DETECTION AND IDENTIFICATION OF SEED BORNE BACTERIA OF MAIZE AND THEIR MANAGEMENT WITH SELECTED Cu-FUNGICIDES

KADAMBARI ROY

DEPARTMENT OF PLANT PATHOLOGY
SHER-E-BANGLA AGRICULTURAL UNIVERSITY
SHER-E-BANGLA NAGAR, DHAKA-1207

JUNE, 2011

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BY

KADAMBARI ROY
REGISTRATION NO. : 05-1829

A Thesis
Submitted to the Faculty of Agriculture,
Sher-e-Bangla Agricultural University, Dhaka,
in partial fulfillment of the requirements for the degree of

Master of Science
In
Plant Pathology

Semester: January-June, 2011

Approved by:

Nazneen Sultana	**Mrs. Nasim Akhtar**
Associate Professor	Professor
Department of Plant Pathology	Department of Plant Pathology
Supervisor	**Co-Supervisor**

Nazneen Sultana
Chairman
Examination Committee
Department of Plant Pathology
Sher-e-Bangla Agricultural University, Dhaka

Nazneen Sultana
Associate Professor
Department of Plant Pathology
Sher-e-Bangla Agricultural University
Dhaka-1207, Bangladesh

Email: nazneensau @yahoo.com
Cell No.01733955171

Ref:…… **Date:…….**

CERTIFICATE

*This is to certify that thesis entitled, "**DETECTION AND IDENTIFICATION OF SEED BORNE BACTERIA OF MAIZE AND THEIR MANAGEMENT WITH SELECTED Cu-FUNGICIDES**" submitted to the Faculty of Agriculture, Sher-e-Bangla Agricultural University, Dhaka, in partial fulfillment of the requirements for the degree of **MASTER OF SCIENCE** in **PLANT PATHOLOGY,** embodies the result of a piece of bona fide research work carried out by **KADAMBARI ROY,** Registration No: 05-01829 under my supervision and guidance. No part of the thesis has been submitted for any other degree or diploma.*

I further certify that such help or source of information, as has been availed of during the course of this investigation has been duly been acknowledged by her.

Dated: 20th September, 2012
Dhaka, Bangladesh

……..
(NAZNEEN SULTANA)
Supervisor

DEDICATED TO
MY
BELOVED PARENTS

ACKNOWLEDGEMENT

First and foremost the author would like to thank Almighty God for what he has given to the author and whose abundant grace and mercy has enabled for successful completion of the research, preparation of manuscript its submission in time as a partial requirement for the degree of MS (Master of Science) in Plant pathology.

And after that the author expresses her greatful respect, wishes, whole hearted gratitude and appreciation to her benevolent teacher and supervisor Associate Professor and Chairman ***Nazneen Sultana****, Department of Plant Pathology, Sher-e-Bangla Agricultural University, Dhaka, for her precious suggestions, constructive criticism, proper guidance and helpful comments through out the study.*

The author expresses with a deep sense of respect to her Co-supervisor ***Mrs. Nasim Akhtar*** *Professor, Department of plant Pathology, Sher-e-Bangla Agricultural University, Dhaka for her cordial inspiration, guidance and helpful suggestions for its improvement. Her scholastic supervision and constant inspiration brought this thesis up to its present standard.*

Cordial thanks and honors to ***Dr. Md. Rafiqul Islam****, Professor, Department of plant Pathology, Sher-e-Bangla Agricultural University, Dhaka for his valuable advice, criticism, suggestions and provision of facilities and support needed to undertake this research work.*

The authoress expresses her sincere appreciation and gratitude to her respectable teachers, Professor ***Dr. M. Salauddin M. Chowdhury*** *and Associate Professor* ***Dr.F.M. Aminuzzaman*** *Department of plant Pathology, Sher-e-Bangla Agricultural University, Dhaka, for their inspiration and encouragement throughout the whole period of the research work.*

The author also conveys her special thanks to Shanjida Haque, Nargis Islam Roni, Mamun-or-Rashid and Matin Sarkar for their cordial co-operation whenever required. The author is greatful to the office staffs of the Department of Plant Pathology for their co-operation and help.

The whole credit for the achievements goes to the author's family and highly gratitude to her great father Late Upendra Nath Roy, mother Jothsna Roy, sister and brother who always stood by her during tough times. They supported, encouraged her continuously to study and blessing in all phases of her academic life; they were motivators from near or far. They were her strength and their constant encouragement was an inspiration.

June, 2011

Place: SAU, Dhaka *The Author*

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ABSTRACT

The experiment was carried out in the Department of Plant Pathology of Sher-e-Bangla Agricultural University, Dhaka during the period of January, 2012 to August, 2012 to determine the prevalence of seed borne bacteria of hybrid maize variety NK-40 and their management with some selected Cu-fungicides. Three bacterial genera viz. *Acidovorax* sp, *Burkholderia* sp and *Ralstonia* sp were isolated from maize seeds. Moreover, three Cu-fungicides viz. Sulcox 50WP, Champion 50WP and Cupravit 50WP were used as treatments for management. Bioassay of these fungicides against different bacteria were done and observed that Sulcox 50WP @ 0.3%, Champion 50WP @ 0.4% and Cupravit 50WP @ 0.3% produced remarkable inhibition zone against *Acidovorax* sp, *Burkholderia* sp and *Ralstonia* sp. Effect of seed treatments were studied using nutrient agar plate method, water agar test tube method and rolled paper towel method. In nutrient agar plate method, germination varied from 94.83-98.67%, where the effect of Cu-fungicides was insignificant. In water agar test tube method, the highest number of normal seedlings (69.00%) were recorded when seeds were treated with Cupravit 50WP @ 0.3% and the lowest was recorded in control (44.67%). The lowest number of abnormal seedlings and diseased seedlings (20% and 5%, respectively) were recorded when the seeds were treated with Cupravit 50WP @ 0.3%. In rolled paper towel method, Cupravit 50WP @ 0.3% showed best performance regarding germination, shoot length, root length and vigor index. The vigor index varied from 885.50-2488.40, where the highest count was recorded in Cupravit 50WP @ 0.3% and lowest in control.

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CHAPTER 1

INTRODUCTION

Maize (*Zea mays* L.) belongs to the family Gramineae is one of the most leading cereal in the world next to wheat and rice (Aldrich *et al*., 1975). The most likely centre of origin of this crop is Central America or Mexico, with a possible secondary origin in South America (Martin and Leonard, 1975). Maize is mostly used as animal feed, but it is an important staple food crop particularly in Africa, Asia and some Central and South American countries. In Bangladesh, it has good potential as a cereal crop due to its low cost of production, wide adaptability and diversified uses. Moreover, it can also be cultivated in both Robi and Kharif season, but mainly in Kharif season. Maize kernels have high nutritive value contains 66.2% starch, 11.1% protein, 7.1% oil and 1.5% minerals (Hulse *et al.*, 1980). Besides, it contains 90 mg carotene, 1.8 mg niacin, 0.8 mg thiamin and 0.1 mg riboflavin per 100 mg grains (Chowdhury and Islam, 1993).

In 2006-2007, 1,51,012 ha of land was under maize cultivation and total production of kernels was about 0.902 million tons having the average yield 5,973 kg/ha (BBS, 2008) which is lower as compared to production of developed countries of the world like USA and North America produce 8,924 kg/ha and 7,268 kg/ha, respectively (FAO, 2003). But in 2010-2011, cultivation area increased to 1, 65,516.57 ha of land while production jumped to 13.08% increased *i.e.* 1.02 million tones (BBS, 2011). Therefore, it is highly important to give special attention for management practices of this crop for obtaining higher yield. There are many factors involved in yield loss of maize of which environmental conditions, yield potential, soil fertility, genetical variation of hybrids, antagonistic action of different diseases, insects and some nutritional scarcity are important point of

consideration (Leon, 1984). Among these factors, diseases play a significant role and seed borne diseases create a great threat to the production of maize in Bangladesh. As many as 490 seed borne diseases are known to attack 756 different crop plants in Bangladesh (Fakir, 2000) and eleven seed-borne diseases have been listed for maize (Fakir, 2001).

High quality seed is not only important for increased crop production, but also for proper establishment of quality seed industry in the country. Among the important characteristics of seed quality, purity, germination, high yielding potentiality and seed health quality are of major importance. Of these major characteristics of quality seed, health is immensely important. Seed health refers to whether a seed or a seed lot is infected by pathogens or not. Infected seed fails to germinate and the pathogen from the infected seeds may be transmitted to seedlings and growing plants in the field causing disease. Therefore, it is important to know whether a seed lot is free from seed-borne infection of pathogen (s) or the lot contains pathogen (s) with its maximum acceptable limit. This has great value to the growers. Because of even a pure viable seed of high yielding variety is of little or no use to the growers, if the seed is unhealthy or infected by virulent pathogens. In fact, under favorable conditions such infected seeds can create disease epidemic in the field resulting partial to total crop failure.

Seed is common carrier of plant pathogens. It carries several destructive pathogens that often take heavy toll causing diseases of crops raised from them. Seed-borne diseases are very important from the following point of view: (i) induction of new pathogens (ii) quantitative and qualitative crop losses (iii) permanent contamination of soil (Anslem, 1981). Seasonal yield loss is significantly correlated with disease incidence of maize (Zhang *et al.*, 1999). Although some works have been done on the incidence of diseases of maize in Bangladesh, but little or no attention has been given yet for the

management of seed and seedling diseases and their occurrence in the country.

As seed is the basic material and vital input of agriculture, healthy seeds that are free from seed borne pathogens are prerequisite for successful crop production. Unfortunately, maize seeds are infected by three major category of pathogens namely fungi, bacteria and viruses that affect seed health and quality (Avinder and Rai, 1991). There are many bacterial diseases in maize. The important diseases are bacterial leaf blight and stalk rot (*Pseudomonas avenae*), bacterial leaf spot (*Xanthomonas campestris)*, bacterial stalk and top rot (*Erwinia carotovora* and *E. chrysanthemi*), bacterial stripe (*Pseudomonas andropogonis*), chocolate spot (*Pseudomonas syringae*), seed rot-seedling blight (*Bacillus subtilis*), Stewart's disease or bacterial wilt (*Erwinia stewartii).* Yield losses of maize are related to systemic infection. Yield is reduced about 0.8% for each 1% incidence of plants infected systemically as seedlings. Bacteria can substantially reduce yields and also economically impact on seed trade.

Bacterial disease can be removed by using some antibiotics and some Cu-fungicides. During the last five years, several antibiotics and synthetic compounds have been developed for disease control. Copper oxychloride performed the best against bacterial leaf blight of rice followed by Cupravit where 43.25% and 48.19% disease incidence, respectively were recorded in comparison to control where 71.08 % disease incidence and these treatments gave the highest yield (3.63 and 3.58 t/ha) (Khan *et al.*, 2005). Streptomycin, deoxy, dehydro streptomycin, streptocycline, choramphenicol and cellocidin are commonly used antibiotics to control bacterial plant pathogens (Tagami and Yoshimura, 1967). On the other hand, when the Cu-fungicides are used in combination with other fungicides or antibiotics, they also give good result.

Considering the above facts the present experiment has been undertaken to achieve the following objectives:

- To identify the important seed borne bacteria of maize from hybrid maize variety NK-40.
- To determine the efficacy of some selected Cu-fungicides against seed borne bacteria of maize.
- To know the transmission behavior of those bacteria from seed to seedling.

CHAPTER 2

REVIEW OF LITERATURE

Bacterial diseases in plants may affect stems, leaves, roots or may carry internally. Generally, they belong to the genera *Erwinia*, *Pectobacterium*, *Pantoea*, *Agrobacterium*, *Pseudomonas*, *Ralstonia*, *Burkholderia*, *Acidovorax*, *Xanthomonas*, *Clavibacter*, *Streptomyces*, *Xyllella*, *Spiroplasma* and *Phytoplasma.* Many researches have been carried out research in relation to seed health status and control with selective Cu- fungicides in different crops. However, some of the available literatures and relevant information on seed health and control have been cited in this chapter.

