CHAPTER IV

4. Pathogenicity of *Macrophomina phaseolina* as a Seed-borne Pathogen of Mungbean and Blackgram

4.1. An Overview

The pathogenicity of an organism is its ability to cause disease, and proof of pathogenicity is achieved by fulfilling Koch's postulates (Kabeere *et al.*, 1997). *Macrophomina phaseolina* (Tassi) Goid., is a pathogen with exceptionally broad host range that includes over 500 species of monocots and dicots (Pratt *et al.*, 1998). Diseases incited by *M. phaseolina* are often referred to as 'charcoal rot' because of a dark coloration imparted in parasitized host tissue. As a seed-borne and soil-borne fungus, *M. phaseolina* is also known to produce seed rot, seedling blight, wilt and root rot in many crops (Sultana *et al.*, 1994). On several crops in the world, *M. phaseolina* causes significant economic losses of blackgram and mungbean by reducing the seed quality as well as declining the quality of edible sprout. Due to this reason, being an exporting commodity, the importers very often complain blackgram of Thailand (Putasamai and Surin, 1988). When the infected seed germinates, a wet and fast spreading black rot develops on sprouts, which makes the sprouts blemish and unfit for consumption (Fuhlbohm *et al.*, 1997).

In many mungbean and blackgram producing countries in the world, *M. phaseolina* has been reported as a seed-borne fungus of these two hosts. However, the pathogenicity of *M. phaseolina* in the seeds of mungbean and blackgram has not been precisely documented and Koch's postulates have not been fulfilled yet. That is why, the extent to which *M. phaseolina* is a major individual pathogen of mungbean and blackgram, with a capacity to infect the seed in the absence of other pathogens, is not clear on the basis of signs and symptoms alone.

Therefore, the present investigation was taken up with the following objectives:

- To determine the pathogenicity of *M. phaseolina* in mungbean and blackgram seed and seedlings following various methods.
- To satisfy the Koch's postulates in the mungbean and blackgram seeds with *M. phaseolina*.

4.2. Materials and Methods

4.2.1. Experimental material

The freshly grown seeds of mungbean variety Chai Nat 60 and blackgram variety Uthong 2 were used as test material for the pathogenicity test of *M. phaseolina*.

4.2.2. Preparation of pure culture of *M. phaseolina*

Seed sample of blackgram named as Uthong 2 was obtained from Chai Nat Field Crops Research Center, Thailand, which was carrying natural infection of *M. phaseolina*. Fifty seeds were soaked with 10.0 percent sodium hypochlorite for 2 minutes followed by rinsed in sterile distilled water for four times. The adhering water around the seeds was soaked by sterile blotter paper. Then the seeds were placed in sterilized 9cm-diameter Petridishes contained 3-layered moist Whatman no. 1 blotting paper at the rate of 10 seeds per plate. The plates were kept under 12 hour alternating daylight and darkness at about 25^{0}C. After 3 days, when the pycnidia and microsclerotia of *M. phaseolina* were observed on seed coat and radicle, only these seeds were transferred in another sterilized Petridish containing about 20ml solidified Difco potato dextrose agar (PDA). In the middle of the each plate, one seed was placed. The plates were sealed by Nesco film to avoid contamination of other aerial microorganisms and were kept again under 12 hour alternating daylight

and darkness at about 25^{0}C. After 2 days, when the plates were covered with the blackish mycelia and microsclerotia of *M. phaseolina*, from the periphery of the plate mycelia and microsclerotia were transferred (after cutting by a 5mm diameter cork borer) in another Petridish contained solidified PDA. The Petridishes were also kept under 12 hour alternating daylight and darkness. After three days, when the plate covered with mycelia and microsclerotia (Plate 4.01), the fungal fragments were used as inocula of *M. phaseolina* for pathogenicity determinations in mungbean and blackgram through different methods.

4.2.3. Preparation of suspension of *M. phaseolina* pure culture

The mycelial and microsclerotial mat (prepared as section 4.2.2) separated by scrapping with a sterilized knife after 3 days. The scrapped mat was mixed with 10 ml sterilized water for each Petridish. That mycelial and microsclerotial suspension was used for different inoculation methods.

