

## On the Possible Role of *Colletotrichum lindemuthianum* Induced Anthracnose Disease in *Gossypium hirsutum* L Seedlings

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### Original Research Article

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**Abstract:** Anthracnose is a major and most common fungal disease of different angiospermic plants throughout the world. In the present study *Colletotrichum lindemuthianum* (COL) culture obtained from MTCC, Chandigarh were subcultured in PCA medium and the broth was inoculated into healthy 45-d-old cotton seedlings. The maximum growth of pathogen was noticed at pH 6.0 at 17°C. Cotton, a short day plant in terms of photoperiodic requirement was maintained at 30°C in green house. The anthracnose symptoms appeared only on the 70<sup>th</sup> day of plant growth (25 days after inoculation). The seedlings were kept under observation for the development of disease symptoms. The presence of pathogen in the seedling was further confirmed by isolating and re-infecting the healthy seedling. The severity disease was at its maximum on 60<sup>th</sup> day after inoculation of *C. lindemuthianum* where almost all the leaves showed infection. The severity of disease was estimated in terms of disease incidence, lesion size and percentage of infection. Similarly, biochemical constituents such as phenol, proline, polyphenoloxidase and superoxide dismutase activities were also studied. Certain parameters showed a hike (phenol and proline) in content whereas other parameters (PPO & SOD activities) decreased upon COL infection.

**Keywords:** Anthracnose, AAS, *Colletotrichum lindemuthianum*, short day, SOD, PPO.

### INTRODUCTION

Plants in their environment face potential deleterious organisms such as fungi, bacteria, viruses, nematodes, etc. Many of them are able to cause plant diseases, responsible of important losses in crop production worldwide.

But often the outcome of these interactions is not disease, since plants have developed multiple mechanisms to protect themselves against pathogens attack [1]. Anthracnose is a major disease of the common bean (*Phaseolus vulgaris*) and can occur on other legumes. It is caused by the fungus *Colletotrichum lindemuthianum*. When environmental conditions are favorable, crop losses can be as high as 100% on susceptible cultivars of beans [2]. *Colletotrichum lindemuthianum* is a fungus which causes anthracnose or black spot disease of the common bean plant (*Phaseolus vulgaris*). It is considered as a hemibiotrophic pathogen because it spends part of its infection cycle as a biotroph, living off of the host but not harming it and the other part as a necrotroph, killing and obtaining nutrients from the host tissues. In the present study to obtain pure culture of *Colletotrichum lindemuthianum* and maintenance of active cultures. To test the pathogenicity of *Colletotrichum lindemuthianum* in cotton seedlings by manual infection at different days after inoculation. Verification of Koch's Postulates by reisolating the pathogen from the infected seedlings and subculturing in PCA medium. To understand the symptoms and severity of disease, disease incidence, size of the infection, percentage of infection caused by *Colletotrichum lindemuthianum* in *Gossypium hirsutum*. To quantify the physiological and biochemical constituents like phenol, proline and other antioxidant enzyme activities such as Polyphenol oxidase, Superoxide dismutase.

### MATERIALS AND METHODS

#### Procurement of seeds

Certified seeds of *Gossypium hirsutum* L. variety SVPR1 were procured from Cotton Research Station, Srivilliputhur. The SVPR1 is 90d plants. The time taken for fruit setting and yield were 90days.

### Cultivation of seedlings

Viable seeds were coated with cow dung and allowed to germinate. The percentage of germination was nearly 92%. Seedlings were raised in earthenpots (125 x 25 cm) filled with a mixer of red soil, black soil and sand (in the ratio of 2:2:1). Twenty seeds were sown at equal distances at a depth of 2cm in each pot.

### Procurement of microbes

The pure fungal cultures were obtained from the Institute of Microbial Technology (IMTECH), Chandigarh, India. They were cultured in Potato Carrot Agar medium

### Infection of the plant using the pathogen

*C. lindemuthianum* cultures were subcultured on Potato Carrot Agar (PCA) for 14 d at 28°C as described by Jia *et al.*, [3]. Conidia were collected and washed three times with sterile distilled water and filtered using a double layer of muslin cloth. The conidia were suspended in sterile distilled water and the suspension was adjusted to a concentration of  $1 \times 10^6$  conidia  $\text{ml}^{-1}$ . The seedlings were grown under greenhouse condition for 45 d. *C. lindemuthianum* inoculations was applied according to Wang *et al.*, [4]. Briefly, Cotton seedlings were spray-inoculated with a conidial suspension of  $1 \times 10^7$  conidia/ml containing 0.02% Tween 20. Plants treated with sterile distilled water containing 0.02% Tween 20 were used as the control. The plants were transferred to a green house.