Smith (1909) reported that *Erwinia stewartii*, *Bacillus stewartii*, *Xanthomonas stewartii*, *Pantoea stewartii* are seed-borne pathogen appeared to have become specialized to exist in two specific hosts, *Zea mays* and *Chaetocnema pulicaria*.

Bergey *et al*. (1948) reported that *Enterobacter dissolvens* and *Pseudomonas aveae* subsp. *avenae* as causal agents of maize. These bacteria survive in maize and sorghum seeds, stalks and residues. The bacteria enter the plants through natural openings; wounds from hail, high winds, or insect feeding (eg. stalk borers) can provide additional entry sites into the plant.

Presence of inoculation during the growing season of the crop and at the right time is important for outbreak of epidemics. Rangarajam and Chakravarti (1967) isolated a strain of *Pseudomonas lapsa* from maize seeds of Ganga 3 but its significance in initiation of maize stalk rot is not known. However, *Erwinia carotovora* could not be isolated from maize seeds. In the present studies *P. lapsa* survived for much longer periods than *E. carotovora* in inoculated seeds under varing environmental condition.

Kodota and Ohuchi (1983) reported that the disease on rice caused by *Acidovorax avenae* (formerly *P. avenae*) showed brown stripes on leaf blades and sheaths extending over the whole leaf blade in the severest infections.

Kuhn (1983) investigated bacterial growth on metals. Small strips of stainless steel, brass, aluminum and copper were inoculated with broths of *Escherichia coli*, *Staphylococcus aureu*s, *Streptococcus* and *Pseudomonas* species. The broths contained very heavy inoculums (~107bacteria/ml). The strips were then air-dried for 24 hours at room temperature, inoculated onto agar plates and incubated for 24 hours at 37°C. The results were striking. The copper and brass strips showed little or no growth, while the aluminum and stainless steel strips produced a heavy growth of all the different types of microbes.

Wysong and Doupnik (1984) worked about Goss's bacterial wilt and blight of maize caused by *Corynebacterium* sp may attack plants at any stage of development and reduce yields by 50% or more. The wilt has been referred to as a "warm weather disease" because the symptoms do not appear until mid to late season, when temperatures are high. Typical symptoms are dark green to black, discontinuous, water-soaked spots (freckles) along the margins and ends of developing lesions. Light greenish-yellow or reddish stripes with wavy and irregular margins occur along the leaf veins. Orange bacterial exudates may appear on the surface of diseased tissue. Seed transmission may spread the disease over large areas. Losses are generally minor, but may be severe in individual fields.

Smidt and Vidaver (1986) found that the bacterium can overwinter in maize crop residues, which are the most important inoculums source. Within fields, the main source of inoculums is plant debris, with the pathogen possibly being dispersed by wind and rain and losses as great as 50% attributable to

this disease have been mitigated in field maize in recent years through the use of resistant germplasm. Variation in pathogenicity and the occurrence of different strains has also been reported.

Goto *et al*. (1987) grain rot in rice was reported to be caused by several bacteria, including *Pseudomonas glumae* (*Burkholderia glumae*), but only *P. glumae* caused seedling blight on inoculated plants.

Guo *et al.* (1987) have shown that the bacterium disappears from maize seed after 200-250 days at 8-15°C and after 110-120 days at 20-25°C and recommend storing seed under conditions suitable for eliminating *P. stewartii* subsp. *stewartii*. Seed treatment with chemicals is not effective.

Suparyono and Pataky (1989a; 1989b) studying with bacterial wilt (*Erwinia* sp, *Pantoa* sp) is the most serious disease of sweet corn, causing yield reduction and susceptibility to stem rot. Serious losses did not arise in the USA until 1930-1931, although the disease had already been known for some 30 years previously. Heavy losses were then reported.

Ellis and Bradley (1992) studied about copper fungicide formulations were available to organic growers. Regardless of the formulation, copper fungicides effectively kill fungi and bacteria.

Kadota (1996) demonstrated *Acidovorax* sp has a wide host range, causing diseases on barley, maize, millet, oats and rice.

Tsushima *et al*. (1996) reported that bacterial pathogens of plants exist ubiquitously in the plant ecosystems and are usually found in the air, soil, and water. Soil distributions of these pathogens are affected by soil type, pH value, plants cultivated and weather conditions. It has been established that pathogenic bacteria exist on the phylloplanes of cereal crops during the growing season.

Garrett and Schwartz (1998) reported the response of epiphytic populations of *Pseudomonas syringae* and other bacteria on dry bean plants to four copper-based bactericides was evaluated. The bactericides showed little difference in efficacy, but epiphytic populations on pinto bean leaflets, flowers and pods were occasionally reduced when compared to populations on non-treated control plants, especially after repeated bactericide applications.

Shahjahan *et al.* (2000) said that symptoms typically caused by *Burkholderia glumae* in Louisiana were panicle blighting with floret discoloration (with a gray-brown color), usually on the lower half of the developing grain, with a clear deep brown border followed by sterility or partial filling of the florets causing the panicles to stand erect and yield losses as high as 40% were observed in some fields.

Lecigne *et al.* (2000) studied on Mancozeb, Copper and bacteriosis. They found that Mancozeb significantly enhances copper activity against several bacteriosis. On tomato, Mancozeb increases the efficacy of copper against *Pseudomonas syringae* and *Xanthomonas axonopodis*. A tank mix of Mancozeb and copper provides complete protection against *P. syringae* on melon. In trials with walnut, Mancozeb improves copper efficacy against *Xanthomonas arboricola*. *In vitro* trials against bacterial necrosis on vine caused by *Xanthomonas ampelina* (*Xylophilus ampelina*) have shown the efficacy of Mancozeb and its synergism with copper. Preliminary tests on young plants confirm the *in vitro* results.

Freeman and Pataky (2001) conducted an experiment to determine the levels of Stewart's wilt resistance necessary to prevent the reduction in yield of sweet corn hybrid. They found that resistant and moderately resistant hybrid maize yield losses occur due to systemic infection of Stewart's disease and 0.8% reduction in yield for each 1% incidence of plants systemically

infected as seedlings. Losses do not occur or are minimal in resistant and moderately resistant hybrids; however, losses frequently range from 40 to 100% when susceptible sweet corn hybrids are grown under epidemic conditions and are infected prior to the 5-leaf stage.

Meirelles *et al.* (2001) reported that bacterial colonies with yellow pigmentation were isolated from the lesions, which reacted positively in hypersensitivity tests in tobacco plants. Maize plants were inoculated with the isolated bacteria. After 72 hrs incubation in a dew chamber, plants were transferred to a greenhouse, where they remained until evaluation. Typical symptoms of the disease were observed 5–7 days after inoculation of plants, only on treatments inoculated with the bacteria. The bacterium was re-isolated, which suggests its involvement in the initial phases of disease. The bacterium was identified as *Pantoea ananas* (synonym *Erwinia ananas*).

Michener *et al.* (2002) detected *Pantoea stewartii* was seed transmitted. The rate of seed transmission of *P. stewartii* was associated with the susceptibility or resistance of the host and the severity of infection of the seed parent plant.

Akhter and Bhutta (2002) conducted an experiment where 680 seed samples of wheat, rice and cotton were collected from major crop growing areas tested for seed borne bacterial pathogens. 31 wheat seed samples, out of 351 samples were found infected by *Xanthomonas campestries*. Using the seedling symptom technique, maximum infection in rice seeds was 7.5% in B-385 from Sahiwal and 11% in IIRI-6 from Lahore with *X. campestries* pv. *oryzae*. Infection by *Pseudomonas avenae* was 13% in B-385 and 40% in IRRI-6. Out of 221 seed cotton samples tested, 28 were found infected with bacteria.

Islam *et al.* (2003) used Streptomycin sulphate, Thiovit 80WP, Sulfuric acid, Dithane M-45 and Cupravit either alone or in combination in controlling bacterial blight and on yield of cotton and observed highest germination (86.31 %) when seed treatment with Streptomycin sulphate (0.15%) and foliar spray with Cupravit (0.2%) + Streptomycin sulphate (150 ppm). The lowest disease index (21.24%) was found in that treatment subsequently after three foliar sprays at 104 DAS. This treatment reduced the disease intensity and increased the yield of seed cotton with 26.02%.

The CIMMYT Maize Program (2004) reported the seed borne bacteria of maize. The pathogens were *Acidovorax avenae* subsp. *avenae*, *Burkholderia andropogonis*, *Clavibacter michiganensis* subsp. *Nebraskensis*, *Erwinia chrysanthemi* pv. *zeae*, *Pantoea stewartii* and *Pseudomonas syringae* pv. *lapsa*.

According to the CIMMYT Maize Program (2004) the most serious disease of sweet corn, causing yield reduction and susceptibility to stem rot; less destructive in other types of maize. Outbreaks are generally sporadic. The disease caused major losses in North America in the 1930s, but subsequently has caused only minor outbreaks, except for a few extensive epidemics on susceptible sweet corn hybrids. The current relatively low economic importance of the disease in North America is due primarily to adequate levels of resistance incorporated into maize hybrids grown where the disease occurs. In sweet corn, no or minimal losses occur in resistant hybrids, but losses can be significant in susceptible hybrids grown where flea beetles occur. Severe losses due to Stewart's wilt were reported in Italy in the 1940s and 1980s, but the disease has not persisted in Europe due to absence of vectors.

Yuan (2004) conducted an experiment about the pathogenecity tests and revealed that 69% or 234/339 isolates caused seedling infection, sheath rot and panicle blighting. Most of the pathogenic strains were in the genera *Burkholderia* and *Pseudomonas.* The four most common species *B. glumae*, *B. gladioli*, B. *multivorans* and *B. plantarii*, comprised 90% of all of the pathogenic bacteria, suggesting that a complex of *Burkholderia* spp. were causing the panicle blight/sheath rot syndrome recently found in Louisiana.

Singh and Mathur (2004) described the histopathplogy of seed borne infections and stated that nonfilamentous phytopathogenic bacteria are generally belong to *Acidovora*x, *Agrobacterium*, *Burkholderia*, *Erwinia*, *Pantoa*, *Pseudomonas*, *Ralstonia*, *Xanthomonas* and *Corineform* plant pathogens. These genera are usually seed borne. They cause seed infection *i.e.* carried on the surface on the seed or seed infection, that occurs on the seed coat and others parts of the seed.

Giester *et al.* (2004) described bacterial diseases of corn and Claflin (2000) described diseases caused by prokaryots. They reported that *Pseudomonas avenae* the causal agent of bacterial leaf blight, bacterial leaf spot caused by *Xanthomonas campestris*, *Erwinia carotovora* and *Erwinia chrysanthemi* that elicit bacterial stalk and top rot, bacterial stripe and leaf spot caused by *Pseudomonas andropogonis* occurs also in many places.

Cheneby *et al.* (2004) determined that the effect of the rhizosphere of maize on the diversity of denitrifying bacteria. Community structure comparison was performed by constructing a collection of isolates recovered from bulk and maize planted soil. A total of 3240 nitrate-reducing isolates were obtained and 188 of these isolates were identified as denitrifies based on their ability to reduce nitrate to N_2O or N_2. 16S rDNA fragments amplified from the denitrifying isolates was analyzed by restriction fragment length polymorphism. A plant dependent enrichment of *Agrobacterium*-related

denitrifies has been observed resulting in a modification of the structure of the denitrifying community between planted and bulk soil. In addition, the predominant isolates in the rhizosphere soil were not able to reduce N_2O while dominant isolates in the bulk soil evolve N_2 as a denitrification product.