4.2.4. Determination of Pathogenicity of *M. phaseolina*

Pathogenicity of *M. phaseolina* was determined by the following methods:

1. Seed inoculation method.
2. Sprout Dipping method
3. Soil inoculation method
4. Damping-off symptom test.
5. Test tube seedling symptom test
6. Toothpick inoculation method

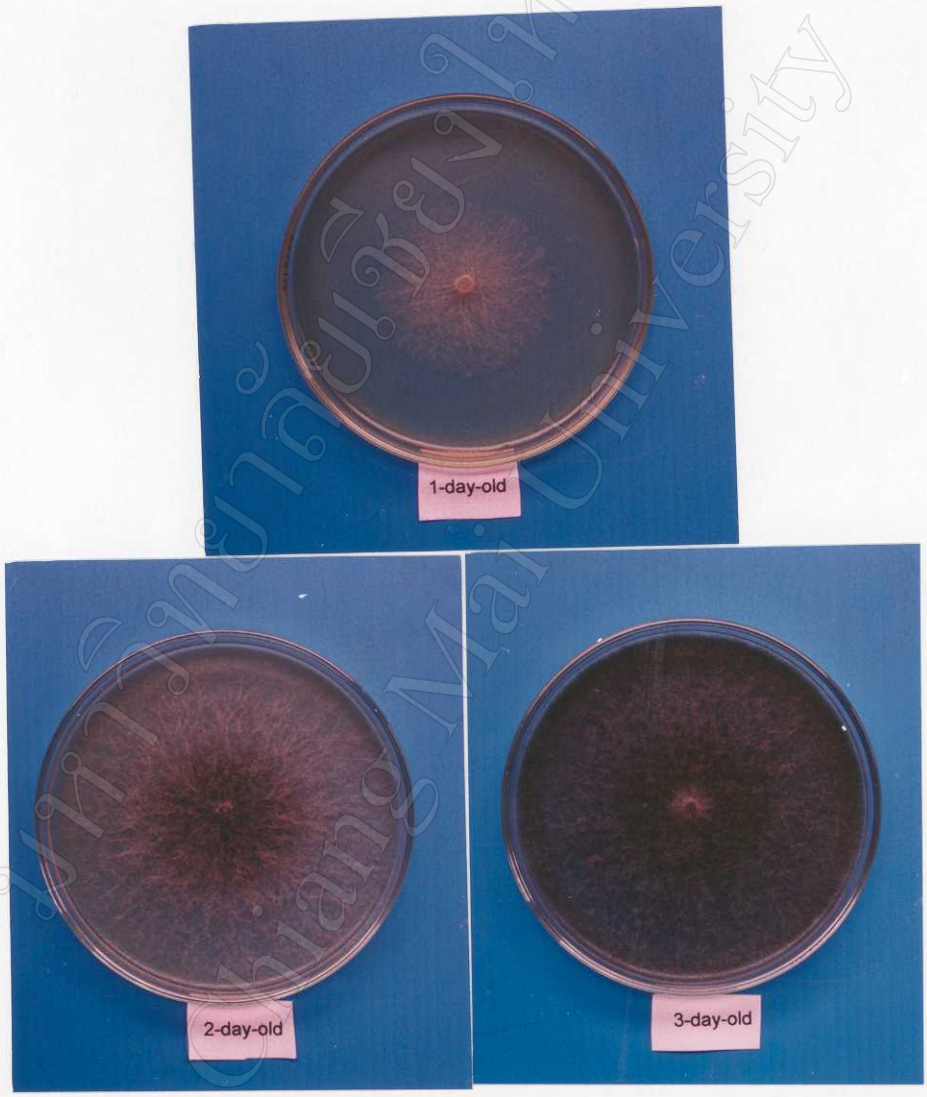

Plate 4.01: Pure culture of *Macrophomina phaseolina* on artificial media (PDA) growing in different days.

4.2.4.1. Seed inoculation method

Ten gram of mungbean and 10g of blackgram seed samples were surfaced sterilized with 10.0 percent sodium hypochlorite solution followed by rinsing with sterilized water. Thereafter, half amount of each sample was dipped in 50 ml mycelial and microsclerotial suspension (prepared as section 4.2.3) for three hours. The remaining seeds were also dipped in 50 ml sterilized water for the same time, which considered as control. Then 400 seeds of each category were put on three-layered moist Whatman no. 1 blotting paper. The blotting papers placed on sterilized glass Petridishes and in each Petridish 10 seeds were placed equidistantly. All the inoculated and control seeds kept under 12 hours alternate NUV light and darkness. After three days, the disease symptoms of seedlings observed and recorded.

