Chapter III

3. Fungi Associated with Mungbean and Blackgram Seeds

3.1. An Overview

Pathogen-free healthy seeds are preferred for sowing to have desired germination, emergence, healthy seedlings, and plant population. Many disease-causing pathogens are seed-borne and seed transmitted. Seed-borne pathogens sometimes cause not only disease on the same crop, but also infects to other crops (Chou and Wu, 1995). It is, therefore, essential to test seeds for determines association of such pathogens with the seeds and to find out the tolerance level or to recommend the suitable chemicals for treating the seeds before planting. Different types of fungi are associated with seeds that may be intraembryal, extraembryal, and contaminant or with inert matter mixed with the seed sample. Lack of symptoms on the seeds does not mean that the seeds are free from infection.

Multitudes of fungal seed-borne pathogens including *Macrophomina phaseolina* were observed in mungbean (Nath *et al.*, 1970; Agarwal *et al.*, 1972; Khan *et al.*, 1977; Saxena and Sinha, 1977; Rath and Routray, 1978; Nik, 1983) and blackgram (Khan *et al.*, 1977; Suhag and Suryanarayana, 1977; Rath and Routray, 1978; Saxena and Sinha, 1979) seeds. However, published reports on the seed-borne fungi associated with mungbean and blackgram in Thailand are very few.

Many methods can be used for the detection of fungi associated with mungbean and blackgram seeds. Among them, 'blotter method' was found to be more effective compared to other methods (Agarwal *et al.*, 1972; Suhag and Suryanarayana 1977; Sadashivaiah *et al.*, 1986). It was, therefore, considered

worthwhile to examine the mungbean and blackgram seeds following the 'blotter method' to meet the objectives below:

- to investigate the frequency of seed-borne *M. phaseolina* and other seed borne fungi associated with mungbean and blackgram seeds;
- to assess the effect of infection of different fungi, especially *M. phaseolina* on seed germination of mungbean and blackgram.

3.2. Materials and Methods

3.2.1. Source of seeds

Nine seed samples were obtained from different research institutes of Thailand. Among them, four varieties (Chai Nat 60, Chai Nat 36, Chai Nat 72 and Khampen saen 2) and three elite lines (MoO 1, No.1, and No.2) of mungbean and two varieties (Uthong 2 and Phitsanulok 2) of blackgram. The seed samples were kept at 4^0C in a refrigerator till they were used for various studies.

3.2.2. Incubation and detection techniques

Seed samples were analyzed by 'blotter method' following International Rules for Seed Health Testing (ISTA, 1993). In this method, three layers of blotter papers named Whatman no.1 were soaked in sterilized water and placed on sterilized glass Petridish of 9 cm diameter. Four hundred seeds from each variety were taken randomly and 10 seeds were placed in each Petridish equidistantly (Nath *et al.* 1970). Findings of ten petridishes i.e, 100 seeds considered as one replication. The experiment was arranged in a Completely Randomized Design (CRD).

The petridishes with seeds were incubated at 20^0C under 12 hours alternating cycles of near ultra violet (NUV) light and darkness. After 7 days of incubation, the germination and the prevalence of the fungi with seeds were

recorded. The pathogens were detected on the basis of their growth character of the incubated seeds in blotter under stereo-binocular microscope, and confirmed after preparing slides and examining under the compound microscope. All the fungi were isolated and cultured in Potato-dextrose agar (PDA) medium. On the infected seed, pycnidia were observed and *M. phaseolina* was identified by observing the pycnidia with conidia.

3.3. Results

3.3.1. Detection of *Macrophomina phaseolina* and other fungi associated with naturally infected seeds of mungbean

Mean percentage of incidence of various seed-borne fungi in different varieties of mungbean seeds are presented in Table 3.01. In all varieties, incidence of *M. phaseolina* was found as a major cause of germination reduction (Figure 3.01). In most of the cases, only development of radicle had started but eventually failed to survive and turn into brownish in color; no hypocotyle extension or cotyledonary leaves developed (Plate 3.01). As a result, the seed and seedlings died. In some cases, the seeds rotted (Plate 3.01). On the dead seed and seedlings, huge number of microsclerotia were observed which were black, spherical to oblong, and occasionally irregular in shape (Plate 3.02).

The frequency of *M. phaseolina* in different varieties ranged from 2.0 to 29.75 percent. After 72 hours of incubation, the symptoms of *M. phaseolina* infection such as microsclerotia, pycnidia, and whitish mycelia manifested on the seed and blemished radicle appeared. These led to die the seedlings or eventual rotting of seeds. In some cases, the seed could not germinate and plenty of pycnidia produced on the surface of seed (Plate 3.03). The pycnidia are blackish and globose with ostiole, and variable in size (Plate 3.04). The pycnidia contain numerous pycnidiospores, which are hyaline and single-celled with a length-to-wide ratio of about 3:1 (Plate 3.06). Also it noticed that when the infected part of seedling like plumule, radicle, seedcoat or cotyledon got touched with the other

Table 3.01: Percentage of fungi associated with the seeds of different mungbean varieties and elite lines (average result of four replications, each replication contained 100 seeds).