### Pathological assessment

The rates of disease were measured by the following assessment. The disease measured the disease incidence and disease severity were assessed randomly the *C. lindemuthianum* infected *Gossypium hirsutum* L. Representative samples, based on visual symptoms of the disease were brought from each at random as per methods described by Yonghao Li [5]. The formulae in calculating the disease incidence and severity are:

### Percentage of disease incidence

$$\text{Percentage of disease incidence} = \frac{\text{Total number of infected plants}}{\text{Total number of plants assessed}} \times 100$$

### Measurement of lesion size

The visual symptoms of the disease were measures as a lesion size. The symptoms were measured in centimeters.

### Percentage of infection

Representative samples, based on visual symptoms of the disease were brought from each at random. The formulae in calculating the Percentage of infection is given below.

$$\% \text{ infection} = \frac{\text{Number of infected leaves}}{\text{Total number of leaves Physiological assessment}} \times 100$$

Total phenol content of the fresh leaf sample was estimated by Folin Ciocalteu method [6]. Free proline content was estimated using the acid ninhydrin method as described by Bates *et al.*, [7].

### Antioxidant Enzyme activities

Polyphenol oxidase activity was analyzed by colorimetric method [8]. Superoxide dismutase activity (SOD) was analyzed by Bowler *et al.*, [9].

### Atomic absorption spectroscopy

The cellular concentration of copper was analysed in control, COL infected cotton seedlings using Atomic Absorption Spectrophotometer (SHIMADZU-AA-6300, Japan). The procedure was followed as given in American Public Health Association (APHA).

### Statistical analysis

All parameters were determined with three independent replicates. The data were reported as Mean  $\pm$ PE.

## RESULTS

The present experiment was proposed to study the pathogenicity of *Colletotrichum lindemuthianum* on *Gossypium hirsutum* L. The concentration of disease alleviation was tested by measuring the symptoms, size of the lesion, disease incidence, enzyme activities and biochemical constituents.

### Verification of Koch's postulates

*Colletotrichum lindemuthianum* pure culture was obtained from MTCC, Chandigarh and sub-cultured on PCA medium. The pathogen was allowed to infect cotton leaves manually by spraying 2ml of culture to the whole leaves of 45-d-old seedlings using an atomic sprayer. The symptoms appeared were confirmed to be anthracnose which usually appears in beans.

After the complete establishment of pathogen, the infected leaf spots were excised to reisolate the pathogen. The spores were confirmed by microscopic examination. Koch's postulates were tested by reisolating the pathogen, verifying under microscopy after lactophenol cotton blue staining (Fig-1). Koch postulates states that the microorganism must be found in abundance in all organisms suffering from the disease, but should not be found in healthy organisms. The microorganism must be isolated from a diseased organism and grown in pure culture. The cultural microorganism must be reisolated from the inoculated, diseased experimental host and identified as being identical to the original specific causative agent [10]. Thus verification of Koch's postulates was carried out successfully.