4.2.4.2. Sprout dipping method

Small quantity (about 10g) of mungbean and blackgram seed samples was surfaced sterilized with 10 percent sodium hypochlorite solution followed by rinsing with sterilized water. Then they placed equidistantly in moist germination paper and rolled. Altogether 400 seeds from each mungbean and blackgram sample were taken and in each germination paper fifty seeds were placed equidistantly. The roles with seeds were kept in a germinator at 25^0C. The humidity was maintained by spraying water whenever required. Forty-eight hours later, when the seeds germinated, half number of each sample i.e. two hundred sprouts were separated and dipped in a suspension of microsclerotia and mycelia fragments of *M. phaseolina* for 30 minutes and then returned in the germinator after rolling by moist germination paper like earlier. The remaining sprouts were dipped in sterile water followed by rolling with germination paper and placed in the germinator in the same way, which was considered as control.

After forty-eight hours, the symptoms were studied which developed on the incubated sprouts. Symptom bearing seedlings were again recultured in PDA after surface-sterilization with ten percent sodium hypochlorite solution as before. The emerged fungal structures were compared with the culture of *M. phaseolina* with the purpose of proving the Koch's postulate.

4.2.4.3. Soil inoculation method

At first, total 40 Petridishes were filled with sterilized soil. In 20 plates, the suspension of mycelia and microsclerotia (made as section 4.2.3) was poured as 20ml per plate. In the remaining 20 plates, 20ml sterilized water was poured per plate instead of suspension. Thereafter, in each plate, 10 seeds of mungbean were planted. The blackgram seeds were also planted in the same manner in another 40 Petridises. All the Petridishes were kept in the green house without giving the cover. After seven days, data were recorded on the basis of germination, seedling damping-off and symptoms on seedlings. The plant parts contained disease symptoms were recultured on PDA in order to prove the Koch's postulate.

4.2.4.4. Damping-off method (Thirumalachar *et al.*, 1977)

Petridishes with 20ml solidified PDA were inoculated with the pure culture of *M. phaseolina* (prepared earlier as Section 4.2.2). The control or check was prepared with only PDA without inoculation of pure culture. All the Petridishes were incubated for 72 hours under 12 hours alternating light of NUV and darkness. When the all Petridishes were covered with grayish-white mycelia, the mycelia mat were overlaid with 0.5cm thick sterilized soil. Then the test material (mungbean and blackgram seeds) surfaced sterilized with 10% sodium hypochlorite and planted as 10 seeds per plate. Total 200 seeds were planted for each sample and also same numbers were planted as control. In each category, 50 seeds i.e, 5 plates were considered as one replication. After 7 days, data were recorded on the basis of ungerminated

seeds, seedlings with symptoms on stem, leaf and cotyledon. The infected plant parts were recultured in PDA and identified whether the organism was *M. phaseolina*, in order to fulfill the Koch's postulate.

4.2.4.5. Test tube seedling symptom test

In Pyrex test tube (20cm x 2.5cm) about 20ml 2 percent water agar was poured. Total 400 test tubes were prepared in the same manner and then autoclaved. After solidification of water agar, in each test tube (total 200) inoculums of *M. phaseolina* (which were made in section 4.2.2) was inoculated with a sterilize needle. After 2 days, 200 seeds of mungbean and 200 seeds of blackgram were surface sterilized with 10 percent sodium hypochlorite solution for one minutes. After that, the seeds were rinsed with sterilized water. The adhering water with the seeds was soaked with sterilized blotting paper. Then all mungbean and blackgram seeds were placed singly in each test tube. One hundred seeds of each mungbean and blackgram were placed in inoculated test tubes. Similarly same amount of seeds were placed in the same way into non-inoculated test tubes. The non-inoculated test tubes were considered as control. All the test tubes were closed with aluminum foil and kept 12 hours alternating NUV light and darkness. After seven days, data were recorded on dead seedlings, symptoms on cotyledonary leaf and symptoms on seedlings. The symptoms bearing plant parts were also recultured on PDA.

