CHAPTER VIII

8. Effect of *Macrophomina phaseolina* on Carbohydrate and Protein Content in Mungbean and Blackgram Seeds

8.1. An Overview

Invasion of pathogen in the seeds results the biochemical deterioration and change in the quality of seed nutrients. Several seed-borne fungi are reported to cause considerable loss in seed contents (Sinha and Prasad, 1977). The loss occurs mainly due to biochemical changes. Depletion and accumulation of important metabolites due to seed-borne infections are also known (Bilgrami *et al.*, 1976). Because of activity of storage fungi, remarkable alter in the chemical composition of seeds has been recorded (Bilgrami *et al.*, 1978). Usually after fungal infection, the nutrient levels of seeds are reduced but in some cases, some nutrients are found to have increased *viz.* due to *Aspergillus flavus* infection some amino acids increased in arhar (*Cajanus cajan*) seeds (Sinha and Prasad, 1977).

The biochemical changes due to fungi invasion in different crops has been studied such as the effect of *Aspergillus flavus, Aspergillus niger*, *Penicillium* sp, *Alternaria alternata*, *Fusarium semitectum*, *Curvularia lunata* and *Helminthosporium hawaiiense* in mungbean and blackgram (Bilgrami *et al.*, 1976); *Alternaria alternata*, *Aspergillus flavus*, *Aspergillus niger*, *Fusarium moniliforme* on sesame and sunflower (Saxena and Karan, 1991); *Aspergillus flavus* on arhar (*Cajanus cajan*) seeds (Sinha and Prasad, 1977). Nevertheless, the effect of *M. phaseolina* infection in mungbean and blackgram seeds has not been taken into account earlier. Therefore, the present investigation was undertaken to:

- find out the effect of *M. phaseolina* infection on carbohydrate and protein content in mungbean and blackgram seeds.

8.2. Materials and Methods

8.2.1. Source of Sample

Macrophomina phaseolina infected seed of mungbean and blackgram were obtained from Chai Nat Field Crops Research Centre, Thailand. The name of mungbean variety was Chai Nat 60 and blackgram variety were Uthong 2 and they were carrying 29.75% and 24.0% infection of *M. phaseolina* according to blotter method.

8.2.2. Infected and healthy seed separation and sample preparation

One hundred seed of each variety were plated in 3-layeded Whatman no.1 blotter paper. The papers were previously soaked with sterile water. In each plate, 10 seeds were placed. The plates were kept under 12 hours alternating NUV light and darkness. Just after two days, the seeds bearing with pycnidia, mycelia and microsclerotia of *M. phaseolina* were separated. After separation, 20 healthy seeds and 20 infected seeds of each variety were dried at 65^0C in air-oven for 3 days. Thereafter, the seeds of each category were grinded finely in a ball mill. The finely grinded samples were used for carbohydrate and protein analysis.

8.2.3. Carbohydrate analysis by 'Anthrone' method (Yoshida *et al.*, 1976)

8.2.3.1. Reagent preparation:

i) Anthrone solution: Two gram of anthrone was dissolved in 1liter concentrate sulphuric acid. The solution was stored in a refrigerator. A fresh solution was prepared for every 2 days.

ii) Glucose stock solution: In a 1000ml volumetric flask, 0.10g of dried glucose were put. The flask was filled with distilled water up to the mark. The prepared solution contains 100ppm glucose. From this 100ppm solution, 1ml, 2ml, 3ml, 4ml and 5ml solution were transferred in five Pyrex test tubes. To make 5ml volume in each test tube, the

required distilled water added. After adding water, the sugar content of the tubes became 0.1mg/tube, 0.2mg/tube, 0.3mg/tube, 0.4mg/tube, and 0.5mg/tube respectively.

8.2.3.2. Procedure:

i) Sample extraction

Into a 15ml centrifuge tube, 100mg of finely grinded sample of each category were placed. In each centrifuge tube, 10ml of 80 percent ethanol was added. Placing a glass ball on the top, the tubes were kept in a water bath at 80^0C to 85^0C for 30 minutes. After that, these tubes were centrifuged at 3500 rpm for 30 minutes and the decance was poured in a 50ml beaker. This extraction was repeated for three times more.

The alcohol extract was evaporated on a water bath at 80^0C to 85^0C until most of the alcohol was removed. After evaporating alcohol, the volume of the extract was made as 25ml with adding distilled water. The carbohydrate content of this extract was analyzed. From each category of the sample, extraction was done in same way for 4 times to make 4 replicates.

ii) Analysis of carbohydrate by Anthrone method:

Five milliliters of extract was transferred to a 100ml volumetric flask and was made the volume up to the mark with distilled water. From this extract, 5ml were put in a Pyrex test tube. These tubes and the tubes containing standards were kept in an ice bath. Thereafter, 10ml of anthrone reagent was added slowly to each tube allowing the reagent to run down the side of the test tube. Then stirring was done slowly with a glass rod.