Ritchie (2004) reported on the bacterial spot pathogen (*Xanthomonas arboricola* pv. *pruni*) is sensitive to copper. There are many factors that affect the efficacy of copper in the control of bacterial plant pathogens. An important characteristic of copper is that it is protective and not curative, thus to be effective copper must be present prior to occurrence of conditions for infection (*i.e.* the presence of moisture such as rainfall or dew). This also means that good spray coverage of the tree is essential. The higher rate should be used on highly susceptible varieties or in orchards where bacterial spot has been damaging or weather conditions are very favorable for disease.

Qiming *et al.* (2004) studied the techniques of diagnosing maize seedling diseases in the fields and greenhouses in China by artificial inoculation and marked plant investigation. The results showed that maize seedling diseases could be categorized into infective and non infective diseases. The infective diseases were caused by *Fusarium* spp., *Helminthosporium* sp and *Pseudomonas zeae*. Non-infective diseases included symptoms influenced by nutrition deficiency, environment and chemical burn.

Ahmad *et al.* (2005) recommended spray of Cupravit or Vitigran Blue (3 g of water) to check further spread of disease. Spraying of copper fungicides alternately with streptomycin (250 ppm) is reported to be effective in controlling the BLB of rice.

Sivakumar and Sharma (2007) conducted an experiment about *Pseudomonas fluorescens* PF-1, isolated from crop rhizosphere and exhibited inhibitory action against *Rhizoctonia solani* f. sp *sasakii* causing banded leaf and sheath

blight disease in Maize. Among the carriers, peat and talc were noted to maintain the population at 19.5×10^7 and 18.3×10^7 colony forming units per gram of the product, respectively after 40 days of storage. The disease was effectively controlled by seed treatment with the peat based formulation @ 16 g/kg or soil application @ 2.5 kg/ha or spraying the liquid formulation twice @ 5 g/l of water on maize foliage. Seed treatment with *P. fluorescens* resulted in increased population of fluorescent *Pseudomonads* in the rhizosphere. Soil application of *P. fluorescens* along with seed treatment resulted in further increase in rhizosphere population of the bacterium in glass house and field conditions though the seed treatment alone was quite effective in minimizing disease incidence.

Hopkins *et al.* (2009) working with bacterial fruit blotch (BFB), caused by *Acidovorax avenae* subsp *citrulli*, is a seed-borne disease of cucurbits that spreads rapidly in the warm, humid environment of the transplant house, often resulting in high numbers of infected plants going into the field. The only control options for BFB once it gets into a transplant house are crop destruction or multiple applications of a copper-containing bactericide/fungicide. The utilization of these transplant house treatments along with the elimination of all transplants with symptoms or near plants with symptoms should greatly reduce the chances of introducing BFB into fields on transplants.

Elphinstone (2009) described about the bacterial wilt disease and the causal agent isolated, in more than 200 plant species belonging to 53 different botanical families. The disease has a worldwide distribution. This unusually wide host range is continuously expanding and so descriptions of new hosts are not uncommon.

Milijasevic *et al.* (2009) showed that three copper-based compounds (copper hydroxide, copper oxychloride, copper sulphate), two antibiotics (streptomycin and kasugamycin) and a plant activator (ASM) significantly reduced population sizes and spread of *Clavibacter michiganensis* subsp. *michiganensis* among tomato seedlings in the greenhouse. Among copper compounds, copper hydroxide was the most prominent in reducing the bacterial population, especially in the region closest to the inoculums focus, while its combination with mancozeb did not improve the effects.

Chase (2010) working with copper as various forms as an algaecide, bactericide, fungicide and water treatment. Copper fungicides are classified as multisite and act by disrupting cellular proteins. For use on ornamentals, copper is often thought of as only a bactericide, perhaps due to the fact that there are very few bactericides.

Tahat and Sijam (2010) studied on *Ralstonia* spp. (race 3 biovar 2) and stated that *Ralstonia spp.* is a bacterial wilt causal agent of many plant species and infect potatoes, eggplant, peppers, tomatoes, geraniums, ginger, corn and a few weed species including bittersweet, nightshade and stinging nettle. *Ralstonia* spp. can be infectious in the soil for years in the presence of a host. Race 3 biovar 2 is most commonly transmitted by contaminated soil, equipment, water and insect or by transplantation of infected seeds or seedlings.

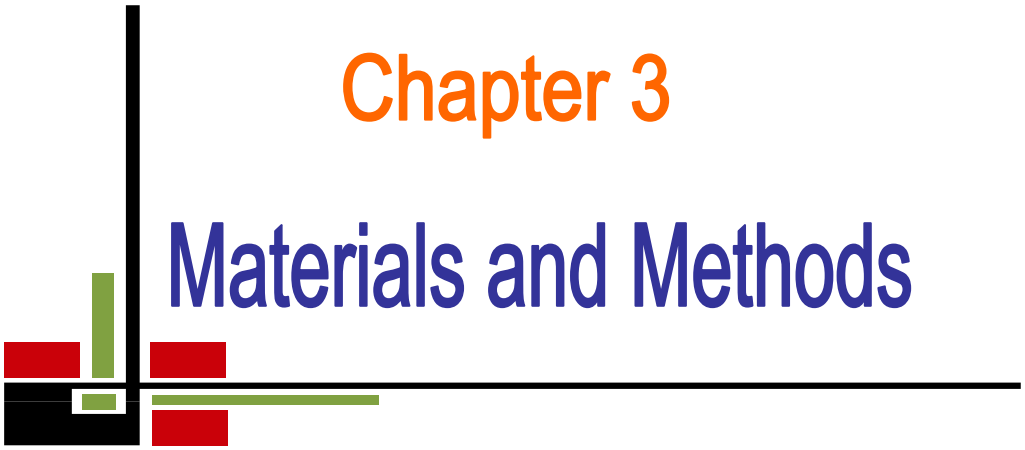
Gijon *et al.* (2011) conducted an experiment on leaf stripe of maize caused by *Burkholderia gladioli* and said that this disease not previously observed, appeared in 2003-2004 in Cosoleacaque, Tlalixcoyan and Paso de Ovejas counties, in the state of Veracruz, Mexico. Initial symptoms on leaves were small white-yellow watery spots, which coalesced into dry necrotic stripes 0.3 wide and up to 8cm long. Reddening sometimes developed on these leaves. Stems developed a rot in the crown. The flag leaf became rot and

necrotic at the base, rolled inwards and dried out. Necrosis developed at the base of the corn ears and their growth was inhibited.

HuiYing *et al.* (2011) studied bacterial dry stalk rot caused by *Pantoea agglomerans,* is a new disease in maize seed production field. The result of systematic detection showed that *P. agglomerans,* inoculated on seeds, can move up to maize plant and it can reach to leaves and seeds through stem along vascular system. All results mean that *P. agglomerans* successfully cycled from seed to new seed and the infection percentage was 80% to 100%.

Chapter 3

Materials and Methods



CHAPTER 3

MATERIALS AND METHODS

3.1. Experimental site

The experiment was conducted at the Seed Pathology Laboratory and Plant Disease Diagnostic Laboratory, Department of Plant Pathology, Sher-e-Bangla Agricultural University, Dhaka.

3.2. Experimental period

The experiment was conducted during the period from January, 2012 to August, 2012.

3.3. Collection of seed samples

Thousand gram maize hybrid seed variety NK-40 was collected from the farmer of Dinajpur district of Bangladesh.

3.4. Detection of seed borne bacteria

To identify the bacteria on maize hybrid variety NK-40, the following two methods were used in this experiment:

3.4.1. Blotter method

The collected seed samples of maize were analyzed for the presence of major seed borne bacterial pathogens by blotter method following the International Rules for Seed Testing (ISTA, 1996). Four hundred seeds were tested. Seeds were surface sterilized by 3% chlorax (seeds were dipped into 3% chlorax for 30 seconds, then wash 3 times with distilled water). Ten seeds were placed on three layered of moist blotting paper (Whatman No. 1) in each glass petridish. The petridishes were incubated at 25±1ºC under 12/12 hrs

light and darkness cycle for seven days. Each seed was observed in order to record the presence of bacterial ooze after seven days of incubation. The presence of bacteria was recorded as percentage. The results were presented as percent incidence. Germination of the seeds was also recorded.

3.4.2. Preparation of nutrient agar medium

Nutrient agar (15 g) was taken in the Erlenmeyer flask containing 1000 ml distilled water. Peptone (5 g) and beef extract (3 g) were added to flask. For mixing properly the nutrient agar was shacked thoroughly for few minutes and pH was adjusted at 7. Flask was then plugged with cotton and wrapped with a piece of brown paper and tied with thread. It was then autoclaved at 121ºC under 15 lbs pressure for 15 minutes. After autoclaving, the liquid medium was poured in the petridishes and solidified.

3.4.3. Nutrient agar plate method

In the agar plate method, 400 seeds were tested. Each plate was containing five seeds. Seeds were dipped into 3% chlorax for 1 minute, then wash 3 times with distilled water. Then five seeds were placed on the NA medium and the plated seeds were usually incubated for 5-7 days at 30ºC under 12hs alternating cycles of light and darkness. After incubation, bacteria growing out from the seeds on the agar medium were examined and streaked on new NA plates to get single colony. Identification was done based on different biochemical characteristics and by using differential media.

Fig. 1. Blotter method

Fig. 2. Nutrient agar plate method

A. Bacterial ooze deposited on seed

3.5. Isolation of bacteria from seed

The bacterial ooze deposited on the surface of the incubated seeds were collected with the help of a wire loop and streaked on nutrient agar plate. Then these NA plates were placed in the incubator at about 30ºC for 48 hrs. After 48 hrs of incubation, one loopfull of bacteria from single colony was collected and restreaked on new NA plate to get pure culture.

3.6. Preservation of bacteria

After isolating bacteria, a number of single colonies of the bacteria were purified by streaking using a wire loop, incubated at 30ºC for 24-48 hours, examined for purity and restreated on NA plates. After purification the bacteria streaked on NA slants and incubated for 48 hours. Then these slants were kept at refrigerator at 4ºC as stock culture.

Fig. 3. *Acidovorax* sp

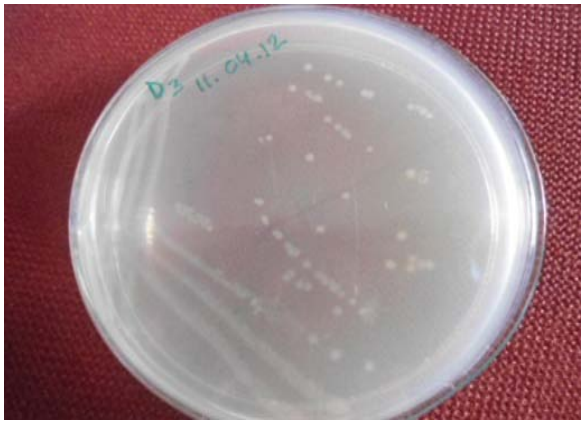


Fig. 4. *Burkholderia* sp

Fig. 5. *Ralstonia* sp

Plate 1. Pure culture of maize seed borne bacterial genera on NA medium

3.7. Gram reaction

3.7.1. KOH test

Twenty four hours old cultures were used for KOH test. One loop bacterial culture was mixed with 2 drops of 3% KOH on a sterile slide and rubbing was done with a wire loop and picked up the loop slowly to test the gummy texture of the colony (Suslow *et al*., 1982).

3.7.2. Gram staining

After purification of bacteria, gram staining is the first step for identification of a bacterium. It was done on a clean slide, dried a thinly speeded bacterial film in air without heat. Then underside of the slide was lightly flamed twice to fix the bacteria to the slide. Then the smear was flooded with Crystal violet solution for 1 minute. It was washed with tap water for a few seconds and excess water removed by air. Then the smear was flooded with Iodine solution (Lugol's Iodine) for 1 minute and then washed with tap water for few seconds and excess water removed by air. After that the smear was decolorized with 95% Ethanol for 30 seconds and again washed with tap water and dried by air. Then the smear was counterstained with 0.5% Safranine for 10 seconds and washed in tap water and excess water was removed by air. Finally, it was examined under microscope at 100X oil imersion objective.