4.2.4.6. Toothpick inoculation method (Grezes-Besset *et al.*, 1996)

The plants of mungbean and blackgram were grown in plastic pot (size 15cm x 12cm) and each pot contained five plants. All the pots were kept under sunny condition and watering was done whenever required. Inoculation was done with toothpicks. Sterile wooden toothpicks were infested with the pure culture of *M. phaseolina* in PDA. The pointed ends of infested toothpicks were inserted into the stem and internodes when the plants became three

weeks old. Non-infested sterile toothpicks were inserted in the plant parts with only PDA following the same manner, which were considered as control. After inoculation, all the inoculated plants and control were covered with transparent polythene packet for three days in order to retain optimum humidity around the experimental plants. In ten plants stem and in other ten plants internode were inoculated. During inoculation, the average temperature was 30^0C. Disease reaction was scored by plant survival and the lesion length of stem and internode. The infected plant parts were recultured on PDA for fulfillment of Koch's Postulate.

4.3. Results

4.3.1. Seed inoculation method

The result of seed inoculation method showed that 100 percent diseased seedlings were developed from the inoculated seeds of both mungbean and blackgram (Table 4.01). On the other hand, the control treatment did not produce any diseased seedlings. After three days of inoculation, brown colored radical initiation was noticed and before plumule development, the radical turned into brownish in color followed by death of radicle (Plate 4.02 and 4.03). Some seeds did not develop radicle and the seed encompassed with whitish mycelia. Mungbean and blackgram seeds showed identical symptom in case of disease development in the inoculated seeds.

Table 4.01: Pathogenicity determination in mungbean and blackgram seeds by seed inoculation method.

Treatment	Healthy seedlings (%)		Diseased seedlings (%)	
	Mungbean	Blackgram	Mungbean	Blackgram
Inoculated	0.0	0.0	100.0	100.0
Control	100.0	100.0	0.0	0.0

Plate 4.02: Mungbean seedling from *Macrophomina phaseolina* inoculated (left) and noninoculated (right) seed.

Plate 4.03: Blackgram seedling from *Macrophomina phaseolina* inoculated (left) and noninoculated (right) seed.

4.3.2. Sprout dipping method

From the result it was revealed that 91.0 and 88.0 percent diseased seedlings were produced in mungbean and blackgram when the sprouts were dipped in mycelial and microsclerotial suspension of *M. phaseolina* (Table 4.02). The infection was started within two days of inoculation. The sprout became deep brown in color. Within seven days, all infected seedlings were died and copious microsclerotia and pycnidia along with mycelia of *M. phaseolina* were noticed. In contrast, no any diseased seedlings were appeared in the control treatment (Plate 4.04 and 4.05). When the infected parts of seed and seedlings were recultured, the characteristic colonies of *M. phaseolina* were evolved in every case.

Table 4.02: Pathogenicity determination in mungbean and blackgram sprouts by sprout dipping method.

Treatment	Healthy seedlings (%)		Diseased seedlings (%)	
	Mungbean	Blackgram	Mungbean	Blackgram
Inoculated	9.0	12.0	91.0	88.0
Control	100.0	100.0	0.0	0.0

Plate 4.04: Mungbean seedlings after dipping the sprout in *Macrophomina phaseolina* suspension (left) and in sterilized water (right).

