Effects of humidity, temperature and inoculum density under controlled environment and fungicide application under field conditions on the development of Cercospora leaf spot of Swiss chard.

By

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CERTIFICATION

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APPROVAL

This thesis has been approved as meeting the required standards for the degree of Master of Science in Applied Microbiology (Biological Science), University of Botswana.

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STATEMENT OF ORIGINALITY

This dissertation is my original work except where due reference is made. It has not been and shall not be submitted for the award of any degree in any other University.

Author's name and signature Date

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DEDICATION

I dedicate my work to my daughter Karyn Hannah Imi. Utmost gratitude is extended to my loving parents Augustines and Uyapo Utlwang for having supported my dreams and their words of encouragement. I give special thanks to my best friend Baboloki Kethobile for the many hours of proofreading my write up. To all those that I might have not mentioned, who took it upon themselves to assist me, guide me and open doors of opportunities for me, I would like to thank each and every one of them. I will always appreciate their dedicated partnership for the success in my life.

ABSTRACT

Cercospora leaf spot (CLS), caused by *Cercospora beticola* is an important foliar disease of Swiss chard in Botswana. Regular outbreaks of the disease in Botswana is a major cause of concern and thus have necessitated the use of fungicides under field conditions in order to control the disease and bring the crop to maturity. The objectives of the study was (1) to analyse the genetic diversity of *C. beticola* isolates from Swiss chard collected from southern Botswana, (2) to determine the effect of humidity temperature and inoculum concentration on plant disease development, under controlled environment and (3) to assess the effectiveness of locally available fungicide in managing CLS of Swiss chard under field conditions in Botswana.

Genomic DNA extraction from *C. beticola* mycelia was done using the MasterPure Yeast DNA purification Kit and PCR amplification utilized ITS1 (5'-TCCGGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') primers in a Techne thermocycler and the phylogenetic tree of ITS rDNA was constructed using version 6.06. Phylogenetic analyses of 87 isolates revealed that *C. beticola* isolates clustered in four major classes, which could not be differentiated by the sampling location. Similarly genetic analysis revealed high genetic diversity of *C. beticola* strains, with diversity accounted for by within population diversity of Swiss chard isolate (greatest pairwise distance = 0.005).

Effect of humidity, temperature and inoculum concentration on disease development over a 4-week period was conducted in the Department of Agricultural Research (DAR) Laboratory, using a modified egg incubator as a controlled environment growth chamber. The

humidity and temperature treatments were run separately and sequentially each for 28 days where the incubator was set at 50%, 70% and 90% relative humidity (RH) and at 25°C and at 15° C, 20°C, 25°C and 30°C, respectively, and plants inoculated with $0x10^4$, $0.1x10^4$, $1.2x10^4$, $2.3x10^5$ and $2.3x10^6$ spores/mL using a hand sprayer. A completely randomised design for the three-factorial experiments (RH x Inoculum Concentration x Time and Temperature x Inoculum Concentration x Time), with ten replicate plants was used. Number of spots per leaf for inoculated plants at 25°C increased significantly ($P \leq 0.05$) when plants were incubated at 50% (0.8), 70% (1.9) and 90% (2.9) RH, respectively. As inoculum increased from 0 to $2.3x10^6$ spores/mL the number of leaf spots also significantly increased from 0.4 to 2.9 spots per leaf. The highest number of spots per leaf was recorded on plants inoculated with $2.3x10^6$ spores/mL and incubated at 90%RH (4.4) followed by $2.3x10^5$ spores/mL at 90%RH and $2.3x10^6$ spores/mL at 70%RH. For each spore concentration the amount of disease significantly increased with increase in humidity. At 90%RH disease development was highest at 25°C (2.9 spots/leaf)) followed by 30°C (2.5 spots/leaf), 20°C (1.9 spots/leaf) and 15°C (no spots). Averaged across inoculum concentrations and humidity number leaf spots significantly increased from 0.760, 1.680, 2.225, to 2.580 spots/leaf from week 1 to 4, respectively. Similarly the interactions of time with inoculum concentrations and humidity resulted in significant increases in number of leaf spots per plant from week 1 to week 4. The results showed that low temperatures and low humidity which prevail during the winter in Botswana are not conducive while temperatures and humidity during the rainy season are more conducive to CLS disease development.

The study confirmed that as RH increases, CLS numbers also increase. Relative humidity assessed alone or interacting with either inoculum concentration or sampling time increased as humidity increased. Cercospora leaf spot increased significantly ($P \leq 0.05$) from 0.795,

1.855 to 2.86 at 50%, 70% and 90% RH respectively. The interaction of humidity with inoculum concentration at 50% was 0.400 CLS for the 0x 10^4 concentration compared with 1.250 for the $2.3x10^6$ concentration. The 70% RH recorded 1.325 CLS for the $0x10^4$ concentration and 1.855 CLS for the $2.3x10^6$ concentration. The 90%RH had higher value of CLS 1.725 for the $0x10^4$ concentration compared with a 4.350 at $2.3x10^6$ concentrations. These values showed that as RH and inoculum concentration increased CLS also increased significantly at $P \leq 0.05$. Moreover Sampling time alone or interacted with RH showed an increase in CLS as both humidity and sampling time increased significantly $P \leq 0.05$.

