

The pathogenicity test, cultural and morphological characterization of anthracnose (*Colletotrichum lindemuthianum*) of green gram (*Vigna radiata* L. Wilczek)

Abstract:

Aims: The objective of this study was to identify the appropriate anthracnose fungus of green gram using symptomatology, identification, pathogenicity test and characterization of samples.

Study design: Pathogenicity test, cultural and morphological characterisation

Place and Duration of Study: The laboratory studies were conducted in the Department of Plant Pathology, N. M. College of Agriculture, Navsari Agricultural University, Navsari, Gujarat during rabi 2020-21.

Methodology: The sample was collected and subjected to isolation, purification and identification of pathogen. Four different pathogenicity tests were conducted to verify the pathogen causing anthracnose of green gram. On the other hand, the cultural and morphological characteristics of pathogen were studied.

Results: The pathogenicity test confirmed that the pathogen as *Colletotrichum lindemuthianum* in all four tests. Spraying inoculum after needle pricking and carborandum powder producing 100% infection in inoculated plants while 96% in spraying spore suspension without any abrasion. The less per cent of infection was observed in seed inoculated plants. The cultural studies of *C. lindemuthianum* shown sub-aerial whitish-to-pink cottony growth and pinkish-to-brown pigmentation under the culture plate. The morphological study of *C. lindemuthianum* observed as single-celled, hyaline, cylindrical, and smooth with oil globules. The acervuli were blackish brown and measure $181.3 \times 254.6 \mu\text{m}$. The setae were visible through the conidial mass with 1-3 septations.

Conclusion: A study on green gram anthracnose confirmed *C. lindemuthianum* as pathogen in all four pathogenicity test with needle pricking and carborandum powder producing 100% infection in inoculated plants while 96% in spraying spore suspension without any abrasion. Cultural studies revealed whitish-pink cottony growth and pinkish-brown pigmentation. *C. lindemuthianum* was morphologically observed as single-celled, hyaline, cylindrical, smooth, with oil globules, blackish brown acervuli, and visible setae through conidial mass with 1-3 septations. Therefore, these findings are important for identifying the anthracnose disease of green gram.

Keywords: Anthracnose, cultural characteristics, green gram, morphology, pathogenicity

1. INTRODUCTION

The greengram [*Vigna radiata* (L.) Wilczek] is a significant Asian legume. This crop is widely used for a variety of purposes, including grazing, green manure, and seeds. It is also known as the "Golden Bean." because it is nutritious and essential for supporting life soil fertility by enhancing the physical characteristics of the soil and stabilising atmospheric nitrogen [1]. Along with India, South Asian nations including Bangladesh, Sri Lanka, Thailand, Cambodia, Pakistan, Vietnam, Indonesia, Malaysia, and South China also cultivate it extensively. In India, 20.89 million tonnes of grains are produced from 2.37 million ha of mungbean crop [2]. It is extensively cultivated in the following Indian states: Rajasthan, Gujarat, Maharashtra, Orissa, Karnataka, Bihar, Madhya Pradesh, Tamil Nadu, Punjab, West Bengal, and Haryana [3]. Anthracnose of green gram has been reported from various regions of India. The disease may occur in mild to severe form. The disease may deteriorate qualitative traits of crop by reducing the seed quality and affect quantitative characteristics with yield decline [4]. The term 'anthracnose' means 'coal like' and the word anthracnose was first coined by Fabra and Dunal (1883) while working on disease of grape that cause blackening of tissue and it was associated with pathogen *Colletotrichum* sp. [5]. Now the genus constituting 66 species which are listed in current literature according to recent reviews [6,7]. Three varieties that has been reported to cause green gram anthracnose in India are *C. truncatum*, *C. lindemuthianum* and *C. dematium* [8,9,10]. The green gram anthracnose was first reported in India from Jorhat of Assam state in 1951 [11]. Anthracnose of mungbean caused by *C. truncatum* in Assam was reported in 2004 [12].