3.8. Morphological and biochemical test

Different biochemical test were done on the following parameters:

i. Catalase test
ii. Starch hydrolysis test
iii. Oxidase test
iv. Gilatin liquification test
v. Citrate utilization test
vi. Pectolytic test

vii. Motility test

viii. Salt tolerance test

3.8.1. Catalase test

One colony of the organism from the agar plate was taken on a slide onto which one drop of 3% H_2O_2 (Hydrogen Peroxide) was added and observed.

3.8.2. Starch hydrolysis

A nutrient agar plate containing 0.2% soluble starch was inoculated with the bacterium isolates to be tested. Then incubated at 30ºC temperature for 48 hrs. After 48 hrs, incubated plate was flooded the plate with Lugol's iodine and observed.

3.8.3. Oxidase test

A portion of the test organism was picked up from the agar plate with a sterile wooden toothpick onto the wet oxidase disk containing tetramethyl-p-phenylene-diamine dihydrochloride. Formation of a dark purple color developed within 5-10 seconds indicted a positive test for oxidase.

3.8.4. Gelatin liquification test

One hundred and twenty gram of gelatin (bacteriological grade) was added to 1L water and adjusted at pH 7.0. Then it was poured in each tube, autoclaved and cooled (not slant) immediately. It was inoculated by stabbing a loop with inoculums into the center of the medium, then incubate the tubes at room temperature. After incubation, the tubes were kept in refrigerator at 4ºC for 20 minutes and observed after 20 minutes.

3.8.5. Citrate utilization test

A portion of the test organism was picked up from the agar plate with a sterile inoculating loop and streaked into Simmon's citrate agar slants. Following incubation at 30^{o}C for 24 hours changing of the green bromothymol blue indicator positive results.

3.8.6. Pectolytic test

Potato tubers were disinfected with 99% ethanol, cut up into slices of about 7-8 mm thick, and then placed on moistened sterile filter paper in sterile Petri dishes. Bacterial cell suspension was pipetted into a depression cut in the potato slices. One potato slice pipetted with sterile water was treated as control. Development of rot on the slices was examined 24–48 h after incubation at 25°C. Examination was done for 5 days after inoculation. Two slices were inoculated for each isolate.

3.8.7. MIU test

One suspected isolated colony was touched with a sterile straight wire and stabbed into agar carefully down the tube, without touching the bottom. The tube was incubated at 30ºC for 18 to 24 hours. Motility of organism was detected by the presence of growth along the time of inoculation on.

3.8.8. Salt tolerance test

Nutrient broth was prepared by amount peptone (5 g) and beef extract (3 g) were added to the Erlenmeyer flask containing 1000 ml distilled water. There were four isolate and every isolate had seven test tubes for 1%, 2%, 3%, 4%, 5%, 6% and 7% NaCl containing nutrient broth. At first, 10 ml nutrient broth was poured in every test tube. For preparing 1% NaCl concentration, 0.1 g NaCl was mixed in 10 ml nutrient broth. Similarly for 2%, 3%, 4%, 5%, 6% and 7% NaCl concentration, 0.2, 0.3, 0.4, 0.5, 0.6 and

0.7 g NaCl were mixed in every 10 ml nutrient broth, respectively and finally autoclaved.

After that, the 1, 2, 3, 4, 5, 6 and 7% salt containing broth tubes were inoculated with one loopfull of bacterial isolate from nutrient agar plate. These inoculated test tubes were transferred in incubating shaker machine maintaining 30ºC temperature and 150 rpm. Data were recorded after every 24 hours for 7 days.

3.9. Use of differential medium

3.9.1. YDC (Yeast extract-dextrose-$CaCO_3$) medium

Yeast extract (10 g), dextrose (20 g), $CaCO_3$ light powder (20 g) and agar (15 g) were needed to prepare1 litre YDC medium. To obtain an even milky white medium, the $CaCO_3$ must be of the finely ground form, otherwise it would precipitate to the bottom. Yeast extract, dextrose and agar were mixed properly and autoclaved at 10 PSI for 1 hr separately. The autoclaved medium was cooled to 50ºC in a water bath and $CaCO_3$ suspended by swirling before pouring the plates. Then streaked the isolates on YDC plate and incubated at 30ºC for 48 hrs. After incubation, data were recorded on colony morphology, colony color, shape, size, elevation etc.

3.10. Pathogenecity test

The pseudostem is inoculated by injecting bacterial cell suspension 10^8 cfu/ml using a syringe. After inoculation, kept in a net house and observed upto 7 days of inoculation. When symptoms appeared, the bacteria were reisolated by using dilution plate technique. The cultural characteristics compared with the pure bacterial culture that was isolated from incubated seed.

3.11. Bioassay of selected Cu-fungicides against bacteria

All the isolated bacteria were inoculated in nutrient broth in test tubes and shaking the culture at 150 rpm and incubated at 30ºC for 24 hours. After 24 hours of growth, nutrient agar plates were swabbed with bacterial isolate. Wells, 4 mm in diameter were punched into the NA medium and different concentration of Cu-fungicides were added in the wells. The plates were then incubated at 30ºC upto 4 days of incubation. The zone of inhibition around the wells measured and recorded.

Table 1. List of selected Cu-fungicides and their trade name

Trade name	Active ingredient (a.i.)	Applied concentrations (a.i. %)	Amount used (µl)
Cupravit 50WP	Copper oxychloride	0.1%, 0.2%, 0.3%, 0.4%	0, 50, 80, 100
Champion 50WP	Copper hydroxide	0.1%, 0.2%, 0.3%, 0.4%	0, 50, 80, 100
Sulcox 50WP	Copper oxychloride	0.1%, 0.2%, 0.3%, 0.4%	0, 50, 80, 100

3.12. Effect of seed treatment with Cu-fungicides

3.12.1. Seed treatment

Hybrid maize variety-NK40 was used in this experiment. Seeds were treated with Sulcox 50WP @ 0.3%, Champion 50WP @ 0.4% and Cupravit 50WP @ 0.3% for one hour. Then seeds were dried and placed.

3.12.2. Nutrient agar plate method (germination, seed infection and dead seed)

Four hundred seeds were treated with Sulcox 50WP @ 0.3%, Champion 50WP @ 0.4% and Cupravit 50WP @ 0.3% by dipping for 1 hour for each treatment. Then seeds were dried properly and transferred to the NA media and kept in growth chamber. Data were recorded on germination percent, seed infection and number of dead seeds.

3.12.3. Water agar test tube method (germination, normal seedling, abnormal seedling, diseased seedling and dead seed)

The agar test tube seedling symptom test developed by Khare *et al.,* (1977) was used in the present evaluation. In this technique, test tube were prepared by pouring 10 ml of 1% water agar in each test tube (2 cm in diameter and 15 cm in length) and then sterilized in autoclave for 15 minute under 15 lbs pressure at 121ºC. The water agar in the test tube was solidified at an angle 60º. One hundred seeds for each treatment *i.e.* Sulcox 50WP @ 0.3%, Champion 50WP @ 0.4% and Cupravit 50WP @ 0.3%, were taken. Seeds were treated with each treatment for 1 hour and then dried and one seed per test tube were placed on solidified water agar. The tubes were then incubated at erect condition in an air cooled room (22ºC) under fluorescent day light tube. The cotton plugs were removed when the seedlings reached the rim of the test tube. Data on germination, number of normal seedlings, number of abnormal seedlings, number of diseased seedlings and number dead seeds were recorded.

3.12.4. Rolled paper towel method (shoot length, root length, vigor index and dead seed)

Seedling infection and seedling vigor test was done in rolled paper towel method (Warham, 1990). In this method, 200 seeds were randomly taken from each treatment and were placed uniformly between a pair of moist paper towels. The towels were rolled and the two ends were closed with rubber band as the moist could not remove easily. Then the rolled papers containing seeds were placed in an upright position for 7 days at room temperature under normal 12/12 light and darkness cycle. Length of shoot was measured from the base of the stem up to the growing point of the youngest leaf. Similarly, length of root was measured from starting point of

the root to the largest available lateral root apex. Vigor of the seedling was determined by the following formula (Baki and Anderson, 1972).

Vigor Index= (Mean of root length + Mean of shoot length) × % of seed germination

Fig. 6. Nutrient agar plate method

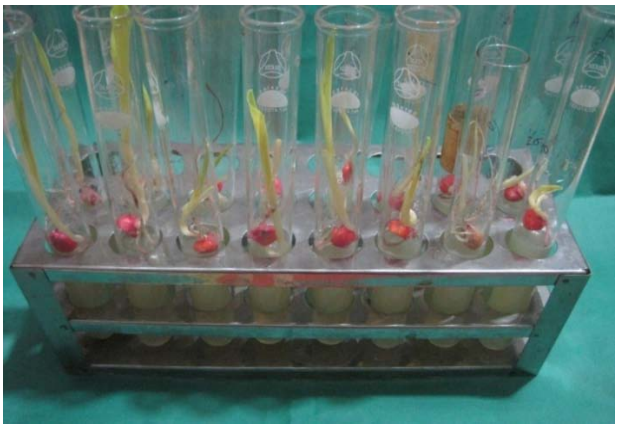


Fig. 7. Water agar test tube method

Fig. 8. Rolled paper towel method

3.13. Statistical analysis

The recorded data on different parameters were subjected to statistical analysis by using MSTAT-C software to find out the significance of variation resulting from experimental treatments. The difference between the treatment means were judged by Least Significance Difference Test following the procedure as described by Gomez and Gomez (1984).

Results

CHAPTER 4

RESULTS

4.1. Seed health test

4.1.1. Blotter method

Seed health test of hybrid maize variety NK-40 was done by blotter method and fungal incidence was 14.24% and germination was 85%.

4.1.2. Agar plate method

Bacterial incidence on seeds of hybrid maize variety NK-40 were varied from 1.25% to 2.25%, where *Acidovorax* sp incidence was 2.25% and incidence of *Burkholderia* sp and *Ralstonia* sp were 1.5% and 1.25%, respectively (Table 2).

Table 2. Frequency and occurrence of various seed borne bacteria of hybrid maize variety NK-40 seeds in agar plate method

Bacteria	Incidence (%)
Acidovorax sp	2.25
Burkholderia sp	1.5
Ralstonia sp	1.25

4.2. Isolation and identification of seed borne bacteria from hybrid maize variety NK-40

The identified pathogens were *Acidovorax* sp (new genus name for *Pseudomonas pseudocaligenes*, *P. avenae*, *P. cattleyae*, *P. rubrilineans*, *P. setariae*), *Burkholderia* sp, *Ralstonia* sp.

4.3. Biochemical test of different bacteria

Biochemical test of isolated bacteria were done and the results were showed (Table 3). In case of gram staining, all three genera were gram negative (Plate 2; Fig. 9) and rod shape. Colonies on differential YDC medium, *Acidovorax* sp produced dark beige color, *Burkholderia* sp produced purple color and *Ralstonia* sp produced beige to light brown color. These genera were catalase positive when H_2O_2 was added, all the isolate produced bubble (Plate 2; Fig. 10). In case of starch hydrolysis test, these genera were showed positive result *i.e.* a clear zone appeared within 10 seconds after adding indols iodine (Plate 2; Fig. 11). In case of oxidase test, when the genera were simply swabbed on oxidase dry slide, color was changed to blue within 30 seconds (Plate 2; Fig. 12). After incubation with gelatin liquification, tubes were kept in the refrigerator for 20 minutes and liquification observed (Plate 3; Fig. 13). In case of citrate utilization test, all these genera were capable of utilizing citrate as a carbon source. They changed the color of the medium from green to blue and the result was positive (Plate 3; Fig. 14). For pectolytic test, 4 days after incubation of bacteria with potato slices, the potato slices became rotten and the result indicated positive (Plate 3; Fig. 15). In case of motility test, after incubating the inoculated test tube for 24-48 hours, bacteria grew away from the stab line and indicated that the bacteria were motile.