In addition temperature assessed alone or interacted with inoculum concentration or sampling time significantly increased CLS development as temperature increased from 20°C to 25°C from 1.925 to 2.835 respectively. However at 15°C there was no CLS development (0.000) and at 30°C CLS development reduced significantly to 2.485 at $P \leq 0.05$. As Inoculum concentration increased CLS also significantly increased from 1.031, 1.325, and 1.594 to 2.769 for the $0x10^4$, $0.1x10^4$, $1.2\ x10^4$, $2.3x10^5$, and $2.3x10^6$ inoculum concentrations per ml. Cercospora leaf spot increased significantly when assessing the effects of Sampling time (7, 14, 21, and 28 days) from 0.760, 1.680, 2.225, to 2.580 respectively showing that the longer the plants are kept the more disease they develop. Furthermore when sampling time is interacted with temperature, the highest means are realised at 28 days for the 25°C, 30°C, 20°C and lastly 15°C where no CLS developed

Assessment of locally available fungicides for the control of CLS was carried out in Sebele using 5m x 2m plots arranged in a randomized complete block design (RCBD) with three blocks and seven fungicide treatments and untreated control Swiss chard. Seedlings were transplanted into the plots spaced 40cm inter-rows and 30cm between plants and natural

infection was used. Once symptoms were observed plants were sprayed at 2-week intervals for 12 weeks with distilled water, copper oxychloride; benomyl + mancozeb; mancozeb + copper oxychloride; benomyl; benomyl + copper oxychloride; mancozeb; benomyl + mancozeb + copper oxychloride using recommended rates. Where more than one fungicide was used, the fungicides were applied at alternating intervals. The average number of leaf spot per leaf was recorded two weeks after each fungicide application and samples were collected prior to spraying. Data was subjected to one-way analysis of variance and means separated using LSD test at p=0.05. Number of spot/leaf and percentage disease reduction in all treatments involving benomyl were 29.3±5.5 and 85%, respectively which was significantly lower than on those treatments involving mancozeb and copper oxychloride used alone or in combination which were 50.85±9.5 and 74.2%, respectively while the had 196.9 spots/leaf. This study confirms that when benomyl is alternated with copper oxychloride, it is much more effective in managing Cercospora leaf spot than mancozeb or copper oxychloride used alone or alternated.

Key words: *Cercospora beticola*, Swiss chard, Cercospora leaf spot (CLS), ITS PCR, genetic diversity, Humidity, Inoculum, Environment, Fungicides, control, Botswana

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LIST OF ABBREVIATIONS

ANOVA……..…..Analysis of variance

ARISA……...Automated ribosomal intergenic spacer analysis

CLS……...Cercospora leaf spot

DAR……..Department of Agricultural Research

DGGE ……...Denaturing gradient gel electrophoresis

DNA……..Deoxyribonucleic acid

LSD……...Least Significant Difference

MEA……..Malt extract agar

MEGA…….. Molecular Evolutionary Genetics Analysis program

MSTAC…….Michigan State University Statistical Package

OA…….Oatmeal agar

PCR……...Polymerase chain reaction

rDNA……..…Recombinant DNA

RH…….Relative Humidity

tRFLP ……...Terminal restriction fragment length polymorphism

1.0. INTRODUCTION

Swiss chard (*Beta vulgaris)* subsp. *cicla* (L.), also known as spinach beet belongs to the Chenopodiaceae or goosefoot family (goose family name because the leaves resemble a goose foot).The vernacular names include Roman kale, strawberry spinach, seakale beet, leaf beet, spinach beet and silver beet. It belongs to the same species as beetroot (Beta vulgaris). It is a biennial but is grown as an annual. It is generally, but mistakenly, called spinach in Botswana. The leaves are prepared like spinach and the leaf stalks sometimes like asparagus or celery. However in Africa the whole leaf blade is usually prepared with the midrib as one dish and eaten as a pot herb or relish. Swiss chard is a common substitute for true spinach *(Spinacia oleracea* L.*)*. It furnishes a considerably higher yield, is easily cultivated and has a similar flavour to spinach. Swiss chard is a form of beetroot which has been selected for the production of large, fleshy leaf stalks and broad leaf blades with pronounced white or red midribs and deeply veined lamina (Tindall, 1988).

There is limited information on the early distribution and development of Swiss chard. Wild forms of *Beta vulgaris* occur along the shores of the Mediterranean, extending eastwards as far as Indonesia, and westwards along the coasts of the Atlantic up to the Canary Islands and southern Norway (Letschert, 1993). *Beta vulgaris* grown for its leaves was taken into cultivation in the eastern Mediterranean or the Middle East and is first mentioned in the literature in Mesopotamia in the 9^{th} century BC. Recipes for roots of beet date from the 3^{rd} century AD, but true garden beets appear in Europe as late as the 16^{th} century. Garden beet is the most important form worldwide and can be found in all African countries, however, it is a relatively minor market vegetable around the big cities. In most African countries spinach beet/Swiss chard is far more important than garden beet, which is primarily consumed by

Europeans and Americans. Two other cultivated forms of *Beta vulgaris*, fodder beet and sugar beet are of no importance in tropical Africa (Lange *et al*, 1999; Bakry *et al*, 2014).

Some varieties of Swiss chard have greenish-white or pretty red foliage which is robust and erect. This plant has deep roots and will regrow even when heavily cut. The main root is long, stout with tampered side roots forming a dense extensive root system in the top 25cm of the soil. Swiss chard can grow in a wide range of soils as long as they are moist and fertile (DAR, 2003). Fertile, sandy loams, high in organic matter is recommended. The plants are sensitive to acidity and the soil should be at least of a pH of 6.0, with an optimum pH of 6.2 - 6.9. As they originate from sea shores, they are tolerant of limited concentrations of salt, therefore can be irrigated with saline water thus the neutral to slightly alkaline pH range (Shannon *et al*, 2000).