Subsequently, in Karnataka same species was identified on green gram in 2006 [13]. The kharif season in south Gujarat is when anthracnose in greengram is most widespread because of the consistent favourable weather during the cropping season [1]. Considering the growing and detrimental nature of *Colletotrichum* spp. the research that follows on the green gram anthracnose fungus highlights the need for a cultural and morphological reexamination of the aforementioned organism as well as for correct identification and confirmation of pathogen through pathogenicity test. The objective of the research was to isolate the causal agent of green gram anthracnose, and perform pathogenicity tests.

2. MATERIALS AND METHODS

2.1 Collection of diseased sample

The green gram sample showing typical symptoms of anthracnose was collected from Pulse Research Station, NAU, Navsari.

2.2 Symptomatology of fungal pathogen

To study symptomatology of fungal pathogen, the visual and microscopic examination of anthracnose sample was carried out. The typical signs and symptoms of anthracnose on leaves and pods under natural condition were observed and critically recorded. The collected sample was placed in blotting paper and preserved for further studies.

2.3 Isolation and purification of fungal pathogen

To isolate the pathogen, the diseased sample was cut into small pieces along with some healthy tissues and surface sterilized with 0.1 per cent mercuric chloride solution for 1 minute followed by three washes with distilled water. The surface sterilized pieces were transferred to 20 ml poured potato dextrose agar (PDA) plates and incubated at $28\pm 2^{\circ}\text{C}$. After seven days of incubation, the fungal growth was transferred aseptically to PDA slants and purified following hyphal tip method.

2.4 Identification of fungal pathogen

The pathogens were tentatively identified through microscopic study of pure cultures through cultural and morphological characteristics. Cultural characteristics such as mycelial growth, colony diameter, colony colour, acervuli and sporulation were observed. Morphological characteristics of the fungal structures were studied in cotton blue stained slides under light microscope. Microscopic observations of size of conidia and conidiophore were made at magnification of 10X and 40X. The cultures were further confirmed and identified with comparing to earlier literatures. The microphotograph was also taken. Cultures were maintained on PDA slants by sub culturing and stored at 5°C for further study.

2.5 Pathogenicity test

2.5.1 Spraying spore suspension using atomizer:

The pathogenicity test of pathogen was proved by artificial inoculation of pathogen by following standard method of inoculation (Koch's Postulate). The pathogenicity test of leaves of green gram was carried out in pot under glass house condition. Ten earthen pots were filled with sterilized soil and FYM in the ratio 3:1. Five seeds of greengram variety were sown and each of the pots were covered with plastic to avoid any infection. The pots were labelled, watered gently and arranged in the green house. The pots were watered up to saturation on the morning of inoculation. The leaves were surface sterilized with 0.1% mercuric chloride solution and washed thoroughly with sterile distilled water to remove the traces of mercuric chloride. Spore suspension (1×10^6 spores/ml) spray inoculation was carried out at 2 to 3 boot leaf stage in the evening hours with the help of atomizer. Ten replications were maintained by keeping one control pot. The control pot without inoculation was sprayed with distilled water. The inoculated and uninoculated pots were covered with polythene bag for 48 h to bring high humidity. The observation on disease development was recorded periodically from initiation of the disease. Re-isolation was made from the artificially inoculated plants showing typical anthracnose symptoms by tissue isolation method and identity of the fungus was confirmed as per the original description. The culture obtained by re-isolation was transferred on PDA slants for comparison with original culture and for further studies.

2.5.2 Seed inoculation with pathogen culture:

The healthy seeds of green gram were surface sterilized by dipping them in 1% solution of sodium hypochlorite for 2 min and then washed in distilled water. These seeds were inoculated with pathogen by soaking in the inoculum. And the inoculated seeds were sown to pot with control pot.

2.5.3 Rubbing fungal suspension with carborandum powder:

The green gram plants were inoculated by fungal suspension with help of cotton swab on both the surface of leaves. The cotton swab was soaked in inoculum carrying some carborandum powder (300 mesh) for making gentle injury and then applied inoculum simultaneously.

2.5.4 Spraying of inoculums after multiple needle pricking:

The leaves were injured with the help of multiple needle prepared by fixing 8-10 fine pins with the help of a rubber band tightly. The injury to the leaves should be gentle so that it does not tear or perforate leaf surface. The fungal suspension was atomized on the leaf surface.