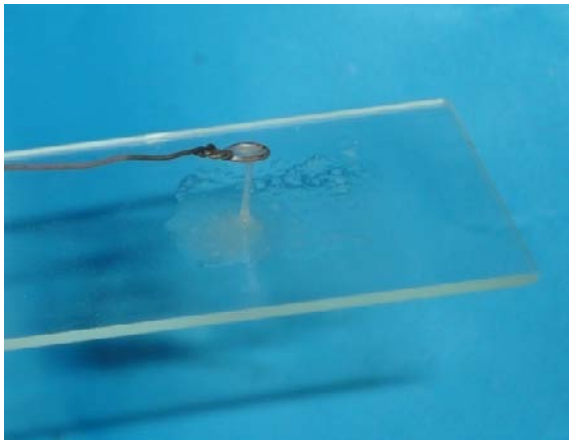


Fig. 9. KOH test

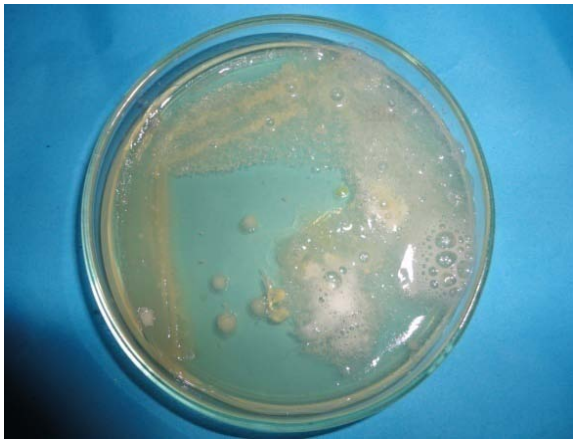


Fig. 10. Catalase test

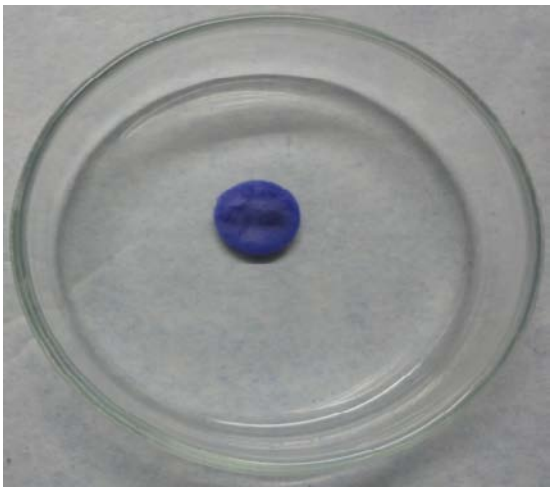


Fig. 11. Starch hydrolysis test

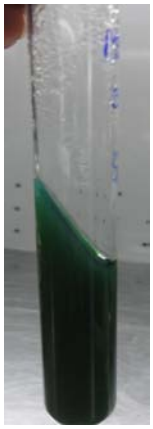
Fig. 12. Oxidase test

Plate 2. Biochemical test results

Negative test

Positive test

Fig. 13. Gelatin lequification test



Negative test

Positive test

Fig. 14. Citrate utilization test

Negative test

Positive test

Fig. 15. Pectolytic test

Plate 3. Biochemical test results

Table 3. Tests used to differentiate genera of plant pathogenic prokaryotes that found on the seeds of hybrid maize variety NK-40

Tests	Isolated bacteria		
	***Acidovorax* sp**	***Burkholderia* sp**	***Ralstonia* sp**
Gram staining	-	-	-
Colonies color on YDC	dark beige	purple	beige to light brown
Catalase test	+	+	+
Starch hydrolysis	+	+	+
Oxidase test	+	+	+
Gelatin liquification test	+	+	+
Citrate utilization test	+	+	+
Pectolytic test	+	+	+
Motility test	+	+	+

4.4. Salt tolerance test on nutrient broth

All the isolate grew well at 2% salt stress. But *Acidovorax* sp and *Ralstonia* sp, failed to grow at 3-7%. In case of *Burkholderia* sp grew well at 3% salt stress, but failed to grow at 4-7% salt stress (Table 4).

Table 4. Growth observation of different isolate of hybrid maize variety NK-40 seeds at 1-7% NaCl nutrient broth (salt tolerance test)

Isolates	Concentration						
	1%	2%	3%	4%	5%	6%	7%
***Acidovorax* sp**	++	+++	-	-	-	-	-
***Burkholderia* sp**	+	++	++++	-	-	-	-
***Ralstonia* sp**	++	+++	-	-	-	-	-

4.5. Bioassay of Cu-fungicides

4.5.1. Comparative efficacy of different concentration of Sulcox 50WP against seed borne bacteria of hybrid maize variety NK-40

Significant variation was observed when bioassay of Sulcox 50WP was done by using 0.1%, 0.2%, 0.3% and 0.4% concentration of Sulcox 50WP. The highest inhibition zone 1.63 cm, 1.27 cm, 1.47 cm was observed at 0.3% concentration for *Acidovorax* sp, *Burkholderia* sp and *Ralstonia* sp, respectively followed by 0.4%, 0.2% and 0.1% concentration.

Table 5. Comparative efficacy of different concentration of Sulcox 50WP against seed borne bacteria of hybrid maize variety NK-40

Concentrations (%)	Inhibition zone (cm) of *Acidovorax* sp	Inhibition zone (cm) of *Burkholderia* sp	Inhibition zone (cm) of *Ralstonia* sp
Control	0	0	0
0.1%	0.77 c	0.77 b	1.20 b
0.2%	1.27 b	0.90 b	1.27 b
0.3%	1.63 a	1.27 a	1.47 a
0.4%	1.53 a	1.27 a	1.43 a
$LSD_{(0.05)}$	0.126	0.303	0.141
CV%	4.62	5.12	9.29

In column, means containing same letter indicate significantly similar under LSD at 5 % level of significance. Values are the means of three replications.

4.5.2. Comparative efficacy of different concentration of Champion 50WP against seed borne bacteria of hybrid maize variety NK-40

Significant variation was observed when bioassay of Champion 50WP was done by using 0.1%, 0.2%, 0.3% and 0.4% concentration of Champion 50WP. The highest inhibition zone 1.17 cm, 1.53 cm, 1.20 cm was observed at 0.4% concentration for *Acidovorax* sp, *Burkholderia* sp and *Ralstonia* sp, respectively followed by 0.2%, 0.3% and 0.1% concentration.

Table 6. Comparative efficacy of different concentration of Champion 50WP against seed borne bacteria of hybrid maize variety NK-40

Concentrations (%)	**Inhibition zone (cm) of *Acidovorax* sp**	**Inhibition zone (cm) of *Burkholderia* sp**	**Inhibition zone (cm) of *Ralstonia* sp**
Control	0	0	0
0.1%	0.30 b	1.00 b	1.10 a
0.2%	1.13 a	1.50 a	1.10 a
0.3%	1.10 a	0.57 c	1.17 a
0.4%	1.17 a	1.53 a	1.20 a
$LSD_{(0.05)}$	0.141	0.245	0.200
CV%	7.43	9.55	8.22

In column, means containing same letter indicate significantly similar under LSD at 5 % level of significance. Values are the means of three replications.

4.5.3. Comparative efficacy of different concentration of Cupravit 50WP against seed borne bacteria of hybrid maize variety NK-40

Significant variation was observed when bioassay of Cupravit 50WP was done by using 0.1%, 0.2%, 0.3% and 0.4% concentration of Cupravit 50WP. The highest inhibition zone 1.63 cm, 2.07 cm, 1.33 cm was observed at 0.3% concentration for *Acidovorax* sp, *Burkholderia* sp and *Ralstonia* sp, respectively followed by 0.4%, 0.2% and 0.1% concentration.

Table 7. Comparative efficacy of different conc. of Cupravit 50WP against seed borne bacteria

Concentrations (%)	**Inhibition zone (cm) of *Acidovorax* sp**	**Inhibition zone (cm) of *Burkholderia* sp**	**Inhibition zone (cm) of *Ralstonia* sp**
Control	0	0	0
0.1%	1.17 c	1.23 d	1.10 b
0.2%	1.27 bc	1.57 c	1.17 b
0.3%	1.63 a	2.07 a	1.33 b
0.4%	1.43 b	1.80 b	1.17 a
$LSD_{(0.05)}$	0.167	0.200	0.089
CV%	6.06	5.92	3.96

In column, means containing same letter indicate significantly similar under LSD at 5 % level of significance. Values are the means of three replications.

4.6. Effect of seed treatment with selected Cu-fungicides on seed of hybrid maize variety NK-40 (nutrient agar plate method)

4.6.1. Germination (%)

The effect of Sulcox 50WP @ 0.3%, Champion 50WP @ 0.4% and Cupravit 50WP @ 0.3% on germination (%) of seeds of hybrid maize variety NK-40 was presented (Table 8). The germination is varied from 94.83% to 98.67%, where significantly the lowest germination (94.83%) was recorded in control and the highest germination (98.67%) was recorded from the seeds treated with Cupravit 50WP @ 0.3% followed by seeds treated with Champion 50WP @ 0.4% and Sulcox 50WP @0.3%, which showed 98.13% and 97.58% germination, respectively.

4.6.2. Seed infection (%)

The effect of Sulcox 50WP @ 0.3%, Champion 50WP @ 0.4% and Cupravit 50WP @ 0.3% on seed infection (%) of seeds of hybrid maize variety NK-40 was presented (Table 8). Significantly the highest seed infection (3.88%) was recorded in control and the lowest seed infection (2.23%) was recorded when the seeds treated with Cupravit 50WP @ 0.3%.

4.6.3. Dead seed (%)

The effect of Sulcox 50WP @ 0.3%, Champion 50WP @ 0.4% and Cupravit 50WP @ 0.3% on dead seed (%) of hybrid maize variety NK-40 was presented (Table 8). Significantly the highest number of dead seed (2.03%) was recorded in control and the significantly lowest number of dead seed (1.28%) was recorded when seeds were treated with Sulcox 50WP @ 0.3%.

Table 8. Effect of seed treatment with selected Cu-fungicides on seedlings of hybrid maize variety NK-40 (nutrient agar plate method)

Treatments	% Germination	% Seed Infection	% Dead Seed
Control	94.83 a	3.88 a	2.03 b
Sulcox 50 WP @ 0.3%	97.58 a	3.25 b	1.28 c
Champion 50 WP @ 0.4%	98.13 a	2.97 b	2.28 a
Cupravit 50 WP @ 0.3%	98.67 a	2.23 c	1.40 c
$LSD_{(0.05)}$	4.365	0.429	0.141
CV%	2.25	6.97	4.12

In column, means containing same letter indicate significantly similar under LSD at 5 % level of significance. Values are the means of three replications.

4.7. Effect of seed treatment with selected Cu-fungicides on seedlings of hybrid maize variety NK-40 (water agar test tube method)

4.7.1. Germination (%)

The effect of Sulcox 50WP @ 0.3%, Champion 50WP @ 0.4% and Cupravit 50WP @ 0.3% on germination (%) of seeds of hybrid maize variety NK-40 was presented (Table 9). The germination is varied from 85.33% to 93.00%, where significantly the lowest germination (85.33%) was recorded in control and the highest germination (93.00%) was recorded from the seeds treated with Cupravit 50WP @ 0.3%. Seeds treated with Cupravit 50WP @ 0.3% was significantly different from the seeds treated with Sulcox 50WP @ 0.3%, Champion 50WP @ 0.4%, which showed 89.00% and 91.67% germination, respectively.