Fungi	Mungbean varieties and clones							
	ChaiNat 36	ChaiNat 60	ChaiNat 72	Khampen Saen2	Mo O 1	No. 1	No. 2	$\pm LSD^{0.05}$
Aspergillus flavus	35.25	5.0	6.0	13.25	4.25	18.5	10.75	1.83
Aspergillus niger	29.25	7.0	12.25	31.75	14.5	14.75	45.25	2.21
Aspergillus terreus	5.5	12.25	7.5	9.75	1.25	3.75	2.25	1.27
Alternaria sp.	20.25	3.25	5.0	5.5	0.5	0.5	4.25	1.31
Cladosporium sp.	13.25	8.0	0.0	0.25	0.0	3.25	0.0	1.05
Curvularia sp.	3.0	4.0	3.5	1.75	2.5	3.5	0.5	0.96
Fusaium sp.	10.5	11.5	2.0	1.75	1.5	1.0	0.5	1.26
Macrophomina phaseolina	6.25	29.75	5.5	9.75	12.5	2.0	3.75	1.24
Penicillium sp.	4.5	21.0	6.0	4.25	1.5	4.25	5.5	1.13
Colletotrichum sp.	0.0	4.5	0.0	0.0	0.0	0.0	0.0	0.32
Drechslera sp.	0.0	0.75	0.0	0.0	0.25	0.0	0.0	0.39
Rhizopus sp.	6.75	4.5	3.0	5.0	0.0	6.25	2.0	1.09
Myrothecium sp.	0.0	0.0	0.0	1.0	0.0	0.0	0.0	-
Seed Germination (%)	**80**	**58.25**	**90.0**	**74**	**71**	**94.25**	**90.0**	**2.40**

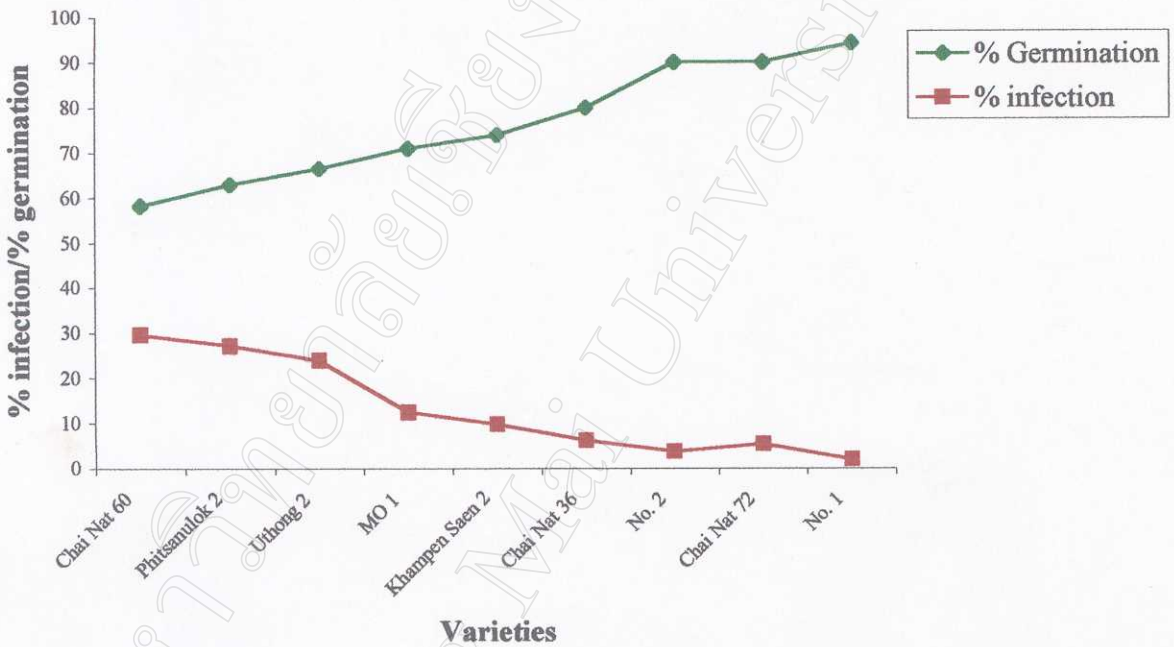

Figure 3.01: Percent infection of *Macrophomina phaseolina* and germination of seeds in different mungbean and blackgram varieties and elite lines

Plate 3.01: 1. Mungbean seed incubated by blotter method.
2. *Macrophomina phaseolina* infection appeared after incubation by blotter blotter method.
B- Mungbean seed with brownish radicle.
R- Rotten seed.
H- Healthy seed.