### *C. lindemuthianum* infection in *G. hirsutum*

*Colletotrichum lindemuthianum* culture at log phase was selected to infect the selected crop viz., *Gossypium hirsutum*. The infection procedure is given in the methodology section. Brown streaks appeared on the vein region of *Gossypium hirsutum* 25 days after *C.lindemuthianum* infection (Fig-2A). *Colletotrichum lindemuthianum* (Sacc & Magnus) Briosi & Cavara is known to result in total yield loss depending on the cultivar environmental conditions [11, 12]. The spots became necrotic after 30<sup>th</sup> days (Fig-2B). The necrotic lesions were with brown in colour and ranged from 0.5 to 5.0 mm in diameter. Mostly lesions were appeared on the vein region (Fig-2C).The spots become brown in color after 40 days of infection. Fig-2D shows the presence of brown spots in the veinal region of *Gossypium hirsutum* leaves. With increase in age of the leaf, the nature of the lesions appeared highly necrotic. The symptoms of the leaves after 45 days of infection with *C.lindemuthianum* are shown in Fig-2E. Disease incidence of the *G.hirsutum* seedlings was assessed under pathogen infection. As far as *COL* is concerned with increase in days after inoculation (Fig-3a).It was found that was *Colletotrichum lindemuthianum* infection caused 95% reduction in plant growth as compared to control (Fig-3b). Incidence of cotton anthracnose showed significant changes after 25-60 days after sowing. The disease became severe after 50, 55 and days of growth. Disease incidence reached to 95%. In the present experiment, disease incidence increased in the end of the infection. The same findings were reported by Dillard and Cobb [13] in areas where beans are consecutively cropped, over seasonal inoculum can initiate epidemics of bean anthracnose. As a measurement of disease severity in cotton, the size of the lesion was measured. The lesion size increased with days and maximum at the end the infection cycle to 60% Fig-3c. Similarly, the percentage of infection increased appreciably at the end of the *C. lindemuthianum* infection Fig-3 (a & b). The primary inoculum from infested debris was relatively more damaging than other inoculum sources, causing early epidemic development and yield reduction [14]. There was no significant variation in size of the lesions (25, 30, 35, 40, 45, 50, 55 and 60 days). Similarly, Mohammed *et al.*, [15] also reported anthracnose disease progression rate in beans in all the days.

### Biochemical constituents in *G. hirsutum* exposed to *C. lindemuthianum* infection

Plant-pathogen interaction is known to bring about a change in the biochemical parameters also the impact of the pathogen on the host species was studied in terms of biochemical parameters such as proline and phenol contents. Proline is a source of energy, carbon and nitrogen for the recovering tissues. From the results obtained, the proline content has been found to increase in *Colletotrichum lindemuthianum* treatment, which indicates its disease resisting capability in host plants. *Colletotrichum lindemuthianum* stressed seedlings have shown 36% amount of increase in proline content as compared to the control (Fig-4a). Results of the present study reveals that, a higher amount of proline was observed in the *Colletotrichum lindemuthianum* infected *G. hirsutum* than the control. Similarly, Lubaina and Murugan [16] reported that the maximum amount of proline was observed in the infected sesame compared to the respective control. Chen and Dickman [17] reported that proline may also act as a potent scavenger of reactive oxygen species this property might prevent the induction of programmed cell death.

Phenolic compounds play a major role in plant defense mechanism to resist diseases and insects, also acts as antioxidants. Many plant phenolic compounds are known to be antimicrobial because increased phenol synthesis of the plants brings about an increase in phenyl propanes, which are lignin precursors. Accumulation of lignin and phenolic

compounds has been correlated with disease resistance in a number of plant pathogen interactions. The phenol content was found to be increased to about 27% in *Colletotrichum lindemuthianum* treated plants of *Gossypium hirsutum* over the control plants (Fig-4b). The increase in total phenolics observed in the present study have been reported by others using different plant pathogen interactions. Girdhari *et al.*, [18] also reported that increased total phenol content was found in rice leaves after treatment with biotic inducers. Kumar and Biswas [19] stated that increased total phenol was found in tomato leaves after treatment with inorganic chemicals. Neisch [20] studied that the accumulation of phenolic compounds in infected host tissues may be related to their release from glycosidic esters by the enzymatic activity of host or pathogen.

#### Antioxidant enzymes

PPO is also an important contributor in plant defense pathway. PPO has a role in catalyzing phenolic oxidation in limiting disease development which may therefore be involved in induction of defense resistance against plant diseases. The present result showed an increase in the polyphenol oxidase activity to about 60% in *Gossypium hirsutum* (Fig-4c). In our findings a Polyphenoloxidase and Superoxide dismutase activity was observed in *Colletotrichum lindemuthianum* in *G. hirsutum* infected seedlings. The Polyphenol oxidase activity was found to be increased in *Colletotrichum lindemuthianum* treated plants. A minimum level of activity was observed in control plants. Our results are in agreement with similar other studies. Sharma and Joshi [21] stated that after treated with *Macrophomina phaseolina* infestation, the PPO activity starts on increasing after 24h and then reached the maximum value after 120 h. Thereafter, the PPO activity gradually decreased and reached the minimum value after 144 h. There was significant difference between pathogen infected plant and the control.

