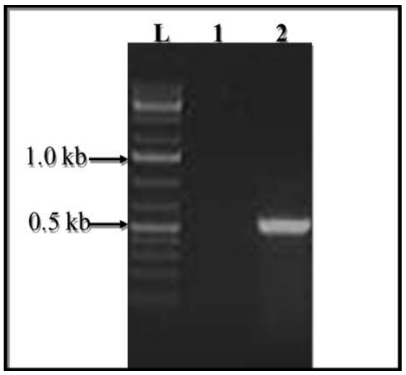

Plate 4.6. PCR amplification of ITS region using ITS 1 and ITS 4 primers.

L: 1 kb plus ladder, 1: Negative control, 2: PCR with fungal DNA

Stagnosporopsis cucurbitcearum. Thus the identity of the test pathogen was established as *Didymella bryoniae* (*Stagnosporopsis cucurbitcearum*). Based on the morpho-cultural characteristics and molecular characterization, the test pathogen is identified and confirmed as *Didymella bryoniae* (*Stagnosporopsis cucurbitcearum*) to be the cause of the disease.

CONCLUSION

Out of the three pathogens associated with wilt complex of bottle gourd, *Didymella bryoniae* proved to be the causal agent of the disease. It produces the characteristics symptoms of the disease when inoculated into healthy plants. It exhibits white cottony aerial myceliuma produces brown pycnidia and hyaline conidia with rounded edges. Pseudothecia produced are dark brown in color. The BLAST results of ITS sequences showed 99 per cent similarity with *Stagnosporopsis cucurbitcearum.* Therefore, based on morphological and molecular characters we were able to identify and confirm the *Didymella bryoniae* as the causal agent of wilt disease of bottle gourd.

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