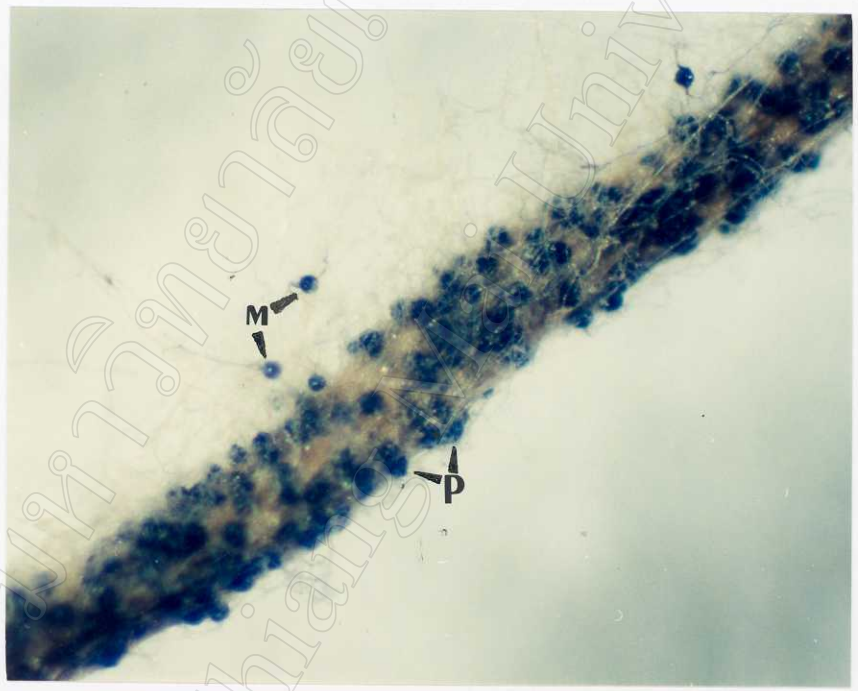

Plate 3.02: Microsclerotia (M) and pycnidia (P) on the *Macrophomina phaseolina* infected radicle of mungbean under stereomicroscope.

Plate 3.03: A. *Macrophomina phaseolina* infected mungbean seed under stereomicroscope with numerous pycnidia.
B. Pycnidia (P) of *Macrophomina phaseolina* on mungbean seed under stereomicroscope.

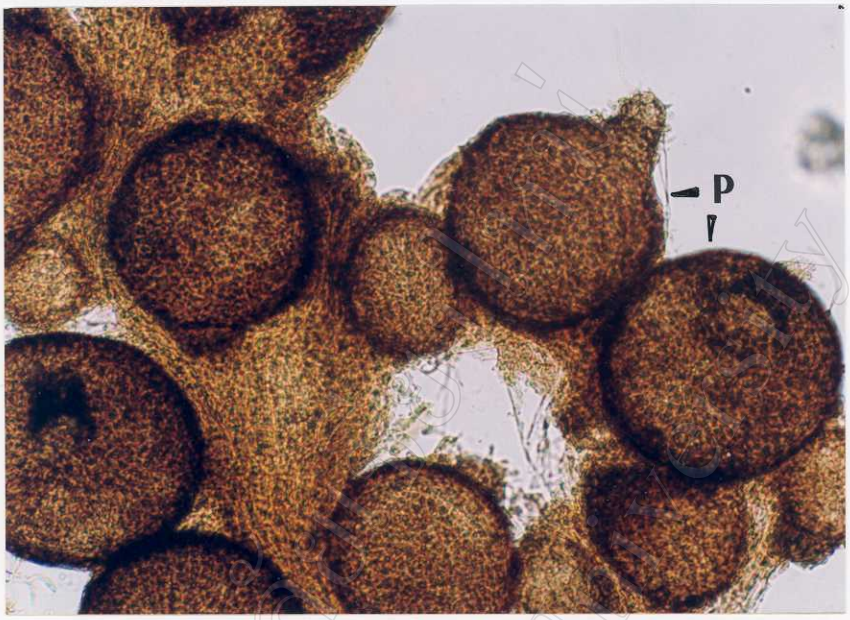

Plate: 3.04: Pycnidia (P) of *Macrophomina phaseolina* from mungbean seed under compound microscope.

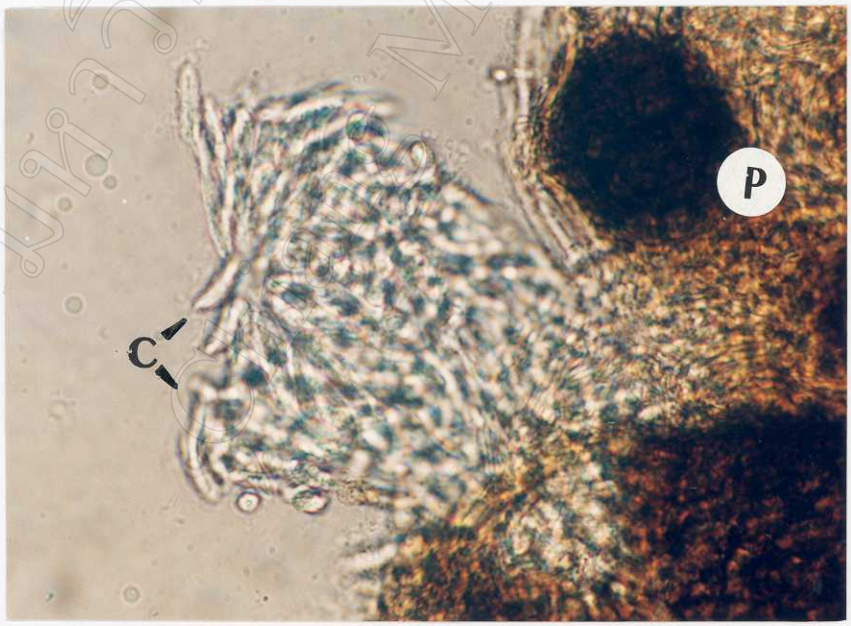

Plate: 3.05: Liberating of conidia (C) from ruptured pycnidium (P) of *Macrophomina phaseolina* of mungbean seed observed under compound microscope.