Swiss chard is a herbaceous biennial plant with leafy stems growing to 30-50 cm tall. The leaves adre heart-shaped, 5-20 cm long on wild plants (often much larger in cultivated plants). The flowers are produced in dense spikes, each flower very small, subtended by minute bracts and bisexual (with stamens 5,1 celled ovary , surrounded by a disk, with 2–3 stigmas.), 3-5 mm in diameter, green or tinged reddish, with five petals; they are wind-pollinated. The fruit is a cluster of hard nutlets enclosed within the swollen corky perianth-bases, 3–7 mm in diameter, 1–6 fruits adhering in glomerules. The seed is kidney-shaped, brown and has a diameter range of about 1.5–3 mm (Kelly and George, 1998; Rashid and Singh, 2000; Zohary *et al*, 2012).

In Botswana, two varieties are of importance, which are Fordhook giant and Lucullus. Other varieties are however available in retail stores but are not very common among farmers.

These would include: bright lights and star 101. Both Fordhook giant and Lucullus can be produced all year round in Southern region and Gaborone (DAR, 2003). A study carried out by Madisa *et al,* (2010) revealed that Swiss chard is grown in most regions of Botswana as shown in Table 1. All farmers that were involved in the study within Ghantsi, Southern and Kgalagadi regions produced Swiss chard. This correlated with results obtained by Obopile *et al*, (2008) which showed that the most commonly grown vegetable in Gaborone region (Kgatleng) and western region (Ghanzi and Kweneng) was Swiss chard. Farmers' ranking of the important vegetable crops generally agrees with reports by and Munthali *et al,* (2004) and Bok *et al,* (2006) that brassicas, Swiss chard and tomato are the most popular vegetable crops grown in Botswana. These crops (except Swiss chard) are significantly associated with major pests of vegetable crops.

Table 1: Percentage farmers producing Swiss chard in each region of Botswana

Region	% of farmers producing swiss chard in the region
Kgatleng	65
Kweneng	42.9
Central	75
Chobe	0
South east	77.8
Ghanzi	100
Southern	100
Kgalagadi	100
North west	50
Average	66.7

Source; Madisa *et al. (*2010)

Most regions have percentage production of above 50% (Table 1) except for Kweneng at 42.9% and Chobe where there is no production (0%)

It is of outmost importance to note that Agriculture contributes 2% of GDP, however an estimated 69% of the total population of 2 098 018 people (Botswana, 2015) live in rural

areas and earn their livelihoods from Agriculture. This lead to the inception of an initiative by the government under the National Strategy for poverty eradication in 2003. Small scale horticulture was pursued as one of the several programs meant to achieve sustainable livelihoods for beneficiaries. The aim of the program being to eradicate poverty, promote food security at household level and small scale entrepreneurship and income generation. Marumo *et al*. (2017) when studying the impact of backyard garden intervention on poverty status of Beneficiaries in Ramotswa found that out of a total of 40 only 13 beneficiaries grew Swiss chard amounting to a relative frequency of 30% and claimed to have the second highest annual sales revenue after maize. The majority of farmers in Botswana are small scale farmers who typically need assistance in capacity building to commercialize agriculture. (Botswana, 2015).

Swiss chard is a good source of calcium magnesium and Vitamin K. Sufficient Vitamin K consumption performs as a modifier of bone matrix proteins and improves calcium absorption preventing bone loss and osteoporosis, a super food for the elderly. It has been also associated with improved red blood cell formation by containing iron and copper. Individuals who are deficient in iron and copper can develop anemia, a condition resulting in weakness, fatigue, stomach disorders and lack of concentration (Pokluda and Kuben, 2002). Swiss chard is a health supportive food containing a compound called syringic acid which has shown to regulate blood sugar levels by inactivating enzyme alpha glucosidase thus inhibiting the breakdown of complex carbohydrates into simple sugars helping to stabilize the body's blood sugar level (Neelwarne and Halagur, 2013). It is also loaded with beta carotene (antioxidant) whose consumption has been linked with improved eye health and risk reduction of macular degeneration glaucoma, night blindness and other vision related conditions (Ninfali *et al*, 2007). Swiss chard also contains biotin, an organic compound that

has been associated with the stimulation of hair follicles and increased hair texture. A super food like Swiss chard is of outmost importance in a country like Botswana facing health challenges associated with HIV/AIDS, cancer, diabetes and tuberculosis. Needless to say people (Batswana) are slowly becoming health conscious and turning to food to boost their immune systems and eat health in order to stay healthy.

The main constraint in the production of Swiss chard is a foliar disease known as *Cercospora* leaf spot, caused by *C. beticola* Sacc. Losses caused by this disease may initially be underestimated, however intense pressure from this pathogen results in increased lesions which would ultimately lead to necrosis of whole tissue. Windels *et al.* (1998) went on to explain that the pathogen survives on infected plant residue as stromata in soil, where it can survive for years. In the following planting season and under optimal conditions, characterized by relatively high humidity or heavy dew, conidiophores and conidia are produced on the stromata which serve to initiate primary infection on a newly planted crop.

Characteristic symptoms of the infection of *Beta* species by *C. beticola* include the randomly distributed leaf spots of typically 2–5 mm in diameter across the surface of mature leaves with tan to ashen grey centers (Duffus and Ruppel, 1993). Unlike many other leaf spot pathogens that necrotize from pin-point lesions and expand outward (Figure 1), lesions produced by *C. beticola* involve the near-simultaneous collapse of cells in an area many millimetres in diameter, as shown in figure 2. This is due to the fact the *C. beticola* enters the host through stomata into the paranchymatous leaf tissue followed by initial colonization of the tissue in an asymptomatic manner and grow intercellularly (Feindt *et al*, 1981; Khan *et al,* 2009). After tissue collapse has occurred, the lesion often becomes encircled by a characteristic reddish-brown ring. Lesions can be observed on leaves and leaf petioles.