Suitable controls were maintained in each case using distilled water in place of inoculum suspension. The plants were inoculated by spray inoculation respective methods and the potted plants were replaced again in humid chamber in green house (RH 78-91%, and $28\pm 2^{\circ}\text{C}$ for 48h) and later kept under natural conditions. The plants were watered at regular interval and frequently observed for disease development. The pathogen was reisolated from inoculated plants as described earlier and then compared with the original culture.

2.6 Study of Cultural Characteristics of pathogen

To study the cultural characteristics, 20ml of sterilized PDA media was poured into Petri plates. Five mm disc was cut from the seven days old fungal culture growth through sterilized cork borer. One disc was placed at the centre of each Petri plate and keep for incubation at $28\pm 2^{\circ}\text{C}$ for seven days. Observation on cultural characteristics viz., mycelial growth, colony diameter, colony colour, acervuli and sporulation were taken.

2.7 Study of Morphological Characteristics

The morphological studies were done on seven day old culture of pathogen culture grown on PDA plates. The observations were recorded using micrometry under light microscope at 40X magnification. Ten observations of each morphological characters were recorded and mean values were calculated. Observation on morphological characteristics viz., length and width of conidia, mycelial width and size of conidiophores shape and conidia were taken.

3. RESULTS AND DISCUSSION

3.1 Collection of diseased samples

During the cropping season 2020-21, the green gram variety GM-6 grown in Pulse Research Station, N. M. College of Agriculture, N. A. U., Navsari depicting typical symptoms of green gram anthracnose were collected and stored in polythene cover and brought into laboratory for further studies. In the laboratory, samples were observed under microscope to confirm the structures of pathogen.

3.2 Symptomatology

During symptomatologic study, anthracnose of green gram was seen in all stages of the crop growth and on all parts of plant viz., leaves, petiole and pods. On hypocotyl region, the disease developed as dark brown to black sunken lesions and caused necrosis of seedlings. Small angular lesions on leaves were seen which later turned into greyish white centre with dark brown or reddish margin. These several spots were coalesced to form necrotic region. Usually, these spots were appeared adjacent to leaf veins. This disease led to serious leaf spotting and produced 'shot hole' symptoms and finally resulted in yield reduction due to defoliation of leaves. Under severe condition, prominent spots were formed on pods. Initially water soaked lesions were produced on pods. Later these spots were changed into brown, enlarged, circular, depressed spots with dark brown centre and bright yellow or red margin [Fig. 1]. Such infected pods produced discoloured seeds. Due to pod infection, direct damages on seed germinability was shown.