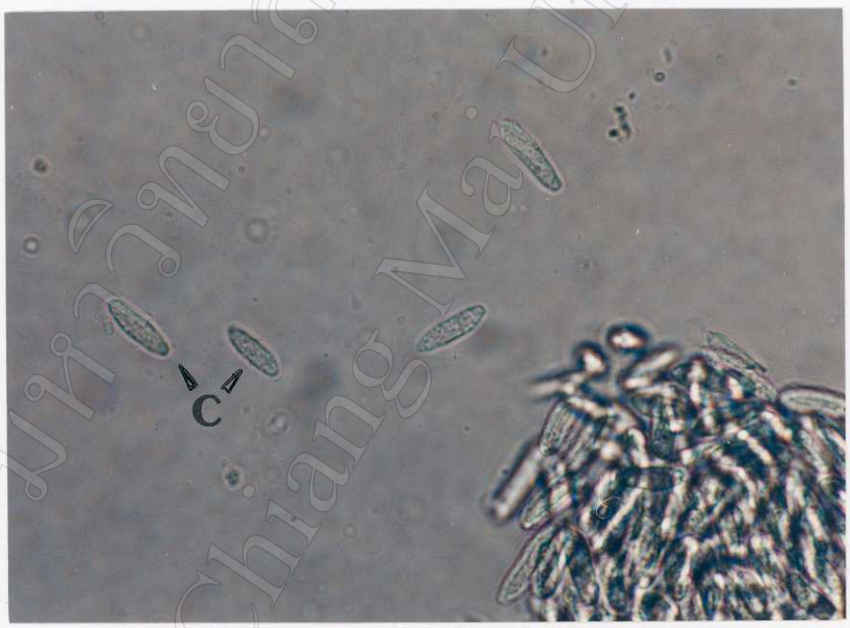

Plate 3.06: Conidia (C) from pycnidium of *Macrophomina phaseolina* from mungbean seed observed under compound microscope.

healthy seedlings, the healthy seedlings became infected and produced the same symptoms of *M. phaseolina* inection (Plate 3.07).

In all, 13 different forms of fungi were found to be associated with the tested samples of mungbean. *Aspergillus flavus, Aspergillus niger, Aspergillus terreus, Alternaria sp., Curvularia sp., Fusarium sp., Macrophomina phaseolina and Penicillium sp.* were associated with all mungbean varieties and elite lines. *Cladosporium sp.* found with the seeds of the variety Chai Nat 36, Chai Nat 60, Khampen Saen 2 and No. 1 by 13.25, 8.0, 0.25, and 3.25 percent, respectively. Among all varieties, *Colletotrichum sp.* caused germination reduction only in Chai Nat 60. However, the presence of this fungus was found by 4.5 percent, which was not quite high.

Association of seed-borne *Drechslera sp.* was rather low. Only 0.75 and 0.25 percent *Drechslera sp.* were detected in the variety Chai Nat 60 and MoO 1, respectively. *Myrothecium sp.* was evolved only in Khampen Saen 2 variety by 1.0 percent. Another prominent fungus is *Rhizopus sp.*, which was observed in all mungbean tested samples except MoO 1. The frequency of *Rhizopus sp* was 6.25, 4.5, 3.0, 5.0, 6.25 and 2.0 percent in the variety Chai Nat 36, Chai Nat 60, Chai Nat 72, Khampen Saen 2, No. 1 and No. 2, respectively.

It was observed that most of the seed-borne fungi could not reduce germination severely except *M. phaseolina* and *Colletotrichum sp.*, even at higher incidence. However, *Alternaria* sp. impeded germination to a lesser extent in some varieties like in Chai Nat 36.

The high incidence of *A. flavus*, *A. niger*, and *A. terreus* did not show any obvious adverse effect on germination. In the incubated seeds, *A. flavus* in Chai Nat 36, Khampen saen 2, No.1 and No. 2, *A. niger* in Chai Nat 36, Khampen saen 2, MoO 1, No.1, and No.2, and *A. terreus* in Chai Nat 60 did not hamper the germination ability, although in all cases they had got high frequency of fungi.

Plate 3.07: *Macrophomina phaseolina* infected mungbean seed spreading infection (I) to healthy seedlings by touching.

3.3.2. Detection of *Macrophomina phaseolina* and Other Fungi Associated with Naturally Infected Seeds of Blackgram

Different fungi and their mean percentage incidence in two varieties of blackgram seeds are presented in Table 3.02. Isolation and recovery of *M. phaseolina* by blotter method undoubtledly ascertained that the pathogen was associated with the seed. Akin to mungbean, incidence of *M. phaseolina* was found to be the major cause of germination reduction in both tested varieties of blackgram (Figure 3.01). In most of the cases, the radicle could not survive although it had emerged and developed neither hypocotyls nor cotyledonary leaves. In the blotter test, the symptom of *M. phaseolina* infection appeared as brownish colored radicle (Plate 3.08). The emerged radicle decayed followed by developing profuse typical pycnidia of *M. phaseolina* on that (Plate 3.09). Due to this reason, the seedlings or edible sprouts of blackgram were turned into blemishes. Similar to mungbean, on the blemished radicle copius microsclerotia were developed which were blackish in color and irregular in shape (Plate 3.10).