Although lesions can expand after initial tissue collapse, the increase in necrotic area on the leaf surface is due primarily to an increase in the number of lesions on that surface. Ultimately, a combination of high lesion number and the accumulation in the leaves of phytotoxins induces complete leaf senescence.

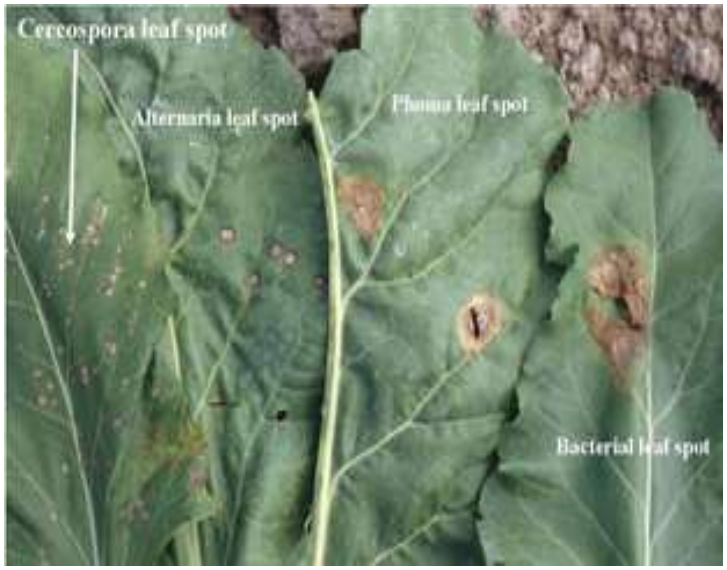

Figure 1: Leaf spots caused by a variety of pathogens including *C. beticola* arranged linearly for comparison adopted from Harveson (2013)

Figure 2: Leaf tissue showing Cercospora leaf spot

Cercospora leaf spot was not of economic importance in furrow irrigated fields, but after growers turned to sprinkler irrigation, medium to severe epidemics occurred (Paulus, *et al*, 1971). An understanding of the optimum environmental conditions for the initiation of *Cercospora* epidemics (elevated temperature, humidity and leaf wetness; (de Nazareno *et al*, 1993b); Shane and Teng, 1983) is of outmost importance. Optimum conditions for sporulation, germination and penetration are day temperatures of 25°C to 35°C with night temperatures above 16°C and prolonged periods of relative humidity of 90 to 95% (Shane and Teng, 1984; Ruppel, 1986) Conidia of *C. beticola* are produced most readily at temperatures from 15 to 23°C and relative humidity (RH) greater than 60%, but do not form at temperatures less than 10°C or above 38°C. This is information that is important to Botswana, a country that experiences summer temperature ranging from 25°C- 27°C during the months from September April. Daytime temperatures can go up to 35°C. The annual rainfall is about 450- 550mm per annum and it rains from September to April. Therefore as such the rainy season in Botswana is accompanied by high temperatures (Veenendaal *et al*, 1996), a condition that is conducive for the development of Cercospora leaf spot. Such information has aided in the development of CLS prediction models for implementation of fungicide spray schedules (Wolf and Verreet, 1997; Denth, 2004).

Khan *et al*. (2009) studied the fluctuations in conidial numbers in relation to the environment and disease severity. This research correlated temporal airborne conidial concentration with average temperature at a relative humidity greater than 87%. The results obtained showed that the potential disease severity can be estimated using conidial numbers. A lower number of conidia were due to unfavorable environmental conditions and exhaustion of conidial sources with high numbers as a result of favorable conditions for sporulation (Khan and Khan, 2003). Khan *et al* (2009) manually inoculated the plants using dried infected leaves.

All plots were inoculated with the same mass of leaf material infected with *C. beticola* inoculum.

Molecular methods for detecting plant pathogenic microorganisms present a more accurate identification. These methods are based on Polymerase chain reaction (PCR). To the author's knowledge, there are no prior studies carried on *C. beticola* identification and genetic diversity in Botswana. The goal of which is to describe and quantify genetic variation and also to make inferences on evolutionary forces such as mutations, migration, genetic drift selection and recombination. This information is of outmost importance to improve disease management and on fungicide resistance studies (Burdon, 1993). Comparison by amplified fragment length polymorphism (AFLP) analysis of single-spore isolates of *C. beticola* in two independent studies in Europe and the USA indicate substantial genetic variation in natural populations (Große-Herrenthey, 2001; Weiland *et al*, 2001). Moreover, subculture of *C. beticola* in the laboratory has been reported to result in chromosome rearrangements as assayed by pulse-field gradient electrophoresis. This apparent genome plasticity in *C. beticola* combined with limited sequence diversity in rDNA regions of related *Cercospora* species contributes to the present confusion in the taxonomy within this genus (Goodwin *et al*, 2001).

1.1 Statement of the problem

Cercospora beticola is endemic in Botswana and has been reported as the major limiting factor in the production of Swiss chard. The outbreaks adversely affect production and ultimately results in economic loss. This study therefore, aims to unravel the effect of temperature humidity and inoculum density on plant disease development in order to bring to the fore possible remedial actions to minimize yield losses associated with the disease.

1.2 Justification of the study

Botswana is at an era where the agricultural sector is establishing quite significantly with more interest on the horticultural sector. In Botswana, Swiss chard cultivation meant for the local market is carried out at a commercial scale in open fields and greenhouses (Obopile *et al*, 2008). Recent efforts aimed at poverty eradication by the government have seen an upsurge in backyard gardens, especially in rural areas (Marumo *et al*, 2017) which meet subsistence demands of poor rural households. Nonetheless, at all levels of production Swiss chard cultivation is subjected to a plethora of insect pests and microbial diseases (Daiber, 1996; Baliyan *et al*, 2014).