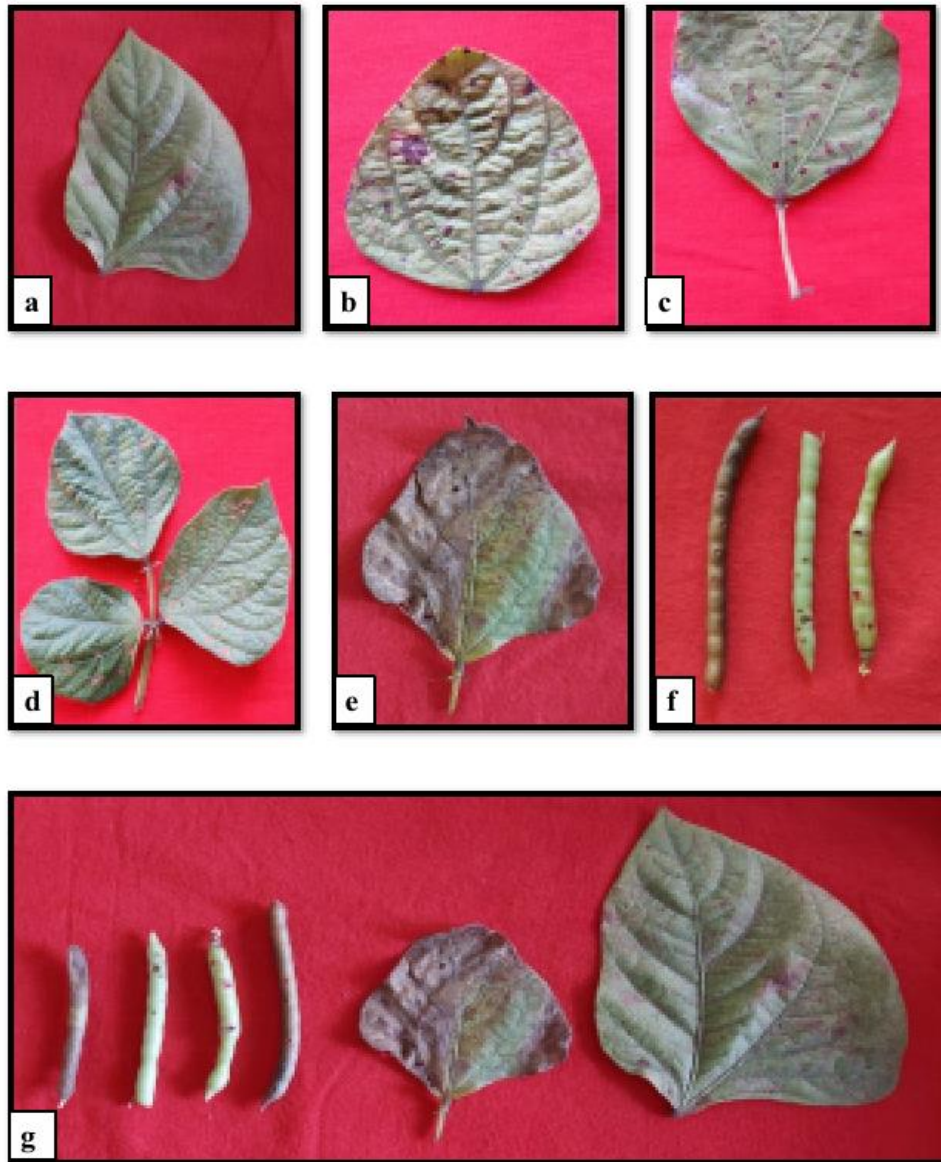


Fig. 1: Typical symptoms of anthracnose on green gram. (a) Initial small spots on leaf; (b) Typical horse shoe shaped spot on leaf; (c) Spot on petiole; (d) Shot hole symptom on trifoliate leaf; (e) Necrosis of leaf; (f) Sunken spots on pods; (g) All set of symptoms in one frame

Similar kind of symptoms on green gram anthracnose were observed by early research carried out by other researchers [12, 13, 14, 15, 16].

3.3 Isolation and purification of fungal pathogen

The fungal pathogen causing anthracnose of green gram was isolated from the diseased sample collected from the Pulse Research Station, N. A. U., Navsari. Tissue isolation technique was employed to isolate fungal pathogen from naturally infected leaves and pods. This method yielded pure culture of pathogen. The obtained pure culture was transferred to fresh PDA slants and stored in refrigerator condition for further investigations.

Previous studies have also used comparable techniques for the isolation and purification of *Colletotrichum* spp. [17, 18, 19, 20, 21, 22].

3.4 Identification of fungal pathogen

The fungal culture isolated and purified was stored for conducting further research work. The hyphal tip method was used to transfer the culture to PDA slants. After required incubation period, these slants were stored at 5°C. In order to maintain viability of pathogen, subculturing was done regularly. The fungus was identified on basis of cultural, morphological and microscopic observation. The microscopic structures were scrutinized under microscope at magnification of 10X and 40X. Based on cultural and morphological characters, the pathogen was recognised as *C. lindemuthianum* [17, 18, 19, 20, 21, 22].

3.5 Pathogenicity test of pathogen

Different methods of pathogenicity tests were carried out in order to prove Koch's postulate. A susceptible variety vaibhav was chosen for pathogenicity test and five seeds were sown in each pot. Ten such pots were maintained along with control pot. The twenty days old seedlings (two to three boot leaf stage) were inoculated. The following were methods performed for pathogenicity tests.