The frequency of *M. phaseolina* in blackgram was 24.0 and 27.25 percent in Uthong 2 and Phitsanulok 2 varieties respectively, which was noticeably high. Like mungbean, the typical symptom of *M. phaseolina* such as enormous blakish pycnidia and microsclerotia including whitish mycelia were appeared on the infected seed after 72 hours of incubation. Simulteniously, the blemished radicles were also observed. These symptoms led the seed or seedlings into eventual death and rotten appearances as mungbean. The color, shape, and size of microsclerotia, pycnidia, and pycnidiospores were also found same like mungbean. Moreover, as mungbean, the touched healthy seedlings by the infected seedlings or seeds got infected in the same manner (Plate 3.11).

Some seeds were not able to germinate. On the surface of these seeds, copius pycnidia and microsclerotia including mycelia were produced. The pycnidia found same as mungbean under compound microscope (Plate 3.12).

Table 3.02: Percentage of fungi associated with the seeds of blackgram varieties (average of four replications. Each replication contained 100 seeds).

Fungi	Blackgram varieties		
	Uthong 2	Phitsanulok 2	± $LSD^{0.05}$
Aspergillus flavus	7.75	5.75	1.30
Aspergillus niger	10.25	6.25	1.29
Aspergillus terreus	1.0	7.0	2.60
Alternaria sp.	2.25	1.5	1.52
Cladosporium sp.	0.5	0.0	0.92
Curvularia sp.	13.25	17.0	2.38
Fusarium sp.	6.25	28.75	3.31
Macrophomina phaseolina	24.0	27.25	2.00
Penicillium sp.	4.25	6.0	1.52
Colletotrichum sp.	0.0	0.75	0.79
Myrothecium sp.	0.0	0.25	0.79
Seed Germination (%)	**66.5**	**63**	**3.31**

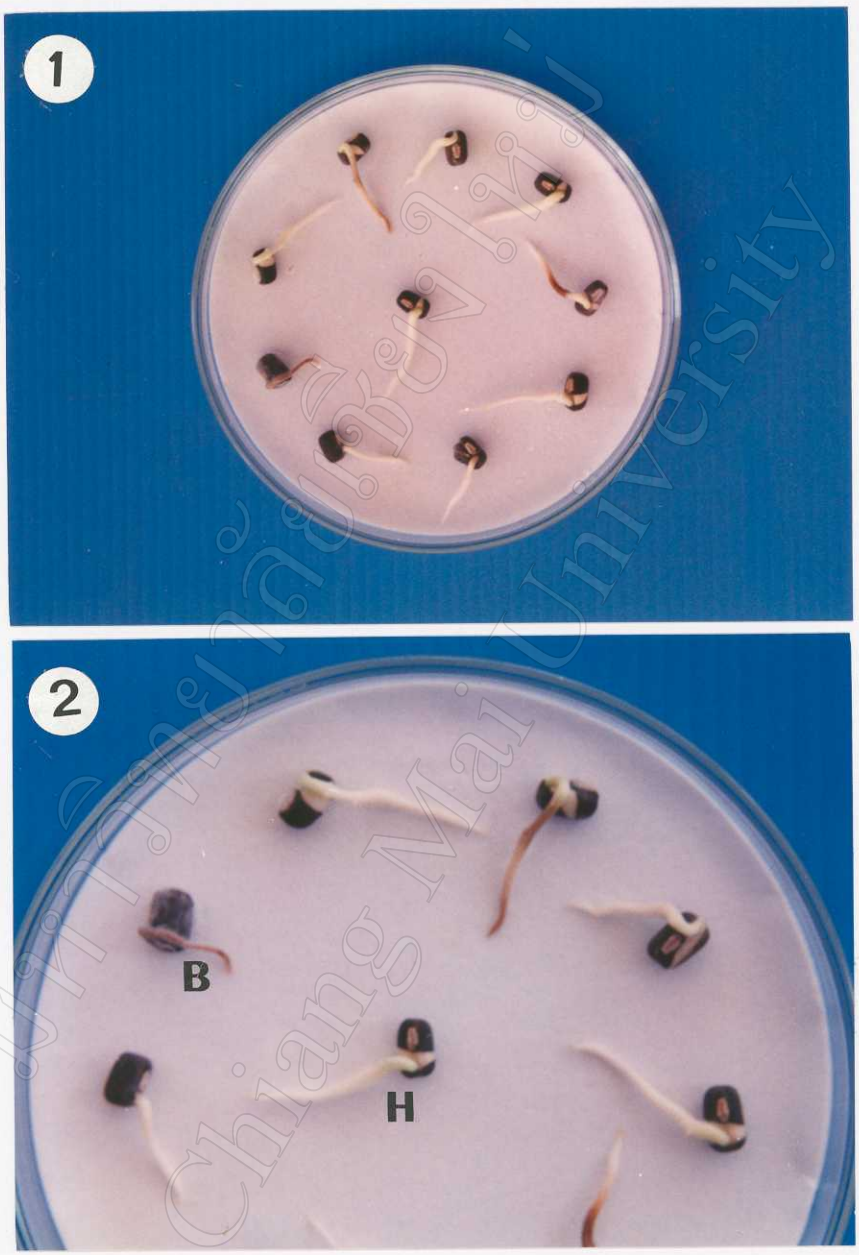

Plate 3.08: 1. Blackgram seed incubated by blotter method.
2. *Macrophomina phaseolina* infection appeared after incubation by blotter method.
B- Blackgram seed with brownish radicle
H- Healthy seedlings of blackgram