The information generated through this research will enable us to establish humidity and temperature conditions as well as the amount of inoculum density that Swiss chard would have been exposed to for Cercospora leaf spot to develop. This would assist local farmers in control measures to adopt; including among others fungicide choice and genetic diversity of *C. beticola* shall also be covered in this chapter because a high genetic diversity is associated with rapid resistance to fungicides. Efforts of the government of Botswana to grow the horticultural sector shall be uplifted as farmers would be in a better position to control infections by *C. beticola*.

1.3 Objectives of the study

The main objective of this research is to assess the role of some of the key epidemiological factors on CLS disease development.

The specific objectives were:

- To isolate and analyse genetic diversity of *C. beticola* isolates from Swiss chard collected from southern Botswana using molecular methods.

- To determine the effect of humidity, temperature and inoculum concentration on disease development under a controlled environment.

- To assess the effectiveness of locally available fungicides in managing Cercospora leaf spot of Swiss chard under field conditions in Botswana.

1.4 Hypothesis

This study tested the following hypotheses:

H_{O1}: Humidity, temperature and inoculum levels have no effect on development of Cercospora Leaf Spot of Swiss Chard under controlled environment

H_{A1}: Humidity, temperature and inoculum levels have a significant effect on development of Cercospora Leaf Spot of Swiss Chard under controlled environment

$H_{O2:}$ Fungicide application has no effect on the development of Cercospora Leaf Spot of Swiss Chard under field conditions

H_{A2}: Fungicide application has an effect on the development of Cercospora Leaf Spot of Swiss Chard under field conditions

2.0. LITERATURE REVIEW

2.1 Taxonomy and diversity of *Cercospora beticola Sacc*

The genus *Cercospora* was described by Fresenius (Groenewald *et al*, 2006a) as one of the largest genera of hyphomycetes. Pollack, (1987) listed more than 3000 names. However Crous and Braun, (2003) reviewed the genus and reduced many species to synonymy, leaving a total of 659 *Cercospora* species. There are 281 morphologically indistinguishable *Cercospora* species, infecting a wide range of plant genera and families, listed as synonyms under *C. apii senso lato* (Crous and Braun, 2003). *Cercospora beticola*, which causes *Cercospora* leaf spot on sugar beet (Groenewald *et al*, 2005; Groenewald *et al*, 2007), is morphologically identical to *C. apii*. Although these two species were considered to be synonymous in the past (Crous and Braun, 2003), a multi-gene phylogenic comparison and cultural characteristics revealed them to be distinct species (Groenewald *et al*, 2005).

The pathogen, *C. beticola* is an imperfect filamentous fungus with no known sexual stage (Duffus and Ruppel, 1993; Ershad, 2009). Interestingly Vaghefi *et al*, (2017) reported the presence of genes that regulate sexual reproduction in the *C. beticola* genome, MAT 1-1 and MAT 1-2. The functionality of these genes would enable isolates carrying the alternate MAT1 genes to mate and sexually reproduce. It is however important to note that sexual reproduction is a major source of genetic variability through recombination and increases the evolutionary potential of a pathogen. Therefore sexually reproducing fungal populations with high genetic diversity due to recombination evolve and become resistant to fungicides more rapidly (Balau and Fraretra, 2010). No sexual stage is known for *C. beticola*, but in its asexual form it overwinters on infected plant debris as pseudostromata.

No sexual stage has been found for *C. beticola*, unlike several *Cercospora* fungi for which a *Mycosphaerella* teliomorph has been characterized. None of the *Cercospora* species considered to be monophylectic with *C. beticola* based on rDNA sequences possesses a known teliomorph, suggesting that this function may have been lost during evolution of the group (Goodwin *et al*, 2001). Nevertheless, hyphal anastomosis or an elusive mating system may promote genome exchange in *C. beticola*, contributing to genetic diversity within natural populations. Surveys revealing diversity in fungicide resistance in populations of *C. beticola* under fungicide pressure are legion (Ioannidis and Karaoglanidis, 2000).

Cercospora beticola infects species of the genus Beta, an important taxonomic characteristic, and a number of species in the Chenopodiaceae, including members of the genera Spinacea, Atriplex and Amaranthus. It has been noted that leaf spotting fungi on weed species having filiform, hyaline conidia have occasionally, if probably hastily, been classified as *C. beticola*. This often could prove erroneous as leaf spot diseases caused by other species of *Cercospora* have been characterized on weeds in the presence of, and on crop species in rotation with, leaf and root beet crops **(**Crous and Braun, 2003; Groenewald *et al*, 2005) Although limited host range studies with *C. beticola* have been performed, systematic investigation confirming the host range of Cercosporoid fungi isolated from the leaf spots of beet, an important characteristic in *Cercospora* taxonomy. Production of the phytotoxin by *C. beticola* and related species has been a useful taxonomic tool in a broad sense, but is too variable in its expression in culture between strains of a species, as well as across culture media formulations, to be relied on for fine taxonomic separation (Goodwin *et al*, 2001).