3.5.1 Spraying spore suspension using atomizer:

The twenty days old seedlings (two to three boot leaf stage) were inoculated with fungal spray suspension during evening hours. Polythene bags were covered to both inoculated and control pots (Fig 2a). The first symptoms were observed in form of small and translucent water soaked lesion after 5-7 days after inoculation. Later lesions changed into dark brown, dark coloured and irregular shaped spots after 10-15 days which is depicted in Fig. 2(b). When two leaved stage seedlings were inoculated, a rapid and massive colonization of pathogen was seen. It was also found that youngest leaves were more susceptible than older leaves. The per cent infection observed was 96% (Table 1).

Table 1: Pathogenicity test of *Colletotrichum lindemuthianum* on green gram plant/ seeds under *in vitro* condition

No.	Inoculation method	Inoculated plants/ seeds	Infected plants/ seeds	Infection (%)
1	Spraying spore suspension with atomizer	50	48	96%
2	Seed inoculation with fungal culture	50	11	22%
3	Rubbing fungal suspension with carborandum powder	50	50	100%
4	Spraying of inoculum after multiple needle pricking	50	50	100%
5	Control	20	0	0%

3.5.2 Seed inoculation with fungal suspension

The pathogenicity test was proven by seed inoculation of fungal suspension. Such inoculated seeds were sown in pot and control pot was also maintained by inoculating with distilled water. The pre-emergence mortality was observed due to seed inoculation with pathogen. And also young seedlings were withered off after germination. The per cent infection development was 22% (Table 1). The mortality of green gram seedlings after emergence is shown in Fig. 2c.