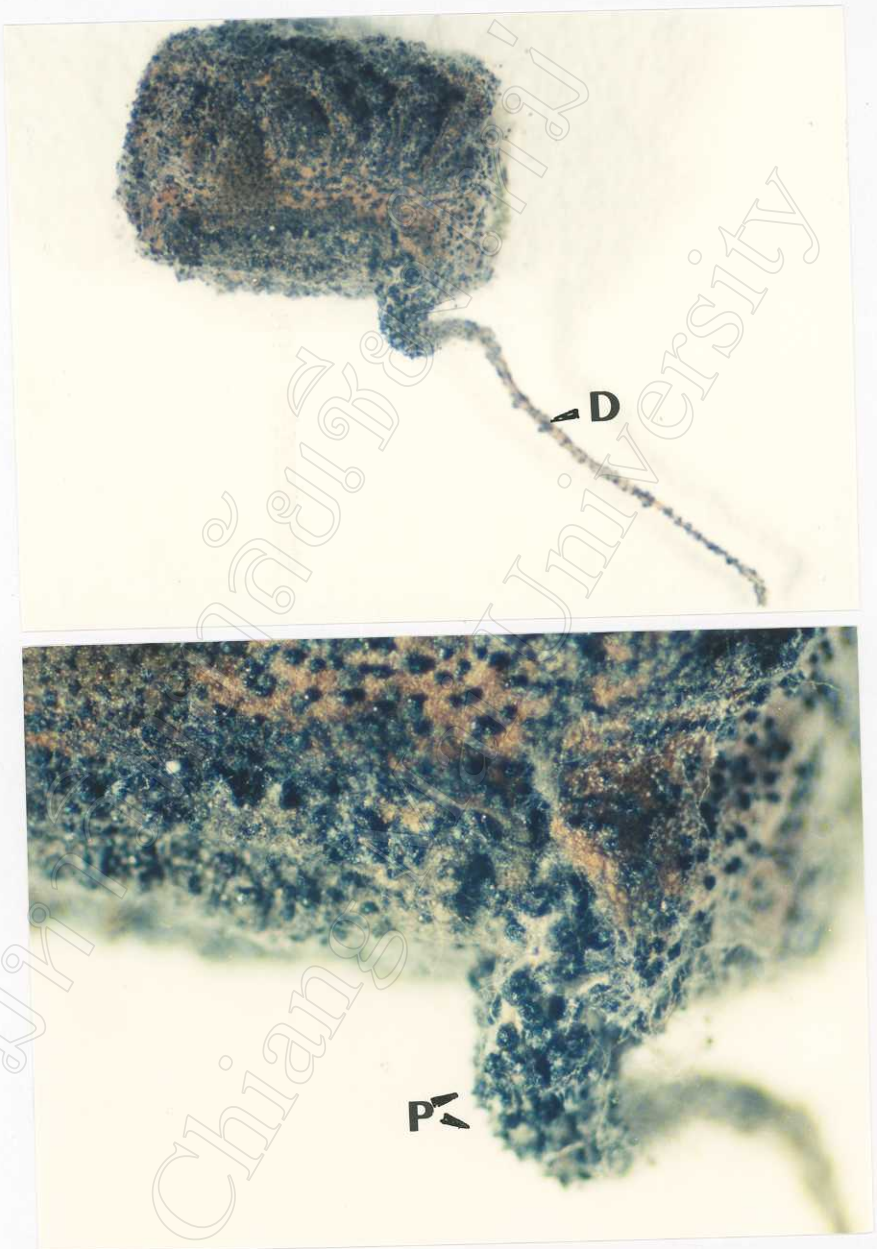

Plate 3.09: A. *Macrophomina phaseolina* infected blackgram seed under stereomicroscope with dead radicle (D) and without plumule
B. Numerous pycnidia (P) of *Macrophomina phaseolina* on blackgram seed.