2.2. Morphological structure and cultural characteristics of *C. beticola*

Different authors (Groenewald *et al* 2013; Poornima and Hedge 2014) have attempted to characterize *C. beticola* through morphological structure and cultural characteristics. The morphological characters that were used include conidial shape and size; presence or absence of mycelium and conidiophore morphology, but unfortunately they are not always reliable as too much intraspecific variation has been found. Pal and Mukhopadhyay, (1984), proved that A small population of *C. beticola* coming from the same lesion can exhibit differences in morphology, cultural characteristics and pathogenicity indeed showing that Physiological and morphological intraspecific differences are well known in *C. beticola* strains with different geographical origin. The reproductive structure can range from 6-10 times in dimension depending on environmental conditions. Groenewald *et al*, (2007) selected reference strains from sugar beet for morphological and cultural characterization. The strains were plated onto 2%Malt extract agar (MEA), Oatmeal agar (OA), V8 agar and Potato dextrose agar (PDA). Colonies on MEA and PDA were grey with patches of dirty white, margins smooth but irregular, however they were reported to be brown to dark brown on V8 agar (Groenewald *et al*, 2013). On OA the colonies were olivaceous grey with smooth but irregular margins. Mycelium was well developed, branched, septate, slender intracellular and brown colored. Only asexual producing bodies were present (conidia). Conidiophores were septate, dark colored structures coming out in turfts of stomata. Conidia developed on geniculate structures. On liberation from conidiophore, each conidium leaves a small scar at the place of attachment. Conidiophores were 22-103µm long X 3-5µm broad. Conidia were hyaline or pale yellow, obclavate, 38- 225µm long X 3-6µm broad. They have 4-14 septa (Moretti *et al* 2004; Crous *et al*, 2006 and Groenewald *et al*, 2013).

2.3 Genome structure/ genes employed in molecular identification of *C. beticola*

The internal transcribed spacer (ITS) region of fungal ribosomal DNA was chosen as marker for identification of fungal species (Lartey *et al*, 2003; Martin and Rygiewicz, 2005). Most approaches such as DGGE (denaturing gradient gel electrophoresis), tRFLP (terminal restriction fragment length polymorphism) or ARISA (automated ribosomal intergenic spacer analysis) employ PCR-based fingerprinting techniques to demonstrate microbial diversity or population shifts in microbial communities (Goodwin *et al*, 2001).

Analysis of ITS Sequences the consensus primers ITS1 5'TCCGTTAGGTGAACCTGCGG 3' and ITS4 5'TCCTCCGCTTATTGAT ATGC 3' (Weiland and Sundsbak, 2000) which are used to amplify a region of the rRNA gene repeat unit, which includes two non-coding regions designated as ITS1 and ITS2 and the 5.8s rRNA gene of the nrDNA operon. Amplification products and gene sequences yields an amplicon corresponding to *C. beticola* approximately 550bp (Crous *et al*., 1999; Crous *et al*, 2001; Crous *et al*, 2004, Crous *et al* 2006 and Hunter *et al*, 2006). However other authors resorted to using the actin specific primers CBACTIN959L and CBACTIN959R (Lartey *et al*, 2003 and Balau and Fraretra, 2010).

The 20 µ1 PCR reaction consisted of 10 µl Extract-N-Amp PCR mix (a 2X PCR reaction mix containing buffer, salts, dNTPs. Taq polymerase and TaqStart antibody),4 µl sample extraction solution and 1.5 µl each of the forward and reverse primers in deionized water. Other controls were a manufacturer provided control and a blank reaction, consisting of extraction solution without plant or fungal extract. Amplification was carried out over 35 cycles using a Mastercycler gradient thermocycler (Eppendorf Scientific Inc. Westbury, NY) at 94°C for I min denaturation. 52°C for 30 sec annealing and 72°C for 1 mm extension

(Lartey *et al*, 2001). The PCR amplified products were resolved by electrophoresis in 1% agarose gels in Loening E buffer. The PCR product sizes were determined by comparing the relative mobility of the amplified fragments to the 1 KB ladder (New England Biolabs Inc., Beverly, MA) in adjacent lanes. At 28 cycles, the primer set ITS3/ ITS4 showed that it can detect as little as 100 pg of *C. beticola* genomic DNA. The result shows that the primer set ITS3/ ITS4 has the highest level of detection. It can detect even the minimum dilution ($1:10^6$), while the primer set Cb-actinF1/ CbactinR2 can detect until the fifth dilution only ($1:10^3$) Moreover, it amplified one fragment with a small size, 223-bp, and therefore, was ensuring its suitability for quantitative detection by real-time PCR. Amplicons derived from PCR using the primer set ITS3/ ITS4 were sequenced and compared to DNA sequence from pure culture *C. beticola*. Alignment of sequences of the amplified products confirmed them to be those of *C. beticola* (Crous *et al*, 2006 and Bakhshi *et al*, 2011).

2.4 Genetic variability and genetic assessment using pairwise distance

Recent studies have attempted to determine population genetic structure of *C. beticola* and thus a substantial amount of genetic variation was found within Cercospora strains isolated from sugar beet fields (Morretti *et al* 2006; Vaghefi *et al* 2017). Genetic variation was also observed in lesions of the same plant (Moretti *et al*, 2004). This is in contrast to the data available for other Cercospora species which have low levels of genetic diversity (Wang *et al*, 1998; Okori *et al*, 2004; Groenewald *et al*, 2006) with little genetic differentiation either within or between populations. Vaghefi *et al*, (2017) used pairwise index of population differentiation between cercospora beticola populations collected from New York and Hawaii on swiss chard, sugar beet and lambsquaters. Total diversity existed within and among populations ($P \leq 0.001$).