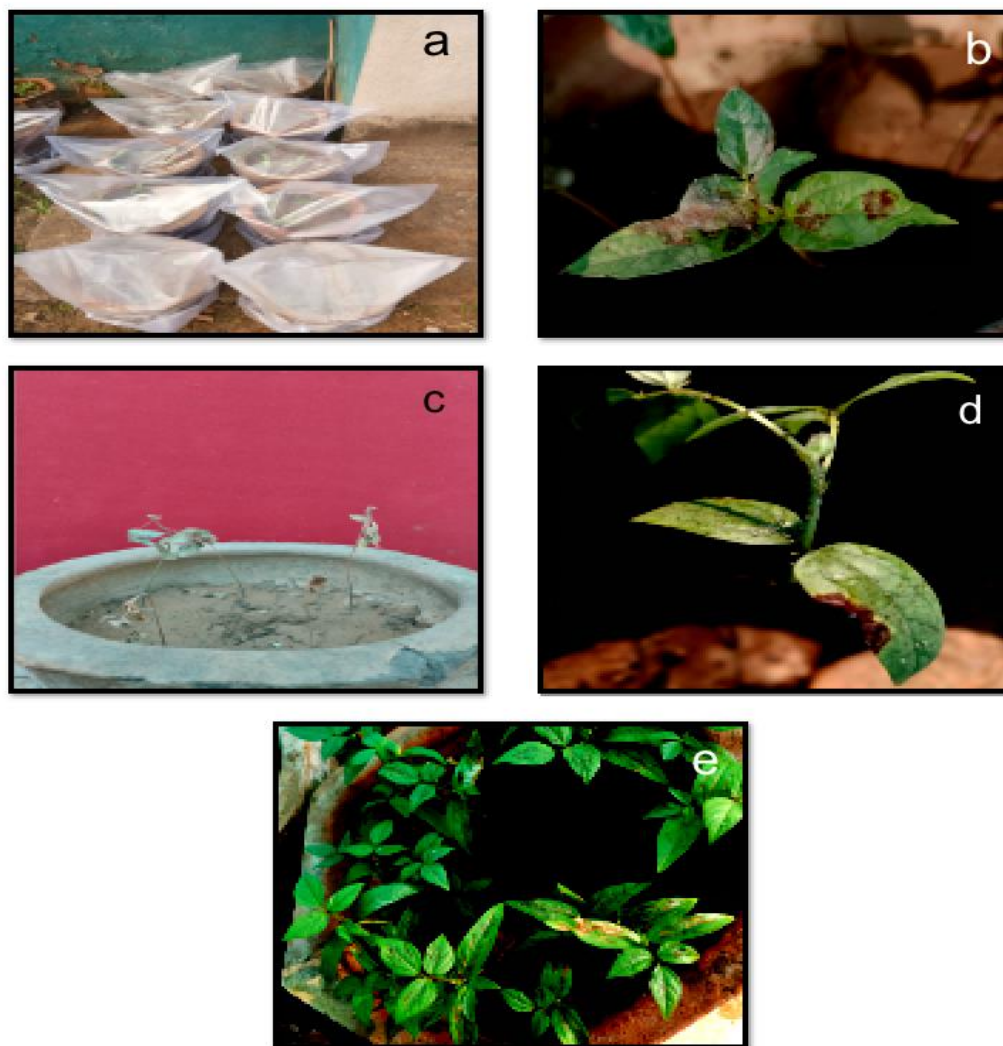


Fig. 2: a) Pots sprayed with fungal spore suspension, b) Typical anthracnose spot appeared on plant sprayed with spore suspension after 10-15 days, c) Green gram plants dying even after germination, d) Disease development in green gram plant after rubbing leaves with fungal suspension and carborundum powder and e) Disease development in green gram plant after pricking with multiple needle and spraying inoculum

3.5.3 Rubbing fungal suspension with carborandum powder:

The green gram plants were inoculated by fungal suspension with carborandum powder. The symptoms started appearing after 3 days of inoculation. Small anthracnose spots appeared throughout the leaf blade depicted in Fig. 2d. The per cent infection was found cent per cent (Table 1). All the inoculated plants were infected by fungal pathogen.

3.5.4 Spraying of inoculum after multiple needle pricking:

The green gram leaves were pricked with the help of multiple needle. Then it was sprayed with fungal suspension. Small brown spots appeared 3-5 days after inoculation and covered whole leaf blade depicted in Fig. 2e. Later these spots coalased and blighted leaves were seen. The per cent infection was found cent per cent. All the inoculated plants were infected by fungal pathogen (Table 1). It is evident from the results of pathogenicity test that anthracnose pathogen *C. lindemuthianum* was able to infect leaves, petioles and pods of green gram plant irrespective of inoculation method. But per cent of infection occurred differs in all inoculation methods. The pathogen was again reisolated from the artificially inoculated plant and obtained the culture similar to earlier culture. Hence, the Koch's postulate was proved.

Equivalent pathogenicity test results were developed in the experiment conducted for seed inoculation of pathogen effective in proving pathogenicity test [19, 20]. Spraying of inoculum of anthracnose pathogen as best method to prove pathogenicity test in black gram cultivars was report in 2019 [22].

3.6 Cultural characteristics of pathogen

The cultural characteristics of pathogen was investigated on culture growing on solid potato dextrose medium at 27°C. The culture was subcultured using hyphal tip technique. The cultural characterization on colony colour, colony diameter, mycelial growth, acervuli formation, acervuli density, pigmentation, zonation and sporulation are recorded in table 2.

Similar cultural characteristics of *C. lindemuthianum* was reported in earlier articles [18,21,23]. The cultural appearance of *C. lindemuthianum* is shown in Fig. 3.

Table 2: Cultural characterization of pathogen

No.	Colony characteristics	
1	Type	Sub aerial
2	Colour	Whitish to pink cottony growth
3	Zonation	Indistinct
4	Radial growth	90.00 cm
5	Acervulus formation	Scattered
6	Acervulus distribution	20-25 days
7	Sporulation	Excellent sporulation
8	Pigmentation	Pinkish to brown colour
9	Growth rate	Very slow
10	Maximum growth rate	13-15 days