4.7.2. Normal seedlings (%)

The effect of Sulcox 50WP @ 0.3%, Champion 50WP @ 0.4% and Cupravit 50WP @ 0.3% on normal seedlings of hybrid maize variety NK-40 was studied and presented (Table 9). Significantly the highest number of normal seedlings (69.00%) were observed when the seeds treated with Cupravit 50WP @ 0.3% followed by Champion 50WP @ 0.4% treated seed and the lowest number of normal seedlings was observed in control.

4.7.3. Abnormal seedlings (%)

The effect of Sulcox 50WP @ 0.3%, Champion 50WP @ 0.4% and Cupravit 50WP @ 0.3% on developing abnormal seedlings of hybrid maize variety NK-40 was determined and presented (Table 9). The effect of Sulcox 50WP @ 0.3% and Champion 50WP @ 0.4% were statistically identical, where the number of abnormal seedlings were recorded 29.33% and 25.67%, respectively.

Fig. 16. Normal seedlings on water agar test tube method

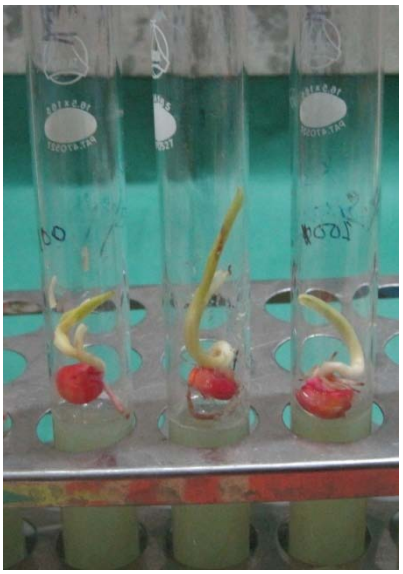


Fig. 17. Abnormal seedlings on water agar test tube method

Fig. 18. Diseased seedlings on water agar test tube method

4.7.4. Diseased seedlings (%)

The effect of Sulcox 50WP @ 0.3%, Champion 50WP @ 0.4% and Cupravit 50WP @ 0.3% on developing diseased seedlings (%) of hybrid maize variety NK-40 was evaluated and presented (Table 9). Significantly the highest diseased seedlings (13.00%) were recorded in control followed by Sulcox 50WP @ 0.3% (9.00%) and Champion 50WP @ 0.4% (8.00%). The lowest diseased seedlings (5.00%) was recorded when the seeds treated with Cupravit 50WP @ 0.3%.

4.7.5. Dead seed (%)

Significantly the highest number of dead seed (7.07%) was recorded in control followed by Cupravit 50WP @ 0.3% (6.00%), Sulcox 50WP @ 0.3% (4.80%) and Champion 50WP @ 0.4% (2.96%), respectively (Table 9).

4.8. Effect of seed treatment with selected Cu-fungicides on seedlings of hybrid maize variety NK-40 (rolled paper towel method)

4.8.1. Shoot length

Efficacy of Sulcox 50WP @ 0.3%, Champion 50WP @ 0.4% and Cupravit 50WP @ 0.3% on hybrid maize variety NK-40 are presented (Table 10). Shoot length ranged from 4.00 cm to 11.43 cm, where the significantly the highest shoot length (11.43 cm) was recorded under Champion 50WP @ 0.4% treated seeds which was statistically identical with Cupravit 50WP @ 0.3% treated seeds (11.24 cm) followed by Sulcox 50WP @ 0.3% (6.33 cm) and lowest shoot length (4.00 cm) was recorded under control.

Table 9. Effect of seed treatment with selected Cu-fungicides on seedlings of hybrid maize variety NK-40 on germination (%), normal and abnormal seedling (%), diseased seedling (%) and dead seed (%) (water agar test tube method)

Treatments	Germination (%)	Normal seedlings (%)	Abnormal seedlings (%)	Diseased seedlings (%)	Dead seed (%)
Control	85.33 b	44.67 d	34.67 a	13.00 a	7.07 a
Sulcox 50 WP @ 0.3%	89.00 ab	56.67 c	29.33 b	9.00 b	4.80 c
Champion 50 WP @ 0.4%	91.67 a	62.33 b	25.67 b	8.00 c	2.96 d
Cupravit 50 WP @ 0.3%	93.00 a	69.00 a	20.00 c	5.00 d	6.00 b
$LSD_{(0.05)}$	5.212	5.577	3.723	0.502	0.712
CV%	2.91	4.85	6.80	2.86	6.84

In column, means containing same letter indicate significantly similar under LSD at 5 % level of significance. Values are the means of three replications.

4.8.2. Root length

Efficacy of Sulcox 50WP @ 0.3%, Champion 50WP @ 0.4% and Cupravit 50WP @ 0.3% on hybrid maize variety NK-40 were evaluated and presented (Table 10). Root length ranged from 5.72 cm to 13.97 cm, where the significantly the highest root length (13.97 cm) was recorded under Cupravit 50WP @ 0.3% treated seeds followed by Champion 50WP @ 0.4% (12.06 cm), Sulcox 50WP @ 0.3% (9.16 cm) and the lowest root length was recorded under control.

4.8.3. Germination (%)

The effect of Sulcox 50WP @ 0.3%, Champion 50WP @ 0.4% and Cupravit 50WP @ 0.3% on germination of seeds of hybrid maize variety NK-40 was presented (Table 10). The germination is varied from 91.00% to 98.67%. The effect of Champion 50WP @ 0.4% and Cupravit 50WP @ 0.3% were statistically identical, where the germination were 97.17% and 98.67%, respectively followed by Sulcox 50WP @ 0.3% and control.

4.8.4. Vigor index

The effect of Sulcox 50WP @ 0.3%, Champion 50WP @ 0.4% and Cupravit 50WP @ 0.3% on vigor index of seeds of maize hybrid variety NK-40 was determined and presented (Table 10). The significantly the highest vigor index (2488.40) was recorded in case of seeds treated with Cupravit 50WP @0.3% followed by Champion 50WP @ 0.4% (2281.20), Sulcox 50WP @ 0.3% (1438.40). The lowest vigor index (885.50) was recorded in control.

Table 10. Effect of seed treatment with selected Cu-fungicides on seedlings of hybrid maize variety NK-40 on germination %, shoot length, root length and vigor index (rolled paper towel method)

Treatments	Shoot length (cm)	Root length (cm)	Germination (%)	Vigor index
Control	4.00 c	5.72 d	91.00 b	885.50 d
Sulcox 50 WP @ 0.3%	6.33 b	9.16 c	92.83 b	1438.40 c
Champion 50 WP @ 0.4%	11.43 a	12.06 b	97.17 a	2281.20 b
Cupravit 50 WP @ 0.3%	11.24 a	13.97 a	98.67 a	2488.40 a
$LSD_{(0.05)}$	1.630	0.778	3.811	6.258
CV%	9.89	3.81	2.01	5.32

In column, means containing same letter indicate significantly similar under LSD at 5 % level of significance. Values are the means of three replications.

Fig. 19. Seedlings raised on paper towel method (treated)

Fig. 20. Seedlings raised on paper towel method (control)

4.9. Comparative efficacy of Sulcox 50WP @ 0.3%, Champion 50 WP @ 0.4% and Cupravit 50WP @ 0.3% on *Acidovorax* sp, *Burkholderia* sp and *Ralstonia* sp of hybrid maize variety NK-40

4.9.1. Comparative efficacy of selected Cu-fungicides on *Acidovorax* sp at different days after incubation

Comparative efficacy of Sulcox 50WP @ 0.3%, Champion 50 WP @ 0.4% and Cupravit 50WP @ 0.3% on *Acidovorax* sp. was done and inhibition zone was measured. Significant variation was observed among the fungicides used (Table 11). At 24 hrs of incubation, the highest inhibition zone (1.77 cm) was recorded by Sulcox 50 WP @ 0.3% followed by Cupravit 50WP @ 0.3% (1.43 cm). At 48 hrs, 72 hrs and 96 hrs of incubation, the highest inhibition zone was recorded by Sulcox 50 WP @ 0.3% and Cupravit 50WP @ 0.3%, which was statistically identical to Champion 50WP @ 0.4%.

4.9.2. Comparative efficacy of selected Cu-fungicides on *Burkholderia* sp at different days after incubation

Comparative efficacy of Sulcox 50WP @ 0.3%, Champion 50 WP @ 0.4% and Cupravit 50WP @ 0.3% on *Burkholderia* sp was done and inhibition zone was measured. Significant variation was observed among the fungicides used (Table 12). At 24 hrs of incubation, the highest inhibition zone (1.97 cm) was recorded by Cupravit 50WP @ 0.3% followed by Sulcox 50WP @ 0.3% and Champion 50WP @ 0.4%, which was statistically identical. At 48 hrs, 72 hrs and 96 hrs, the highest inhibition zone was recorded by Cupravit 50WP @ 0.3% followed by Champion 50 WP @ 0.4% and Sulcox 50WP @ 0.3%, which was statistically identical.

Table 11. Efficacy of selected Cu-fungicides on *Acidovorax* sp of hybrid maize variety NK-40 at different days after incubation

Treatments	Inhibition zone after 24 hrs (cm)	Inhibition zone after 48 hrs (cm)	Inhibition zone after 72 hrs (cm)	Inhibition zone after 96 hrs (cm)
Control	0.00 d	0.00 c	0.00 c	0.00 c
Sulcox 50 WP @ 0.3%	1.77 a	1.70 a	1.67 a	1.60 a
Champion 50 WP @ 0.4%	1.07 c	1.23 b	1.23 b	1.03 b
Cupravit 50 WP @ 0.3%	1.43 b	1.63 a	1.90 a	1.70 a
$LSD_{(0.05)}$	0.203	0.313	0.3362	0.176
CV%	6.20	9.37	9.40	5.29

In column, means containing same letter indicate significantly similar under LSD at 5 % level of significance. Values are the means of three replications.

Table 12. Efficacy of selected Cu-fungicides on *Burkholderia* sp of hybrid maize variety NK-40 at different days after incubation

Treatments	Inhibition zone after 24 hrs (cm)	Inhibition zone after 48 hrs (cm)	Inhibition zone after 72 hrs (cm)	Inhibition zone after 96 hrs (cm)
Control	0.00 c	0.00 c	0.00 c	0.00 c
Sulcox 50 WP @ 0.3%	1.36 b	1.47 b	1.47 b	1.53 b
Champion 50 WP @ 0.4%	1.57 b	1.58 b	1.77 b	1.67 b
Cupravit 50 WP @ 0.3%	1.97 a	2.07 a	2.17 a	2.23 a
$LSD_{(0.05)}$	0.344	0.313	0.359	0.259
CV%	8.99	8.31	8.95	6.24

In column, means containing same letter indicate significantly similar under LSD at 5 % level of significance. Values are the means of three replications.