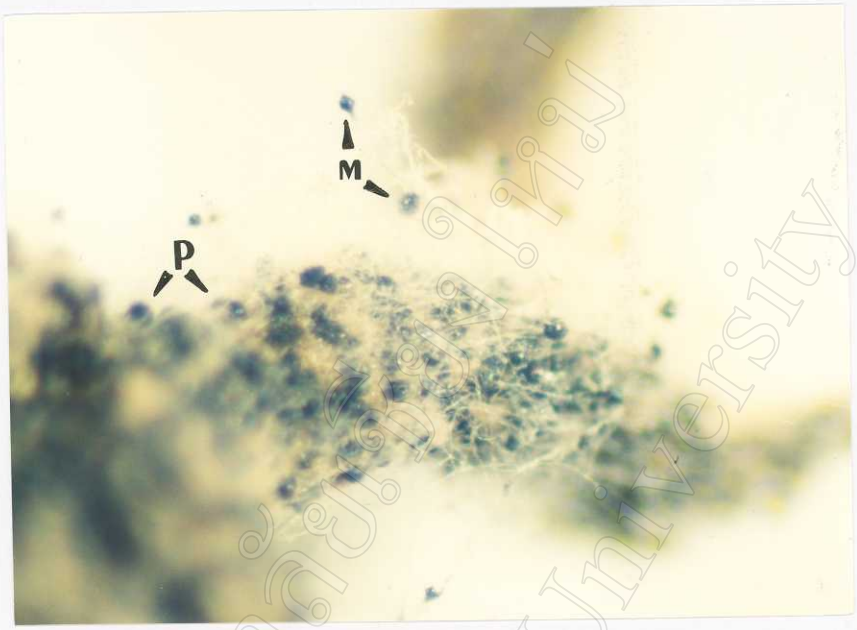

Plate 3.10: Microsclerotia (M) and pycnidia (P) on the *Macrophomina phaseolina* infected radicle of blackgram under stereomicroscope.

Plate 3.11: *Macrophomina phaseolina* infected blackgram seed spreading infection (I) to healthy seedlings by touching.

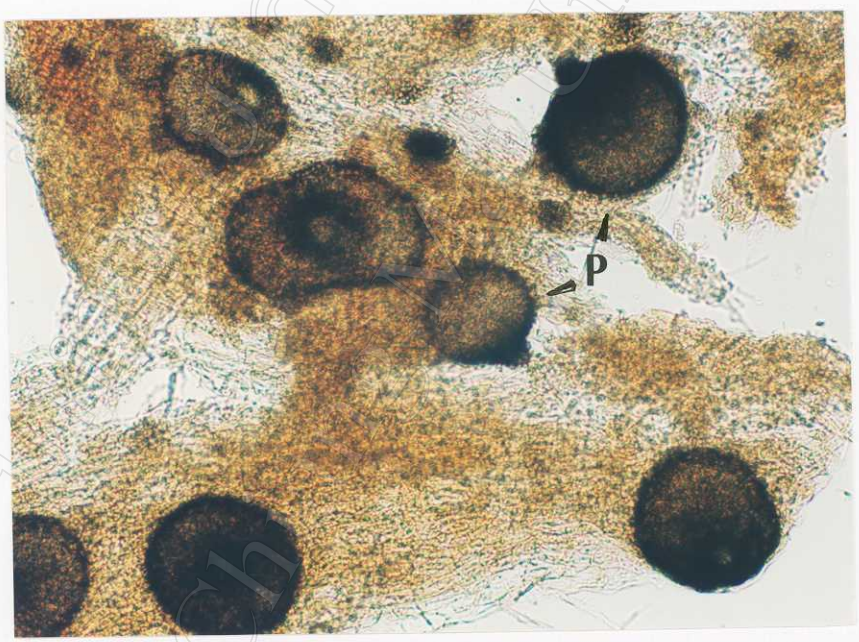

Plate: 3.12: Pycnidia (P) of *Macrophomina phaseolina* from blackgram seed under compound microscope.

When the pycnidia were ruptured, numerous oblong conidia were observed (Plate 3.13). The color and size of conidia appreared to be hyaline like mungbean (Plate 3.14).

With the tested two blackgrams varieties, total 11 different forms of fungi were found to be associated. *Aspergillus flavus, Aspergillus niger, Aspergillus terreus, Alternaria sp., Curvularia sp., Fusarium sp., M. phaseolina and Penicillium sp.* were detected with both the varieties. *Cladosporium sp.* was found in Uthong 2 variety only in 0.5 percent, *Colletotrichum sp.* and *Myrothecium sp* were found in Phitsanulok 2 variety as 0.75 and 0.25 percent respectively (Table 3.02) which were quite low.

From the germination of blackgram, like mungbean it was observed that only *M. phaseolina* could reduce germination greatly, but the other fungi could not exhibit any considerable adverse effect on germination. The high incidence of *Curvularia* sp. and *Fusarium* sp. sometimes decreased the germination, which was not significant. The presence of *A. flavus*, *A. niger,* and *A. terreus* did not menifest any noticeable harmful effect on germination.

3.4. Discussion

The findings of the present study clearly showed that mungbean and blackgram seeds were heavily associated with a number of pathogenic and saprophytic fungi. Among them, *M. phaseolina* was the major seed-borne fungi, which was responsible for germination reductin. It was observed that the higher incidence of *M. phaseolin* resulted the lower germination percentage or vice versa (Figure 3.01). Therefore, it can be inferred that the presence of *M. phaseolina* reduces germination of mungbean and blackgram greatly.