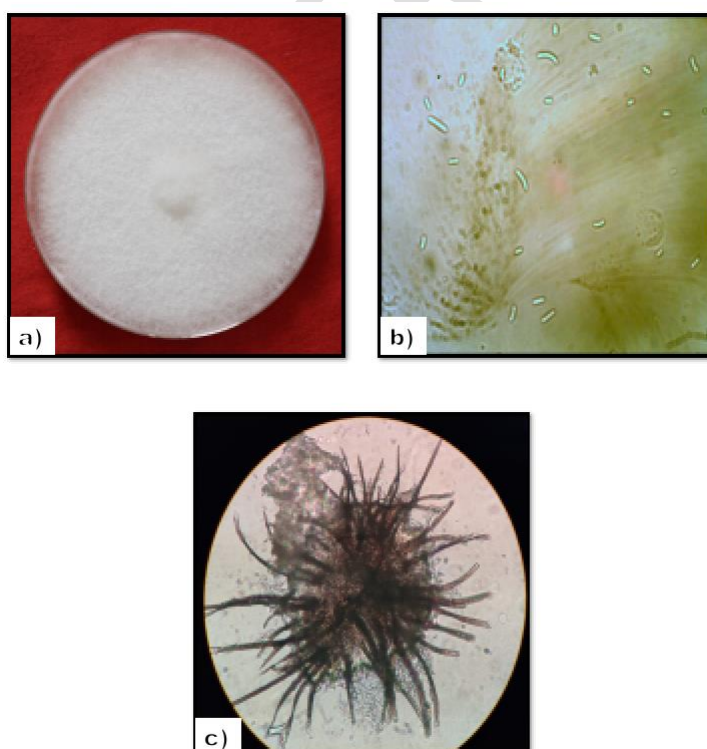


Fig. 3: Cultural and morphological characterization of *C. lindemuthianum*
 (a) White cottony growth of *C. lindemuthianum* on PDA Petriplate
 (b) Cylindrical, hyaline and smooth conidia of *C. lindemuthianum*
 (c) Asexual fruiting body acervulus of *C. lindemuthianum*

3.7 Morphological characteristics of pathogen

The morphological characteristics of the pathogen *C. lindemuthianum* has been investigated with the help of compound microscope. The mycelium was noticed as hyaline, septate and highly branched. The acervuli was found singly and in groups, blackish brown in colour and measured 181.3 × 254.6 µm. The acervuli arose from the stroma beneath the epidermis. The setae was seen through the conidial mass with 1-3 septation and measuring about 80-115.7 × 4.2-7.3 µm. They were found broad at the base and narrower at the tip and dark brown to black in colour. The asexual conidia were single celled, hyaline, cylindrical and smooth with oil globules and measured 15.8-20.1×3.3- 4.1 µm in size. Light honey coloured conidial spore mass with many number of fruiting bodies were found in centre of Petri dish. The morphological structures such as mycelium, acervulus and conidium of *C. lindemuthianum* are represented in fig. 3

Morphological characteristics of *C. truncatum* was examined and reported that acervuli as oval to conical, blackish brown in colour, appeared singly and dimension of 178.6 × 256.0 µm. The conidia were single celled, hyaline, curved and contain oil globules and measured 20-23.10 x 3.8- 4.10 µm in size [24]. Morphological character of *C. lindemuthianum* conidia was reported as hyaline, sickle shaped and single celled. The length of conidia was measured ranging between 10.5-15.5 µm and a breadth of 3.5- 4.5 µm [18]. *C. gleosporoidies* spores were observed as cylindrical and straight with smooth round end with dimension of 3.0 – 5.0 µm in width and 10.3 – 18.2 µm in length [21].

CONCLUSION

Anthrachnose of green gram is the major disease that affected all plant parts and cropping seasons, with dark brown to black sunken lesions on the hypocotyl region causing seedling necrosis. Small sunken spots on leaves turns greyish white with dark brown or reddish margins, coaling to necrotic spots later in the season. Similar spots appears on petiole, stem, and pods. Four pathogenicity tests were used to prove Koch's Postulate, with spraying inoculum after needle pricking and rubbing fungal suspension with carborandum powder producing 100% infection in inoculated plants, 96% infection in spraying spore suspension with atomizer to green gram, and less in seed inoculated plants. *C. lindemuthianum* was studied on potato dextrose agar culture, revealing whitish to pink cottony growth and pinkish to brown pigmentation. The fungal pathogen's morphology included single-celled, cylindrical conidia with oil globules, blackish brown acervuli, and setae with 1-3 septations. These results are crucial in identifying the pathogen on other hosts.

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