All the tubes were put into a boiling water bath for exactly 7.5 minutes and then immediately kept them in ice in order to become cool. After cooling,

the absorbance was measured at 630nm of wave length in a spectrophotometer (Model: Beckman, DU 7500) by setting the spectronic at zero with blank prepared in the same manner except adding distilled water instead of sample extract.

8.2.4. Protein analysis by Micro-Kjeldahl method (Yoshida *et al.*, 1976)

8.2.4.1. Reagent preparation

i) Salt mixture: Two hundred fifty gram of sodium sulphate (Na_2SO_4), 50g copper sulphate ($CuSO_4$ $5H_2O$) and 5g metallic selenium were mixed together.

ii) Boric acid solution (4 percent): Fourty grams H_3BO_4 were mixed in 1 litre of distilled water.

iii) Mixed indicator: In 400ml of 90 percent ethanol, 0.3g of bromcresol green and 0.2g of methyl red were dissolved. In color of indicator was changed into red in acid solution and blue in alkaline solution.

iv) Sodium hydroxide solution (40 percent): Under a fume hood, 400g of technical grade sodium hydroxide (HaOH) were dissolved in a beaker containing 600ml of distilled water. Then the beaker was placed in a cold-water bath to dissolve the produced heat. After cooling, the solution was stored in a screw-top bottle.

v) Sodium carbonate: Ten to 20g of AR grade Na_2CO_3 were transferred to a Pyrex beaker and was heated at 270^0C for 3 hours. Thereafter, the beaker was allowed to cool in desiccators.

vi) Methyl orange indicator: In 100ml distilled water, 0.1g of methyl orange was dissolved.

vii) Standard hydrochloric acid solution (0.1N): Nine milliliters of concentrated hydrochloric acid (HCl) were dissolved with distilled water and the final

volume was made into 1 litre. This approximate 0.1N HCl solution was standardize as follows:

Exactly 0.530g of sodium carbonate reagent was dissolved in 20ml of distilled water. This solution was diluted in 100ml. In a 125ml Erlenmeyer flask, 10ml of this 0.1N sodium carbonate solution was transferred and two drops of methyl orange indicator was added. The approximate 0.1N HCl solution was titrated into the 0.1N sodium carbonate solution until the methyl orange indicator turned into reddish orange color. The solution was boiled gently for one minute and then cool to room temperature under running tap water over the outside of the flask. If the color changed back to orange, more HCl was titrated until the first faint but permanent reddish-orange color appears in the solution. The normality of HCl solution was calculated by the following formula-

$$Normality\ of\ HCl = \frac{0.1x10}{ml\ of\ HCl\ titrated}$$

viii) Standard hydrochloric acid (0.05N): Five hundred milliliters of standardized 0.1N HCl were transferred to a 1-litre volumetric flask and was made up to the volume of the flask with distilled water.

8.2.4.2. Procedure:

i) Digestion:

Two hundred milligrams powdered sample of each variety were taken in 100 ml Kjeldahl flask. Approximately the same weight of salt mixture and 3ml of concentrated H_2SO_4 were added. The Kjeldahl flask was placed in an empty tin can of suitable size and was heated over a flame to digest the sample. When the sample was clear, the flask was allowed to cool and then 10ml of distilled water was added. After mixing thoroughly, the sample was allowed to cool again.

ii) Distillation:

The distilled sample was transferred from Kjeldhal flask to micro-Kjeldahl distillation apparatus by ringing the flask for three times. In every time, minimum water was used. Then with the quick delivery pipette, 10ml of 40 percent NaOH solution was added to the distillation apparatus slowly.

In a 125ml Erlenmeyer flask containing 10ml of 4 percent boric acid reagent, 3 drops of mixed indicator were added. The flask was placed under the condenser of the distillation apparatus and the tip of the condenser outlet was put in beneath the surface of the solution in the flask. Thereafter, the steam from the boiler was allowed to pass through the sample for distilling off the ammonia into the flask containing boric acid and mixed indicator solution. The sample was distilled in this way for 7 minutes. The flask was lowered and was allowed the solution to drop from the condenser into the flask for about 1 minute. Then the tip of the condenser outlet was washed with distilled water.

iii) Titration:

The solution of boric acid and mixed indicator containing the "distilled off" ammonia with the standardized HCl were titrated. Titrate value of blank solution of boric acid and mixed indicator without distilled off ammonia.

8.2.4.3. Calculation:

The nitrogen value of the sample was calculated by the following formula:

$$\%nitrogen\,in\,sample = \frac{(sample\,titre - blank\,titre)\,x\,normality\,of\,HCl\,x14\,x100}{sample\,weight(g)\,x100}$$

$$\%\,protein = 6.25\,x\,\%nitrogen\,in\,the\,sample$$

8.3. Result

8.3.1. Carbohydrate content

The percentage of carbohydrate content in healthy and *M. phaseolina* infected mungbean and blackgram seeds were showed in Table 8.01. In the diseased seeds of both mungbean and blackgram, carbohydrate content declined greatly. In healthy and infected mungbean seeds the carbohydrate content found by 22.50 and 8.25 percent respectively (significant at P=0.05). Similarly, in blackgram seeds, carbohydrate content depleted significantly (P=0.05) from 22.50 percent to 12.25 percent due to *M. phaseolina* infection.