The presence of *M. phaseolina* in mungbean and blackgram causes not only germination reduction but also declines the quality of sprouts. Blemishness of sprout is responsible for quality deterioration. Because of this reason, the

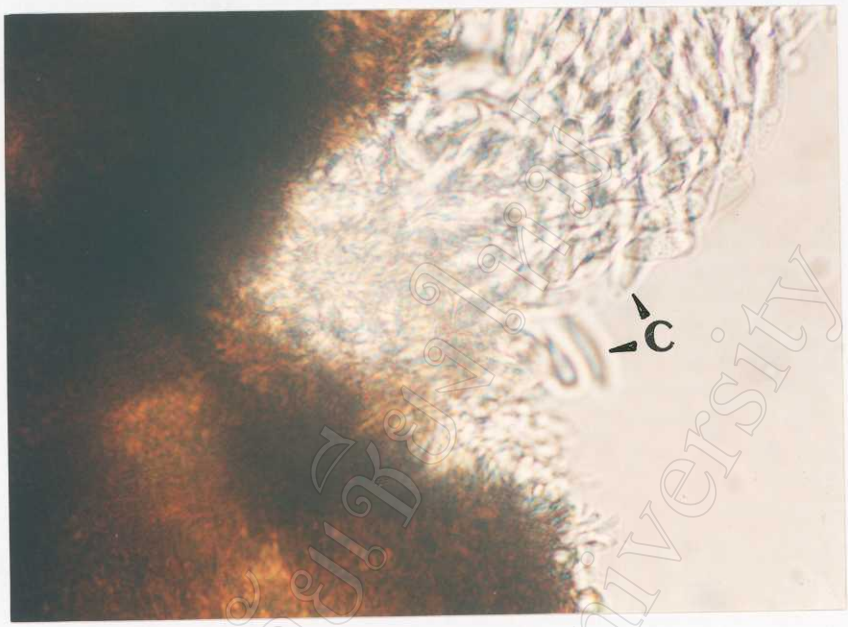

Plate: 3.13: Liberating of conidia (C) from ruptured pycnidium of *Macrophomina phaseolina* of blackgram seed observed under compound microscope.

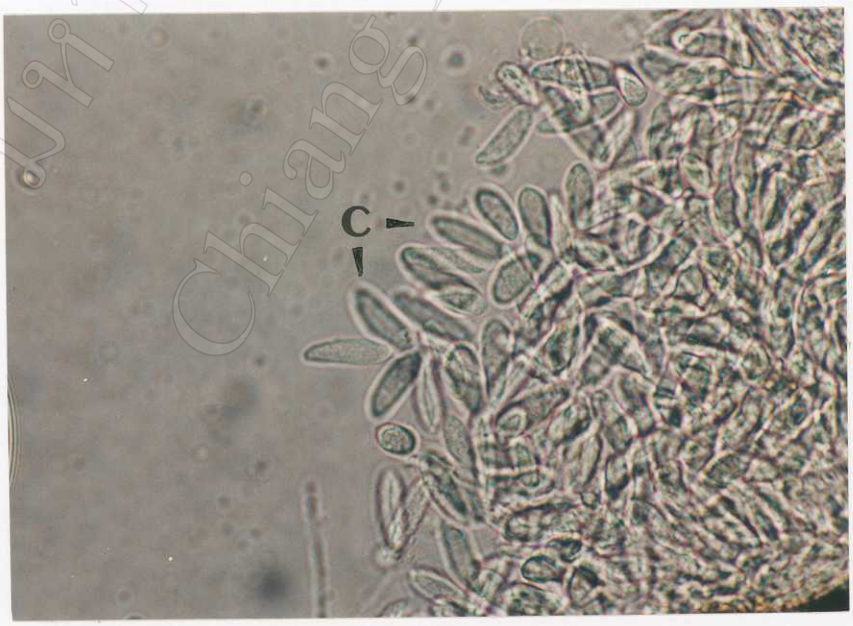

Plate 3.14: Conidia (C) from pycnidium of *Macrophomina phaseolina* from blackgram seed observed under compound microscope.

blackgram of Thailand is often complained by the importers (Chainnuvati *et al.*, 1987; Putasamai and Surin, 1988; Pichitporn and Thavarasook, 1990). In soybean (Gangopadhyay *et al.*, 1970) and sunflower (Fakir *et al.*, 1976; Raut, 1983) seeds, the effect of *M. phaseolina* was reported and described with similar symptoms.

It was also observed that in most of the mungbean varieties and clones did not have high frequency of *M. phaseolina* infection except for the variety Chai Nat 60. On the other hand, both blackgram varieties, which were used in this experiment, showed high incidence of *M. phaseolina.* The cause of high infection in blackgram seeds by *M. phaseolina* may be due to contact of pods with soil as blackgram always lodges on the ground at the maturity stage, which was mentioned by Fuhlbohm *et al.*, (1997) and Ellis *et al.*, (1976). According to Fuhlbohm *et al.*, (1997) and Ellis *et al.*, (1976), the higher infection in mungbean and dry bean respectively when their pod contacted in the soil at maturity stage.

The other fungi, except *Colletotrichum* sp, were not apparently harmful for mungbean and blackgram seeds. *Colletotrichum sp.* caused germination reduction. Nevertheless, fortunately the presence of this fungus was found to be very low. Germination reduction was also encountered seldom by the high incidence of *Alternaria* sp., *Curvularia* sp. and *Fusarium* sp. In case of mungbean and blackgram, the presence of *Aspergillus flavus*, *Aspergillus niger*, and *Aspergillus terreus* were not harmful in germination even at high frequency.

Although these *Aspergillus* spp. do not reduce the germination of mungbean and blackgram, but their presence as a mycotoxin producer might be harmful for human consumption. There is therefore, further study is needed to know the toxic effect of *Aspergillus flavus* in mungbean and blackgram. Moreover, as the seed infection of *M. phaseolina* can reduce the germination greatly and cause the declining of yield, so infected seeds must be treated before plantation.