4.9.3. Comparative efficacy of selected Cu-fungicides on *Ralstonia* sp at different days after incubation

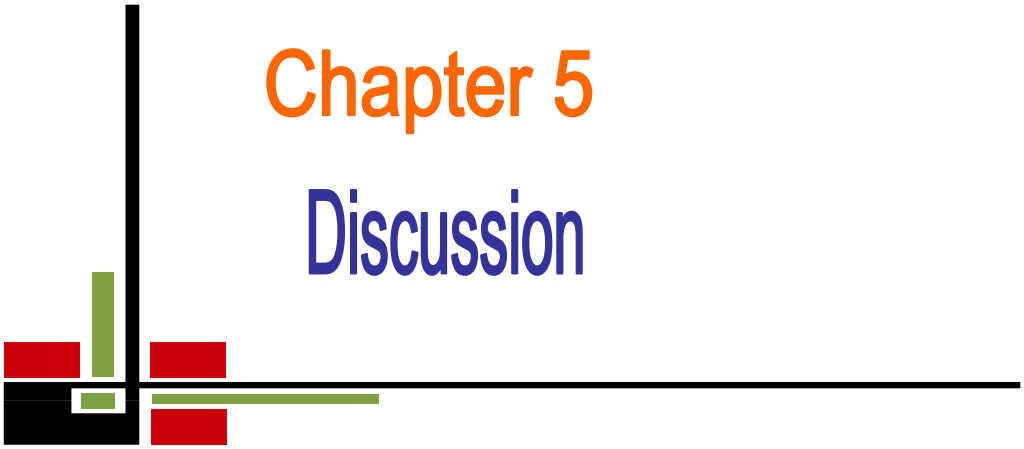
Comparative efficacy of Sulcox 50WP @ 0.3%, Champion 50 WP @ 0.4% and Cupravit 50WP @ 0.3% on *Ralstonia* sp were done and inhibition zone was measured. Significant variation was observed among the fungicides used (Table 13). At 24 hrs of incubation, the highest inhibition zone (1.30 cm) was recorded by Sulcox 50WP @ 0.3% followed by Cupravit 50WP @ 0.3% (1.17 cm) and Champion 50WP @ 0.4% (1.10 cm). At 48 hrs of incubation, the highest inhibition zone (1.27 cm) was recorded by Sulcox 50WP 0.3% followed by Champion 50WP 0.4% (1.20 cm) and Cupravit 50WP 0.3%

(1.17cm). At 72 hrs and 96 hrs of incubation, the highest inhibition zone was recorded by Cupravit 50WP @ 0.3%, followed by Champion 50WP @ 0.4% and Sulcox 50WP @ 0.3%.

Table 13. Efficacy of selected Cu-fungicides on *Ralstonia* sp of hybrid maize variety NK-40 at different days after incubation

Treatments	Inhibition zone after 24 hrs (cm)	Inhibition zone after 48 hrs (cm)	Inhibition zone after 72 hrs (cm)	Inhibition zone after 96 hrs (cm)
Control	0.00 c	0.00 c	0.00 c	0.00 c
Sulcox 50 WP @ 0.3%	1.30 a	1.27 a	1.20 b	1.13 b
Champion 50 WP @ 0.4%	1.10 b	1.20 ab	1.20 b	1.23 b
Cupravit 50 WP @ 0.3%	1.17 ab	1.17 b	1.37 a	1.67 a
$LSD_{(0.05)}$	0.143	0.072	0.160	0.304
CV%	5.61	2.75	5.30	9.92

In column, means containing same letter indicate significantly similar under LSD at 5 % level of significance. Values are the means of three replications.



Chapter 5

Discussion

CHAPTER 5
DISCUSSION

The experiments were conducted in the laboratory of the Department of Plant Pathology, Sher-e-Bangla Agricultural University, Dhaka to determine the seed borne bacteria of hybrid maize variety NK-40 and to manage these bacteria by applying selected Cu-fungicides. The selected Cu-fungicides were Sulcox 50WP, Champion 50WP and Cupravit 50WP. In this study, the seed health test was conducted by blotter method and revealed three species of seed borne bacteria viz. *Acidovorax* sp, *Ralstonia* sp and *Burkholderia* sp. The CIMMYT Maize Program (2004) reported the seed borne bacteria of maize and the pathogens were *Acidovorax avenae* subsp. *avenae*, *Burkholderia andropogonis*, *Clavibacter michiganensis* subsp. *nebraskensis*, *Erwinia chrysanthemi* pv. *zeae*, *Pantoea stewartii and Pseudomonas syringae* pv. *lapsa.* Hebber *et al.* (1992) also reported that *Burkholderia* spp. was found on sorghum, corn, mucuna, trifolium, dolichos, vicia. Similar findings were also reported by Goto *et al*. (1987), Yuan (2004). Tahat and Sijam (2010) studied on *Ralstonia* spp. (race 3 biovar 2) and stated that *Ralstonia spp.* is a bacterial wilt causal agent of many plant species and infect potatoes, eggplant, peppers, tomatoes, geraniums, ginger, corn and a few weed species including bittersweet, nightshade and stinging nettle.

Pathogens were identified by using differential biochemical medium (YDC *i.e.* Yeast extract-dextrose-$CaCO_3$) and biochemical test viz. gram staining, catalase test, starch hydrolysis test, oxidase test, gilatin liquification test, citrate utilization test, pectolytic test, motility test and salt tolerance test following Bergey's manual of determinative bacteriology. *Acidovorax* sp, *Burkholderia* sp and *Ralstonia* sp were also identified by using differential YDC medium (Yeast extract-dextrose-$CaCO_3$). On YDC medium, colonies of *Acidovorax* sp were convex, 2-3mm in diameter after 2 days at 30ºC, dark

beige in color and became very sticky and colonies of *Ralstonia* sp were mucoid, beige to light brown in color. On the other hand, colonies of *Burkholderia* sp on YDC medium were circular and purple color. *Acidovorax* sp and *Ralstonia* sp grew well at 2% salt stress and *Burkholderia* sp grew well at 3% salt stress. Similar findings are also reported by Schaad *et al.* (2000), Cowan (1974).

In blotter method, fungal incidence was 14.24% and germination was 85%. In agar plate method, the incidence of *Acidovorax* sp was recorded 2.25%, *Burkholderia* sp was recorded 1.5% and *Ralstonia* sp was recorded 1.25%.

There were three Cu-fungicides viz. Sulcox 50WP, Champion 50WP and Cupravit 50WP. Among the treatments, the effect of seed treatment with different Cu-fungicides on germination, seed infection, normal seedlings, abnormal seedlings, dead seed and vigor index were recoded and Cupravit 50WP @ 0.3% performed the best result. Ritchie (2004) reported on the bacterial spot pathogen is sensitive to copper. There are many factors that affect the efficacy of copper in the control of bacterial plant pathogens. Ellis and Bradley (1992) studied about copper fungicide formulations were available to organic growers that can effectively kill fungi and bacteria.

In nutrient agar plate method, the effect of seed treatment with Sulcox 50WP @ 0.3%, Champion 50WP @ 0.4% and Cupravit 50WP @ 0.3% on germination, seed infection and dead seed of hybrid maize variety NK-40 was studied. The germination varied from 94.83% to 98.67%, where significantly the lowest germination (94.83%) was recorded in control and the highest germination (98.67%) was recorded from the seeds treated with Cupravit 50WP @ 0.3%. Significantly the highest seed infection (3.88%) was recorded in control and the lowest seed infection (2.23%) was recorded when the seeds treated with Cupravit 50WP @ 0.3%. On the other hand,

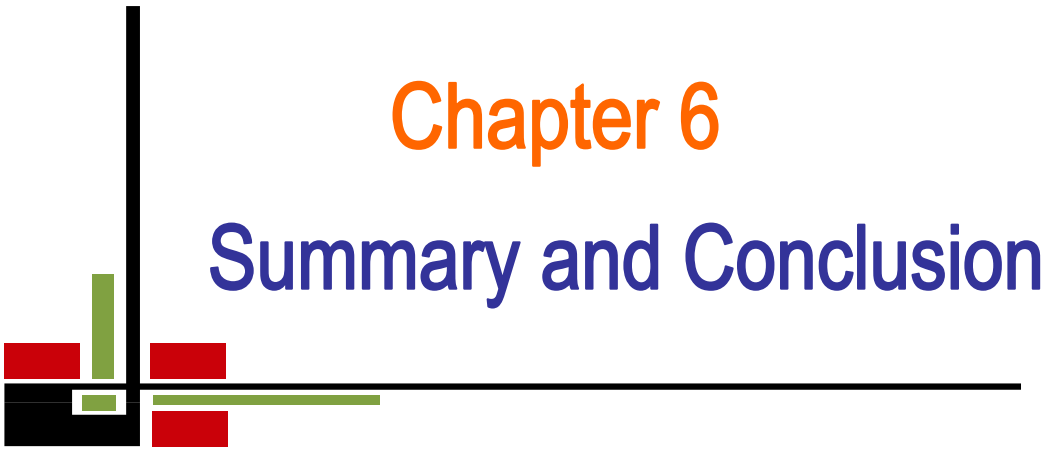
significantly the highest number of dead seeds (2.03%) was recorded in control and the significantly lowest number of dead seeds (1.28%) was recorded in case of seeds treated with Sulcox 50WP @ 0.3%. Milijasevic *et al.* (2009) tested three copper-based compounds against *Clavibacter michiganensis* subsp. *michiganensis* in tomato seedlings and observed Cu-hydroxides were the most prominent in reducing bacterial population where mancozeb did not improve the effects. Spraying with Cu-fungicides alternatively with streptomycin (250 ppm) was found effective in controlling bacterial leaf blight of rice (Ahmed *et al.*, 2005).

In water agar test tube method, the effect of seed treatment with Sulcox 50WP @ 0.3%, Champion 50WP @ 0.4% and Cupravit 50WP @ 0.3% on germination, normal seedlings, abnormal seedlings, diseased seedlings and number of dead seeds of maize hybrid variety NK-40 were recorded. The germination is varied from 85.33% to 93.00%, where significantly the lowest germination (85.33%) was recorded in control and the highest germination (93.00%) was recorded from the seeds treated with Cupravit 50WP @ 0.3%. Significantly the lowest normal seedlings (44.67%) were recorded in control and the highest (69.00%) was recorded from the seeds treated with Cupravit 50WP @ 0.3%. Abnormal seedlings ranged from 20.00% to 34.67%, where significantly the highest abnormal seedling was recorded in control and the lowest abnormal seedlings were recorded from the seeds treated with Cupravit 50WP @ 0.3%. In case of diseased seedlings, significantly the highest diseased seedlings (13.00%) were recorded in control and the lowest diseased seedlings (5.00%) were recorded when the seeds treated with Cupravit 50WP @ 0.3%. Islam *et al.* (2003) used Streptomycin sulphate, Thiovit 80WP, Sulfuric acid, Dithane M-45 and Cupravit either alone or in combination for controlling bacterial blight and on yield of cotton and observed highest germination (86.31%) when seed treatment with Streptomycin sulphate (0.15%) and foliar spray with Cupravit (0.2%) +

Streptomycin sulphate (150 ppm). The lowest disease index (21.24%) was found in that treatment subsequently after three foliar sprays at 104 DAS. This treatment reduced the disease intensity and increased the yield of seed cotton with 26.02%.

In case of rolled paper towel method, efficacy of Sulcox 50WP @0.3%, Champion 50WP @ 0.4% and Cupravit 50WP @ 0.3% on maize hybrid variety NK-40 were studed. Shoot length ranged from 4.00 cm to 11.43 cm, where the significantly lowest shoot length (4.00 cm) was recorded by control and the highest shoot length (11.43 cm) was recorded by Champion 50WP @ 0.4%. Root length ranged from 5.72 cm to 13.97 cm, where the significantly lowest root length was recorded under control and the highest root length (13.97 cm) was recorded by Cupravit 50WP @ 0.3%. The germination is varied from 91.00% to 98.67%, where significantly the lowest germination (91.00%) was recorded in control and the highest germination (98.67%) was recorded from the seeds treated with Cupravit 50WP @ 0.3%. On the other hand, the significantly minimum vigor index (885.5) was determined in control and the maximum vigor index (2488.4) was recorded in case of seeds treated with Cupravit 50WP @ 0.3% followed by Champion 50WP @ 0.4% and Sulcox 50WP @ 0.3%. In bioassay test, of these three Cu-fungicides, the highest inhibition zone was recorded in case of Cupravit 50WP followed by Champion 50WP and Sulcox 50WP.

From the findings of the present investigations, it has been understood that Sulcox 50WP, Champion 50WP and Cupravit 50WP has promising potentiality in controlling different seed borne bacteria of maize with increasing seedling stand. In the world, Cu-fungicides has bright prospect for controlling bacterial diseases of maize. The findings of the present study need to be applied under field condition for its potentiality in the farmer’s plots. Therefore, further research in this discipline is advocated.