2.5 Mycotoxin Production

One of the main features of the genus *Cercospora* is the production of cercosporin (Assante *et al*, 1977), a photosensitive phytotoxin with a perylenequinone skeleton (Lousberg *et al*, 1971), which has been demonstrated to be involved in the pathogenic process (Daub and Ehrenshaft, 2000). Other secondary metabolites produced by *C. beticola* are beticolins and a group of molecules as *Cercospora beticola* Toxins (CBT), whose contribution to the pathogenic process is less understood. Since the production of these phytotoxins can vary widely among strains grown under the same conditions, the metabolic profile can be used as a further tool to test variability (Assante *et al*, 1981). *Cercospora* species, including *C. beticola*, are considered to be necrotrophs, producing low molecular weight phytotoxins and hydrolytic enzymes that debilitate cells in advance of fungal growth. The mode of action of the photoactivated, nonspecific toxin cercosporin in the generation of singlet oxygen has been known for over two decades (Daub and Ehrenshaft, 2000). Indeed, even the molecular basis for the protection of *Cercospora* and other fungi from the effects of cercosporin are being revealed. Thus, production of both an ABC transporter-like protein and elevated levels of pyrridoxal are important in the protection of *Cercospora* from the toxic effects of cercosporin (Daub and Ehrenshaft, 2000). Knowledge of these protectants is being used in strategies to generate transgenic plants with reduced phytotoxin sensitivity with a goal of reducing or preventing leaf spot disease. In addition to cercosporin, other classes of phytotoxins such as the beticolins from *C. beticola* have been shown to debilitate normal plant cell function (Goudet *et al*, 1998). Beticolins are a family of polycyclic molecules sharing a common core structure but differing in the functional groups on the aromatic rings (Goudet *et al*, 2000). Like cercosporin, the beticolins are known to destabilize membranes resulting in electrolyte leakage (Gapillout *et al*, 1996). Additionally, the beticolins inhibit ATP dependent proton

transport and chelate magnesium (Mikès *et al*, 1994). To date, the evidence suggests that phytotoxins produced by *Cercospora* species are virulence factors.

Many phytopathogenic fungi are equipped with various arsenals such as secretion of cell-wall-degradation enzymes and formation of phytotoxins in order to invade their hosts (Schafer, 1994). Host-selective toxins kill plant cells by targeting a specific cellular enzyme or component, and thus are toxic only to a limited range of host cultivars (Walton, 1996). In contrast, non-host-selective toxins, targeting various cellular components, enable the producing pathogens to have wide host ranges. When cercosporin absorbs light energy it is converted to an excited triplet state. In this state it can react with molecular oxygen, producing oxygen radical superoxide, hydrogen peroxide and hydroxyl radical and non-radical like singlet oxygen (Spikes, 1989). Reactive oxygen species (ROS) production induced by cercosporin in the infected plants results in lipid peroxidation and membrane rupture (Daub, 1982), leading to leakage of nutrients from cells and thus allowing fungal growth (Daub and Ehrenshaft, 2000). Although both growth morphology in culture and virulence have been used as characters for the grouping of *C. beticola* isolates, use of this information in the description of unique field strains has not gained acceptance (Duffus and Ruppel, 1993).

2.6 Epidemiology

Agrios, (2005) defines plant disease epidemiology as the study of diseased plant populations. Much like disease of humans and animals, plant diseases occur due to pathogens such as bacteria, viruses, fungi, and protozoa. Epidemiologists attempt to understand the cause and effects of disease and development strategies to intervene in situation where crop losses may

occur and for establishing whether or not there is an excessive amount of a particular disease occurring in a specific geographic area (Madden *et al*, 2007).

Epidemiological data has been used to predict incidence, prevalence and survivorship of the pathogen over time. Data obtained from past occurrences of the pathogen has been used with some success in the containment and control of *C. beticola* in several countries and situations (Gado, 2007: Kaiser and Varrelmann, 2009).Shane and Teng. (1983) developed a scale that measures disease severity by counting the number of spots. Scale ranged from 0-10 categories where: O; no visual infection; 1-5 spots/leaf, 0.1% severity; 6-12 spots ,0.35 % severity; 13-25 spots/leaf, 0.75% severity; 26-50 spots/leaf, 1.5% severity; 51-75 spots/leaf , 2.5 % severity. At higher disease incidences, the average affected area per leaf was estimated from standard area diagrams, and categories 6 through 10 represented 3, 6, 12, 25, and 50% disease severity, respectively. Spatial disease patterns have been studied over time in order to improve understanding of CLS epidemiology and ecology (Lannou and Savary, 1991; Munkvold, *et al*, 1993; Larkin *et al*, 1995). This involved studying disease incidence (Number of diseased plants) and disease severity (using maps of disease severity developed by different authors: Jacobsen *et al*, 2004; Larson, 2004; Wolf and Verreet, 2005).

Rossi (2000) included incubation length, infection efficiency and lesion size on their epidemiological studies of *Cercospora* leaf spot. Resistance delayed the appearance of Cercospora leaf spot by a maximum of 12 days compared to susceptible cultivars. This concurs with Parlevliet, (1979) that in epidemiological terms, resistance reduces the apparent infection rate as an effect of changes in the basic infection rate, due to a reduced effectiveness of each propagule in causing a new infection and a less abundant number of propagules produced per lesion per unit time (due to both reduced sporulation capability and reduced

lesion size), in the latent period (that is the time between the arrival of a propagule on a susceptible plant surface and the formation of the next generation of propagules) and in the infectious period (that is the period of spore production on a lesion). Therefore, the components of resistance that reduce the rate of epidemic development are: infection frequency, latency period, lesion size, spore production, and infectious period.