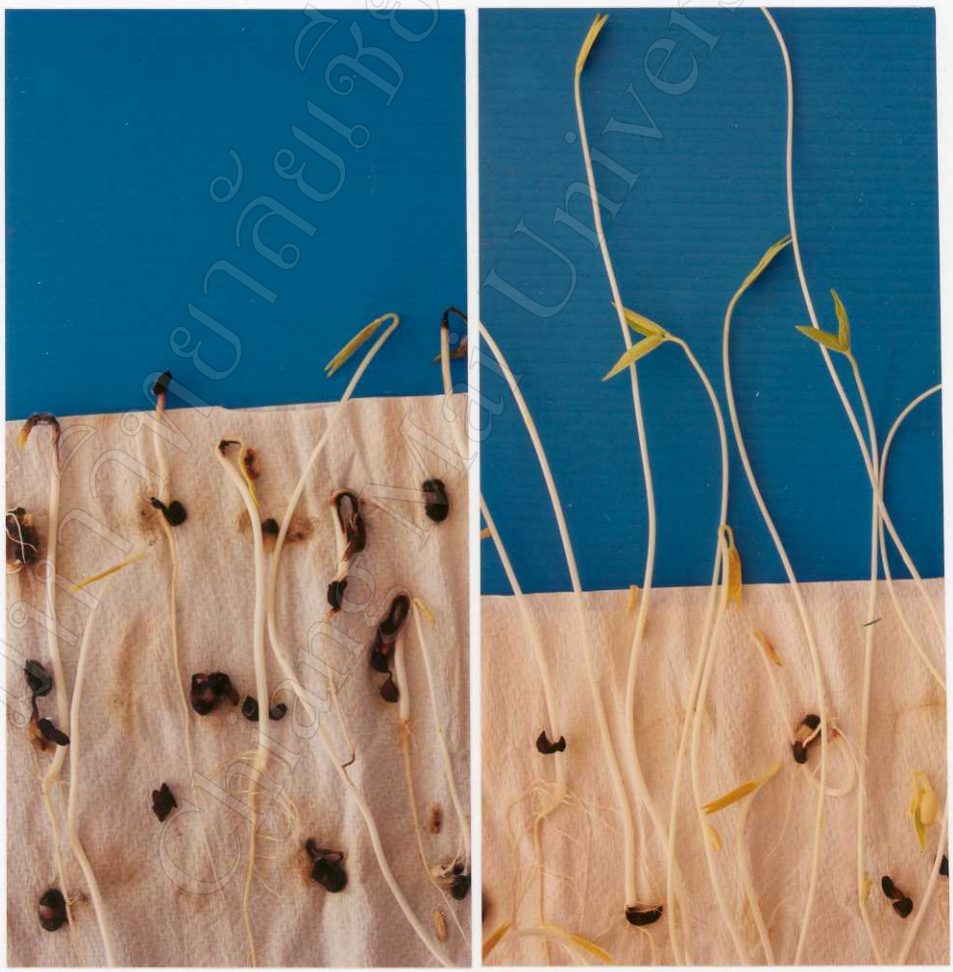

Plate 4.05: Blackgram seedlings after dipping the sprout in *Macrophomina phaseolina* suspension (left) and in sterilized water (right).

4.3.3. Soil inoculation method

Result of soil inoculation method presented in Table 4.03. The inoculated Petridishes produced 86.0 and 79.0 percent diseased plants in mungbean and blackgram respectively. In contrast, the plants from non-inoculated Petridishes showed any diseased plants neither in mungbean nor in blackgram. In the inoculated Petridishes, 11.0 and 7.0 percent seed rot were manifested in mungbean and blackgram respectively (Plate 4.06). Besides that, 64.0 and 59.0 percent seed produced infected cotyledonary leaves in mungbean and in blackgram respectively (Plate 4.07). The infection of cotyledonary leaves extended towards stem followed by the death of whole plant (Plate 4.08). The lesion in stem and leaf due to *M. phaseolina* found by 12.0 and 16.0 percent in mungbean and blackgram respectively (Plate 4.09 and 4.10). Moreover, 18.0 and 15.0 percent plants appeared to be died just after emergence of seedlings (Plate 4.11).

4.3.4. Damping-off method

Results showed cent percent infection was observed from the seeds of inoculated Petridishes. On the other hand, the seeds from non-inoculted or control Petridishes did not produce any disease symptom in case of both mungbean and blackgram (Table 4.04). In the inoculated Pteridishes, within seven days, all seeds observed to be died and mostly rotted. In addition, all seeds were encompassed by mycelia of *M. phaseolina* before emergence (Plate 4.12 and 4.13). In contrast, the seeds from control Petridishes produced cent percent healthy seedlings showing without any disease symptoms. The reculture of diseased seeds showed characteristic symptom of *M. phaseolina* in PDA medium.

Table 4.03: Pathogenicity determination in mungbean and blackgram seeds by soil inoculation method.

Treatment	Symptoms developed (%)									
	Seed rot		Infection						Total disease development	
	Mungbean	Blackgram	Cotyledonary leaf		Stem and leaf spot		Dead seedling after emergence		Mungbean	Blackgram
			Mungbean	Blackgram	Mungbean	Blackgram	Mungbean	Blackgram		
Inoculated	11.0	7.0	64.0	59.0	12.0	16.0	18.0	15.0	86.0	77.0
Control	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Plate 4.06: Seed rot from *Macrophomina phaseolina* inoculated soil.

Plate 4.07: *Macrophomina phaseolina* infected cotyledonary leaves.

Plate 4.08: Infection of *Macrophomina phaseolina* is extending from infected cotyledonary leaves.

Plate 4.09: Lesion after infection of *Macrophomina phaseolina* on the stem.