Chapter 6

Summary and Conclusion

CHAPTER 6
SUMMERY AND CONCLUSION

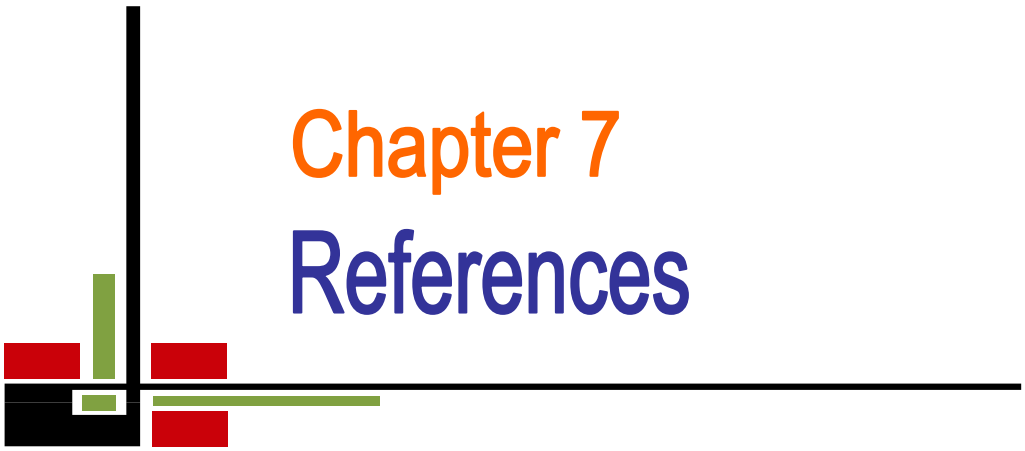
Maize (*Zea mays* L.) is an important and promising crop having good potential as a cereal crop in Bangladesh. Area under its cultivation, demands and popularities in different industries are gradually increasing day by day in the country. The production of maize in the country is very low because of proneness of the plants to different stresses, existing of diseases and other unfavorable environmental conditions.

The present piece of research work was carried out in the Seed Pathology Centre, Department of Plant Pathology, Sher-e-Bangla Agricultural University, Dhaka to find out the incidence of seed borne bacteria of maize and to manage these bacteria by seed treating with some selected Cu-fungicides. Seed health test in agar plate method yielded three bacterial genera. The bacteria were identified by conducting different biochemical test and using differential medium. According to the result of different biochemical test and growth on differential medium revealed that the bacteria were *Acidovorax* sp, *Burkholderia* sp, *Ralstonia* sp and pathogenecity of these bacteria were done. Three Cu-fugicides viz. Sulcox 50WP, Champion 50WP and Cupravit 50WP were selected as treatments and bioassay of Cu-fungicides were tested against *Acidovorax* sp, *Burkholderia* sp and *Ralstonia* sp. The highest inhibition zone observed in Cupravit 50WP @ 0.3% followed by Champion 50WP @ 0.4% and Sulcox 50WP @ 0.3%. Effects of seed treatment with Cu-fungicides on prevalence of seed borne bacteria were tested in nutrient agar plate method. Significantly highest germination (98.67%) was found by Cupravit 50WP @ 0.3% followed by Champion 50WP @ 0.4% (98.13%) and Sulcox 50WP @ 0.3% (97.50%). Significantly lowest seed infection (2.23%) was recorded by

Cupravit 50WP @ 0.3% treated seed followed by Champion 50WP @ 0.4% and Sulcox 50 WP @ 0.3%.

Effects of seed treatment with Cu-fungicides on prevalence of seed borne bacteria were tested on germination, normal seedlings, abnormal seedlings, diseased seedlings and dead seeds were recorded in water agar plate method. Germination varied from 89.00-93.00%, where the highest germination was observed in Cupravit 50WP @ 0.3% treated seeds. The highest number of normal seedlings (69.00%) were recorded by Cupravit 50WP @ 0.3% followed by Champion 50WP @ 0.4% and Sulcox 50WP @ 0.3%. The highest number of abnormal seedlings (34.67%) and the highest number of diseased seedlings (13.00%) were recorded in control followed by Sulcox 50WP @ 0.3%, Champion 50WP @ 0.4% and Cupravit 50WP @ 0.3%. Effects of seed treatment with Cu-fungicides on shoot length, root length, germination and vigor index were recorded in rolled paper towel method. The highest shoot length (11.43 cm) was recorded in Champion 50WP @ 0.4% followed by Cupravit 50WP @ 0.3% and Sulcox 50WP @ 0.3%. The highest root length (13.97 cm) was found in Cupravit 50WP @ 0.3% followed by Champion 50WP @ 0.4% and Sulcox 50WP @ 0.3%. The highest vigor index (2488.40) were recorded when the seeds were treated with Cupravit 50WP @ 0.3% followed by Champion 50WP @ 0.4% and Sulcox 50WP @ 0.3%.

It was observed that seed treatment with Cu-fungicides showed remarkable performance in controlling *Acidovorax* sp, *Ralstonia* sp and *Burkholderia* sp and among the fungicides Cupravit 50WP @ 0.3% showed best performance in controlling seed borne bacterial diseases of maize. Thus, it may be concluded that seed treatment with Sulcox 50WP, Champion 50WP and Cupravit 50WP could be an effective option for controlling seed borne bacteria of maize.



Chapter 7
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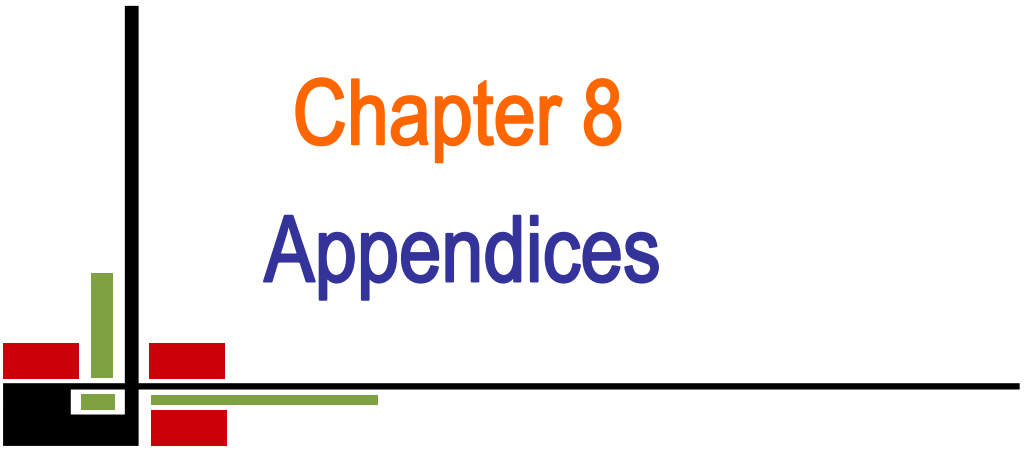
Chapter 8

Appendices



CHAPTER 8
APPENDICES

APPENDIX-A

ANALYSIS OF VARIANCE

1. Analysis of variance of the data on effect of some selected Cu-fungicides of seedlings in nutrient agar plate method

Sources of Variation	Degrees of Freedom	Mean Square Value		
		% Germination	% Seed Infection	% Dead Seed
Replication	2	1.67	0.28	0.18
Cu-fungicides	3	8.73	1.40	0.71
Error	6	4.77	0.05	0.01

2. Analysis of variance of the data on effect of some selected Cu-fungicides of seedlings in water agar test tube method

Sources of Variation	Degrees of Freedom	Mean Square Value				
		% Germination	% Normal seedlings	% Abnorml seedlings	% Diseased seedlings	% Dead seeds
Replication	2	5.25	3.08	0.58	3.06	0.39
Cu-fungicides	3	34.31	319.22	114.31	32.75	9.27
Error	6	6.81	7.97	3.47	0.06	0.18

3. Analysis of variance of the data on effect of some selected Cu-fungicides of seedlings on germination, shoot length, root length & vigor index (rolled paper towel method)

Sources of Variation	Degrees of Freedom	Mean Square Value			
		Shoot Length (cm)	Root Length (cm)	% Germination	Vigor Index
Replication	2	0.12	0.87	3.08	6641.24
Cu-fungicides	3	40.83	38.76	38.81	1669069.09
Error	6	0.67	0.15	3.64	8912.81

4. Analysis of variance of the data on Sulcox 50 WP interaction with isolated bacteria at different concentration at 48 hrs

Sources of Variation	Degrees of Freedom	Mean Square Value		
		Isolated Bacteria		
		***Acidovorax* sp**	***Burkholderia* sp**	***Ralstonia* sp**
Replication	2	0.022	0.076	0.142
Concentrations	3	0.451	0.050	0.197
Error	6	0.004	0.005	0.023

5. Analysis of variance of the data on Champion 50 WP interaction with isolated bacteria at different concentration at 48 hrs

Sources of Variation	Degrees of Freedom	Mean Square Value		
		Isolated Bacteria		
		***Acidovorax* sp**	***Burkholderia* sp**	***Ralstonia* sp**
Replication	2	0.045	0.023	0.063
Concentrations	3	1.569	0.632	0.010
Error	6	0.028	0.015	0.010

6. Analysis of variance of the data on Cupravit 50 WP interaction with isolated bacteria at different concentration at 48 hrs

Sources of Variation	Degrees of Freedom	Mean Square Value		
		Isolated Bacteria		
		***Acidovorax* sp**	***Burkholderia* sp**	***Ralstonia* sp**
Replication	2	0.152	0.028	0.043
Concentrations	3	0.125	0.376	0.030
Error	6	0.007	0.010	0.002

APPENDIX-B

PREPARATION OF MEDIA AND REAGENTS

Preparation of Gram staining reagents:

i) Gram's Crystal violet (Hucker's modification):

Solution A: Crystal violet (90% dye content)	2.0 g
Ethyl alcohol	20.0 ml
Solution B: Ammonium oxalate	0.8 g
Distilled water	80.0 ml

Solution A and B in equal volume to prepare crystal violate solution.

ii) Gram's Iodine (Gram's modification of Lugol's solution):

Iodine	1.0 g
Potassium iodide (KI)	2.0 g
Distilled water	300.0 g

Add iodine after KI is dissolved in water to prepare Gram's Iodine solution.

iii) Gram's alcohol (decolorizing agent)

Ethyl alcohol (95%)	98 ml
Acetone	2 ml

iv) Safranin (counter stain)

Safranin (2.5% solution in 95% ethanol)	10 ml
Distilled water	100 ml

Preparation of KOH solubility reagent:

3% aqueous solution of KOH was prepared from the KOH granules

Preparation of Starch hydrolysis media and reagent:

i) Culture medium

Nutrient broth (Difco)	8.0 g
Soluble potato starch	10.0 g
Bacto agar (Difco)	15.0 g
Distilled water	1000 ml

ii) Reagent (Lugol's iodine)

Iodine	5.0 g
Potassium iodide	10.0 g
Distilled water	100 ml

Preparation of Catalase reagent:

3% aqueous solution of H_2O_2 was prepared from the H_2O_2 absolute solution

Preparation of Oxidase reagent:

1% aqueous solution of N,N,N,'N-tetramethyl-p-phenylene dihydrochloride

Preparation of Citrate utilization test medium:

$MgSO_4.7H_2O$	0.2 g
$NH_4H_2PO_4$	1.0 g
K_2HPO_4	2.0 g
Sodium citrate	2.0 g
NaCl	5.0 g
Bromothymol blue	80.0 mg
Agar	15.0 g
Distilled water	1000 ml

Preparation of Gelatin medium:

Gelatin	120 g
Distilled water	1000 ml

Preparation of MIU medium:

Peotone	30.0g
NaCl	5.0 g
Urea	20.0 g
Monopotassium phosphate	2.0 g
Phenol red	0.005 g
Agar	4.0 g
Distilled water	1000 ml
pH	7.0