SOD is an important enzyme involved in scavenging the superoxide radical. In the present study on superoxide dismutase activity showed a linear increase in activity. *Colletotrichum lindemuthianum* treatment caused an increase in SOD activity to about 79% in cotton. The increase in SOD activity under *Colletotrichum lindemuthianum* treatment indicates that the constant detoxification of the superoxide radical which has been generated (Fig-4d). The superoxide dismutase activity was high in *Colletotrichum lindemuthianum* treated plants over the control plants. Similar to our results, increased levels of SOD are reported from *Nicotiana benthamiana* to infection with two strains of pepper mild mottle virus [22]. Tariq *et al.*, [23] reported that the activities of CAT, POX and SOD were increased in the leaves on account of MeJA treatment. They also mentioned that the application of MeJA further enhanced the activities of all antioxidant enzymes, both in non-stressed and stressed plants, by supplementing the ROS scavenging mechanism. To counteract the toxicity of reactive oxygen species, plants have developed a highly efficient antioxidant enzymes defense system, mainly including SOD, CAT and POD, increasing tolerance to different stress factors [24].

#### AAS analysis

Metals play an essential role in many biological processes but are toxic when present in excess. This makes their transport and homeostatic control of particular importance of living organisms. Within the context of plant pathogen interactions, the availability and toxicity of transition metals can have a substantial impact on disease development. Among the heavy metals, copper is considered to be important heavy metals as many of the enzymes possess copper as cofactor. Under *COL* treatment, the level of copper as assayed by AAS increased to 15% when compared to the untreated and uninfected control. The analysis of copper contents under various treatments is given in Table-1. Copper containing laccases can be important virulence factors for many plants-pathogenic fungi, enabling them to degrade physical barrier and detoxify phytoalexins and tannins [25]. There are reports indicating that copper is an essential micronutrient for pathogen as well. Copper deficiencies in *Colletotrichum lindemuthianum* can result in the pathogen becoming a virulent to bean. Thus copper is not only an essential micronutrient for crops but also for pathogens like *C.lindemuthianum*.

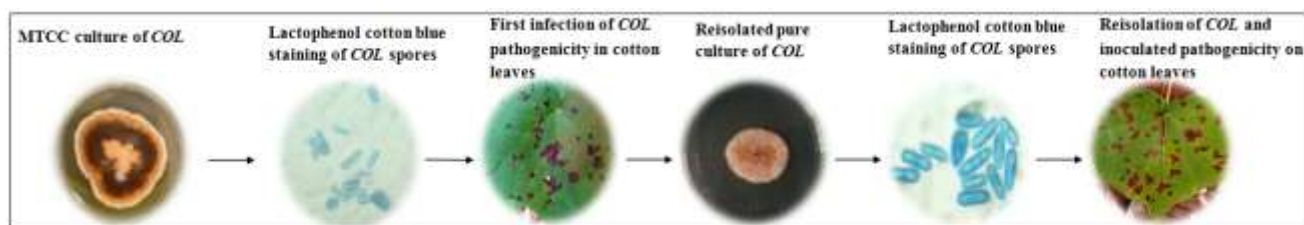


Fig-1: Verification of Koch's Postulates

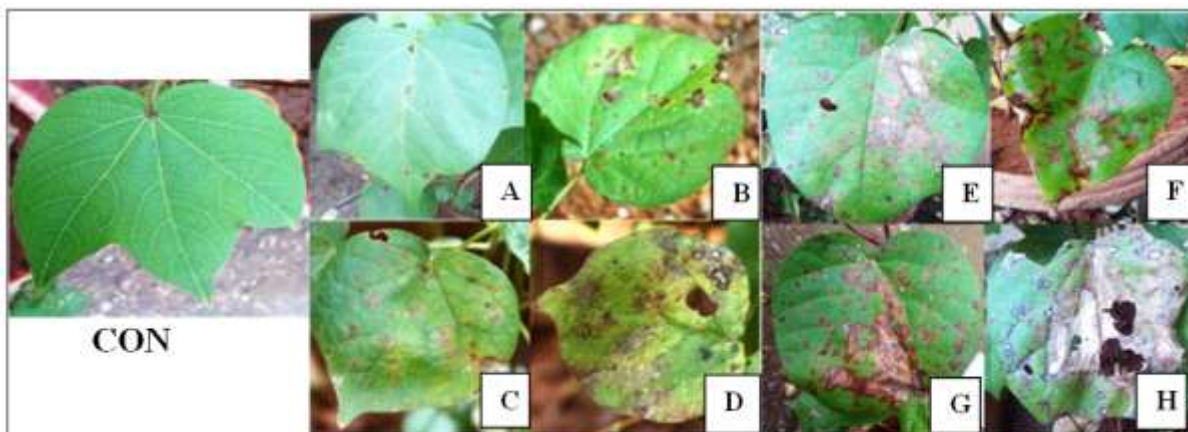


Fig-2: Symptoms of *Colletotrichum lindemuthianum* infection in *Gossypium hirsutum* leaves examined after different time periods. A: 25, B:30, C:35, D:40, E:45, F:50, G:55 and H:60 days of inoculation