8.3.2. Protein content

The change in protein content due to *M. phaseolina* infection in the seeds of mungbean and blackgram is presented in Table 8.02. Owing to *M. phaseolina* infection, protein content in seeds of mungbean and blackgram were increased significantly (P=0.05). The protein content was uplifted from 22.05 to 28.77 percent in mungbean seeds, because of infection. Correspondingly, the upsurge in protein content revealed by 27.13 to 32.81 percent from healthy to diseased blackgram seeds (significant at P=0.05).

8.4. Discussion

From the present investigation, it is revealed that the carbohydrate content has severely reduced due to *M. phaseolina* infection in mungbean and blackgram seeds. However, the reduction of carbohydrate content due to *M. phaseolina* was higher in blackgram than mungbean. The carbohydrate reduction appeared as almost two-third and half in mungbean and blackgram seeds respectively compared to healthy seeds because of *M. phaseolina* infection. Decline in carbohydrate content in mungbean and blackgram

Table 8.01: Percentage of carbohydrate content in healthy and diseased seeds of mungbean and blackgram.

Mungbean		Blackgram	
Healthy	Diseased	Healthy	Diseased
22.50	8.25	22.25	12.25
LSD = 0.683 at P=0.05			

Table 8.02: Percentage of protein content in healthy and inoculated seeds of mungbean and blackgram

Mungbean		Blackgram	
Healthy	Diseased	Healthy	Diseased
25.05	28.77	27.13	32.81
LSD = 0.190 at P=0.05			

diseased seeds are due to their consumption of that fungus and its break down into carbon dioxide and water. Research reports on this aspect either for mungbean or blackgram are not available. However, reports on other crops under this research discipline are available. Saxena and Karan (1991) found the reduction in carbohydrate content in sesame and sunflower seeds due to infection of seed-borne fungi, viz. *Alternaria alternata*, *Aspergillus flavus*, *Aspergillus niger* and *Fusarium moniliforme.* They indicated the probable causes of carbohydrate reduction were the utilization and conversion of carbohydrate into carbon dioxide and water by storage fungi. Different types of carbohydrates like fructose, glucose, sucrose, and ribose were greatly reduced and even in some cases disappeared such as *Geotrichum candidum* infection in round gourd, *Citrullus vulgaris* var. *fistulosus* (Sumbali and Mehrotra, 1982). Sumbali and Mehrotra (1982) described that the decrease in sugars in the infected round gourd was likely to be related to the breakdown of carbohydrates by fungal enzymes or host carbohydrates that being used as substrate. Sandhu *et al.*, (1998) reported that the total sugars and starch content were higher in healthy stem and root tissues as compared to plants infected by *M. phaseolina.* The cause of reduction in carbohydrate content was due to the activity of α-amylase and invertase enzymes in the infected tissues. Sinha and Prasad (1977) observed a considerable decrease in carbohydrate contents (three soluble sugars viz. glucose, fructose and sucrose) of arhar (*Cajanus cajan*) seeds because of *Aspergillus flavus* infection. They reported complete utilization of glucose and fructose in infected seed by the infecting fungi. Similarly, Srichuwong (1992) found less carbohydrate in the *Colletotrichum truncatum* infected soybean seeds in comparison to healthy ones. He mentioned the probable cause of carbohydrate depletion was utilization of carbohydrate as a substrate or energy source, resulting in chemical break down of nutrients.

In the present study, higher protein content was found compared to healthy check in the diseased seeds of both mungbean and blackgram. The increase in amount of protein content in the *M. phaseolina* infected seed can

be described as the additional protein that might be added from the fungal mycelia. Although reports regarding the effect of *M. phaseolina* infection on protein content in mungbean and blackgram are not available, but increase of protein content in infected seed by other fungi has been investigated and reported. Bilgrami *et al.*, (1978) found higher protein in moong seed as a result of fungal infection. Vidhyasek *et al.*, (1973) reported in rice grain due to *Helminthosporium oryzae* infection, the protein content was increased. Similarly, in lima bean stem, because of *M. phaseolina* infection, higher protein content was explored (Jadeja and Patel, 1989). In soybean, due *to Colletotrichum truncatum* infection, the protein concentration was thrived (Srichuwong, 1992). Sinha and Prasad (1977) when studied the deterioration of arhar (*Cajanus cajan*) seeds by *Aspergillus flavus*, they observed some amino acids like glutamic acid and β-alanine were higher in the infected seeds. They reported that increase in amino acid in infected seeds might be due to growth of fungal organisms in the seed. That means, mycelia in seed may play an important role in increasing amino acid content in seed.