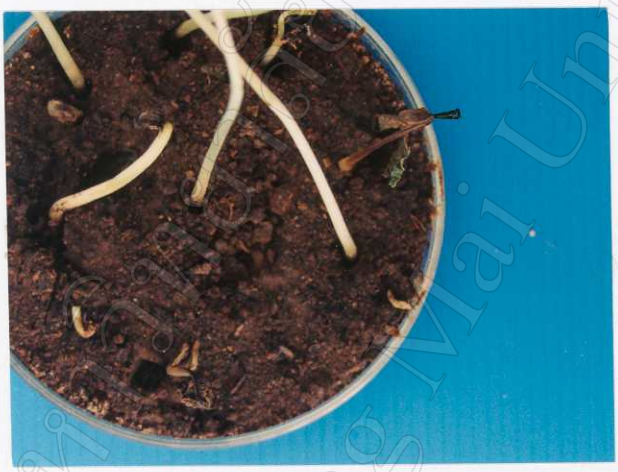

Plate 4.10: Lesion on the leaf after *Macrophomina phaseolina* infection.

Plate 4.11: Seedling died just after emergence due to *Macrophomina phaseolina* infection.

Table 4.04: Pathogenicity determination in mungbean and blackgram by damping-off method.

Petridishes	Healthy seedlings (%)		Diseased seedlings (%)	
	Mungbean	Blackgram	Mungbean	Blackgram
Inoculated	0.0	0.0	100.0	100.0
Control	100.0	100.0	0.0	0.0

Plate 4.12: Emerging seedling of mungbean from *Macrophomina phaseolina* inoculated plate (left) and from noninoculated plate (right).

Plate 4.13: Emerging seedling of blackgram from *Macrophomina phaseolina* inoculated plate (left) and from non-inoculated plate (right).

4.3.5. Test-tube seedling symptom test

The result showed, from the inoculated test-tube no any healthy seedlings were found either in mungbean or in blackgram (Table 4.05). On the other hand, non-inoculated test-tube or control treatment produced absolute healthy seedlings incase of both mungbean and blackgram (Plate 4.14 and 4.15). All the seedlings died followed by producing microsclerotia and mycelia including pycnidia within seven days. Some seeds could not germinate and eventually rotted. The remaining seeds although germinated but could not survive. In some cases, the infection spread from the infected cotyledonary leaves to the other parts. The recultured diseased plant parts produced colony of *M. phaseolina* in PDA culture.

4.3.6. Toothpick inoculation method

In Table 4.06, the result on pathogenicity test by toothpick method has been presented. After 7 days of inoculation, the average lesion length on stem was found to be 2.5 and 2.6 cm in mungbean and blackgram respectively whereas in control treatment the average lesion length was appeared to be 0.24 and 0.21cm in mungbean and blackgram respectively (Plate 4.16 and 4.17). It was also noticed that within 14 days the lesion spread and caused break down of stem followed by producing numerous pycnidia and microsclerotia on the stem (Plate 4.18 and 4.19). Within 21 days, 82 and 81 percent inoculated plants of mungbean and blackgram respectively were died.

From the internode inoculation, after 7 days 1.3 and 1.1cm average lesion length was observed in mungbean and blackgram respectively which turned into blackish necrosis (charcoal like black structure), while control treatment showed only 0.22 and 0.23cm lesion in mungbean and blackgram respectively showing without any necrosis (Plate 4.20 and 4.21). Within 14 days, the lesion spread upwards and downwards

Table 4.05: Pathogenicity determination in mungbean and blackgram seeds by test-tube seedling symptom test.

Treatment	Symptoms developed in seedlings (%)									
	Seed rot		Seedling infection (%)						Total disease development	
	Mungbean	Blackgram	Cotyledonary leaf		Stem		Dead seedling		Mungbean	Blackgram
			Mungbean	Blackgram	Mungbean	Blackgram	Mungbean	Blackgram		
Inoculated	21.0	17.0	27.0	25.0	61.0	65.0	79.0	83.0	100.0	100.0
Control	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Plate 4.14: Mungbean seedling in *Macrophomina phaseolina* inoculated agar medium (left and middle), mungbean seedling in non-inoculated medium (right).

Plate: 4.15: Blackgram seedling in *Macrophomina phaseolina* inoculated agar medium (left and middle), blackgram seedling in non-inoculated medium (right).