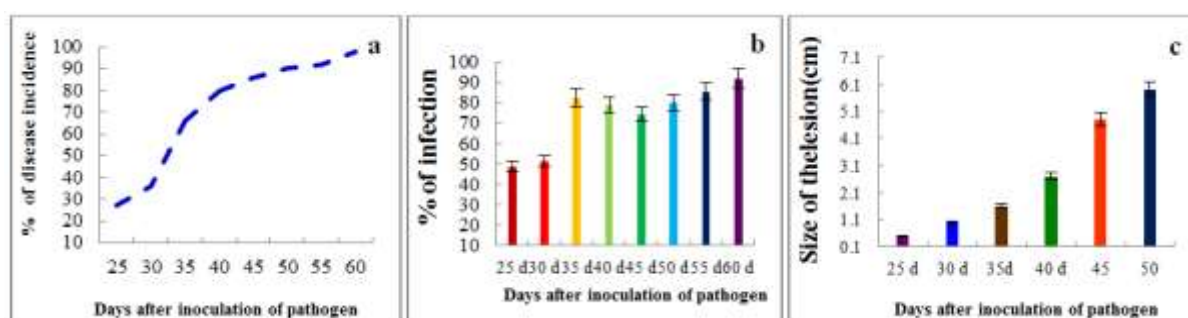


Fig-3: Percentage of disease incidence (a), percentage of infection (b) Size of the lesion (c) of *Colletotrichum lindemuthianum* in *Gossypium hirsutum* seedlings after different days of inoculation

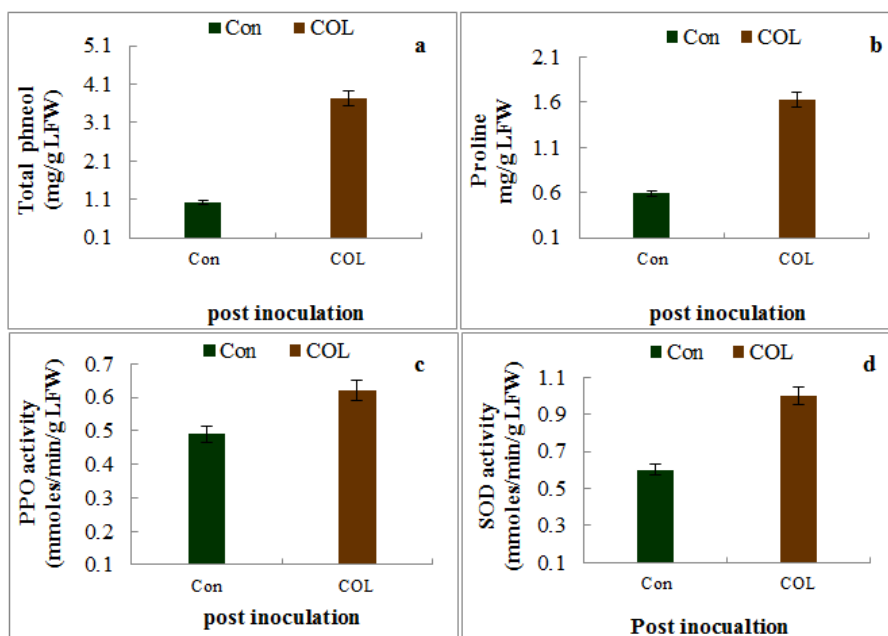


Fig-4: Changes total phenol content and proline content antioxidant enzyme PPO and SOD of *Gossypium hirsutum* treated *C. lindemuthianum*

Table-1: AAS analysis of copper content in the leaves of *Gossypium hirsutum* seedlings exposed to pathogen. The analysis was carried out in 45 days old seedlings

Samples	Con (ppm)	%
Control	1.177	--
+ COL	1.355	+15

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