Table 4.06: Pathogenicity determination in mungbean and blackgram plants by toothpick inoculation method (mean results of ten plants).

Treatment	Lesion length (cm) on stem after 7 days		Lesion length (cm) on internode after 7 days		Death of plant (%) within 21 days	
	Mungbean	Blackgram	Mungbean	Blackgram	Mungbean	Blackgram
Inoculated	2.5	2.6	1.3	1.1	82.0	81.0
Control	0.24	0.21	0.22	0.23	0.0	0.0

Plate 4.16: Mungbean stem inoculation with *Macrophomina phaseolina*. Control treatment (left) and inoculated stem (right).

Plate 4.17: Blackgram stem inoculation with *Macrophomina phaseolina*. Control treatment (left) and inoculated stem (right).

Plate 4.18: Spreading the lesion causing break down of stem followed by producing numerous pycnidia and microsclerotia of *Macrophomina phaseolina* in mungbean.

Plate 4.19: Spreading the lesion causing break down of stem followed by producing numerous pycnidia and microsclerotia of *Macrophomina phaseolina* in blackgram.

Plate 4.20: Mungbean internode inoculated with *Macrophomina phaseolina*. Control treatment (left) and inoculated internode resulted charcoal like necrosis (right).

Plate 4.21: Blackgram internode inoculated with *Macrophomina phaseolina*. Control treatment (left) and inoculated internode resulted charcoal like necrosis (right).

directions, which caused eventual death of plant (Plate 4.22 and 4.23). Moreover, in control treatment no any plant was died due to infection either in mungbean (Plate 4.24) or in blackgram (Plate 4.25).

4.4. Discussion

Results obtained from different types of pathogenicity test revealed that *M. phaseolina* is highly pathogenic to the seeds, seedling (sprouts) and even to the mature plant of mungbean and blackgram. Among all pathogenicity tests, seed inoculation method, damping-off method and seedling symptom test showed 100 percent infection. The remaining methods also resulted remarkable percentage of infection. From the all types of pathogenicity tests it was proved that *M. phaseolina* has the ability to kill mungbean and blackgram seeds and seedlings leading the symptoms viz. seed rot, damping off, seedling blight, blemishness of sprouts, necrosis (charcoal like black appearance), death of mature plants (by secondary infection). Therefore, the results of this study demonstrate that *M. phaseolina* is a pathogen of mungbean and blackgram not only for seed but also the mature plants, which may incite severe symptoms of disease leading to death of whole plant in the absence of other pathogens. The pathogenicity of *M. phaseolina* has been investigated earlier by several scientists in various hosts. According to Nath *et al.*, (1970) and Scholefield and Griffin (1979) *M. phaseolina* is a very destructive pathogen of mungbean, which has got detrimental effect on seed and sprouts. Nayak and Behera (1994) reported the pathogenicity of *M. phaseolina* in blackgram seeds. They observed remarkable germination reduction and rotting of radicle due to infection. Gangopadhyay *et al.*, (1970) and Ellis *et al.*, (1979) found *M. phaseolina* to be highly pathogenic in soybean seeds as well as in soybean plants. Noticeable diseased seedlings were appeared from the *M. phaseolina* inoculated kidney bean seed (Watanabe, 1972). Fakir *et al.*, (1976) reported the pathogenicity of *M. phaseolina* in

Plate 4.22: Spreading of lesion due to *Macrophomina phaseolina* infection to upward and downward directions in mungbean causing death of whole plant.

Plate 4.23: Spreading of lesion due to *Macrophomina phaseolina* infection to upward and downward directions in blackgram causing death of whole plant.

Plate 4.24: Control (left) and *Macrophomina phaseolina* inoculated plant (right) of mungbean after 21 days of inoculation.

Plate 4.25: Control (left) and *Macrophomina phaseolina* inoculated plant (right) of blackgram after 21 days of inoculation.

sunflower seeds. They noticed damping-off and necrotic linear lesions on the inoculated stems. Moreover, pycnidia and microsclerotia were produced on the infected parts. Thirumalachar *et al.*, (1977) studied the pathogenic effect of *M. phaseolina* in the seed of sesame, sunflower, okra, chilli, and horse gram. In all cases they found significant percent damping-off of seedlings.

In all methods, the reviving of the fungal colony in PDA after reculturing fulfills the Koch's postulates. Moreover, presence of microsclerotia and pycnidia of *M. phaseolina* also supports the Koch's postulates.