2.7 Etiology of the pathogen

Cercospora leaf spot caused by *Cercospora beticola* is considered to be the most injurious foliar pathogen of Swiss chard and beetroot in the world (Kerr and Weiss, 1990; Avila *et al*, 2005) including Botswana reducing quality, quantity and marketability of swiss chard (Khare and Moeng, 2015). Before the beginning of the 20th century, investigators gave various names to the fungus causing leaf spot diseases of sugar beet, most of which by description were almost certainly *C. beticola* (Crous and Braun, 2003; Groenewald *et al*, 2008) *Cercopora beticola* infects different hosts like Swiss chard, tobacco, soybean, coffee, rice, corn, and peanut. It occurs wherever these crops are grown. *C. beticola* produces conidiophores growing from the stomata. The conidiophores are simple, unbranched, pale brown near the base and hyaline near the apex sparingly septate and mildly geniculate with small conidial scars at the geniculations and the apex (Ershad, 2000). Conidia (usually 2 µm - 3 µm ×36 µm -107µm) are borne singly, straight to titium shaped and hyaline and have 3 to 14 septa in culture (Kim and Shin, 1998; Soylu *et al*, 2003). However conidial morphology varies considerably according to environmental conditions and sizes of 2 µm -4 µm × 50 µm -400µm and up to 27 septa have been reported by various researchers. The fungus can be grown in vitro on beet leaf juice agar. It has no known perfect state (Daub and Ehrenshaft, 2000; Ershad, 2002).

2.8 Symptomology

Characteristic symptoms of *C. beticola* include individual spots which are almost circular, measuring 3-5mm in diameter at maturity (Khan *et al*, 2008). The lesions are tan to light brown with dark brown to reddish purple boarders, the color depending on the anthocyanin production by the host (Jacobsen and Franc, 2009). As the disease progresses, individual spots coalesce and heavily infected tissue becomes first yellow then brown and necrotic. Conidia are often visible as darkly pigmented black dots or structures called pseudostromata scattered in the necrotic centers of the spots (Weiland and Koch, 2004; Khan *et al*, 2009). During periods of high relative humidity or heavy dew, the necrotic spots become grey and furry due to the accumulation of conidiophores and conidia on the stromata (Lapaire and Dunkle, 2003). Blighted leaves soon collapse and fall to the ground but remain attached to the crown. The heart leaves remain green and are less severely affected or lesion free. Lesions similar to the leaf spot also appear in the petioles but are very long and elliptic rather than circular. Sunken circular lesions have also been reported in sugar beet crown not covered by soil (Wolf and Verreet, 2005).

2.9 Disease Cycle

In most beet growing areas, the leaves are cut and left in the field. Before the next crop is grown the arable layer is tilled and infected leaf material is buried into this layer. Natural inoculum of *C. beticola* in a sugar beet field begins as stroma in infected leaf debris. It is postulated that sporulation may occur directly from overwintered stroma in organic matter or it may be preceded by saprophytic, vegetative growth of fungal mycelia. Pseudostromata of CLS surviving in the soil are capable of infecting beet plants in later years. Vereijssen *et al* (2005) reported that the first infections may occur from the soil or through the roots. Under conditions of high relative humidity, conidial germ tube grows towards stomata apparently in

response to tropistic attraction and forms numerous appressoria (Beckman and Payne, 1982). Following penetration of the epidermis through the stomata by the conidia of this pathogen (Berger *et al,* 1997), fungal hyphae ramify the parenchymous tissue of these structures, growing intercellularly (de Nazareno *et al,* 1993a; Khan *et al,* 2009). Toxins then are produced in order to necrotize the cells in the vicinity of the branched hyphae. The pathogen absorbs nutrients from the host necrotized tissue primarily on the abaxial leaf surface because it possess a greater number of stomates than adaxial surfaces, the opportunity for invasion of leaf parenchyma through open stomates by an elongating hypha of *C. beticola* is maximized (conidiophore and conidial development). Conidia deposited on host leaf, vein or petiole surfaces germinate under conditions of high humidity and leaf wetness and grow toward stomates during the pre-infection stage (Shane and Teng, 1983; de Nazareno *et al,* 1992). Conidia again are dispersed by wind and rain splash to other leaves and plants thus initiate new cycles of infections(Rossi and Battilani, 1989) which take place on higher younger leaves both by autoinfection (re infection of the same plant) and alloinfection where inoculum spreads to adjacent plants (Vereijssen *et al*, 2005). The fungus survives on infected crop residues as spores (conidia) and stromata, thus becoming an important source of inoculum for subsequent crops (Figure 3). Under humid condition, new conidiophores and conidia are formed on stromata and are carried by wind or splashing rain to host leaves where germination occurs. The cycle from penetration to first symptoms takes a few days (Vaghefi *et al* 2017) Penetration is only through leaf stomata with or without the formation of appressoria.

Conidia and stromata can also be carried on seed produced in regions where the disease is severe (Racca and Jorg, 2007). Weed host may also be a source of inoculum (Racca and Jorg, 2007).Once conidiation has commenced, water-splash, wind and insects are culprits in

distributing spores on to leaf surfaces of the host (Berger *et al*, 1997). Lapaire and Dunkle, 2003) detest the use of zero tillage or conservation tillage as it favors the survival of the pathogen because it overwinters on crop debris. They however recommend that full tillage be practiced, as the fungus becomes exposed to soil microorganisms that outcompete it.

The fungus is polycyclic within a beet growing season as depicted in figure 3. One cycle of sporulation typically takes 12 days depending on field conditions (Rossi *et al*, 2000; Weiland and Koch, 2004; Vaghefi *et al* 2017). Secondary infection occurs within 7 to 21 days of the production of conidia on primary leaf spot (Crous and Braun, 2003; Groenewald *et al*, 2006). Conidia of *C. beticola* are dispersed from their source of inoculum by wind, water splash, running water, and insects. However, wind has been considered the major component of *C. beticola* dispersal (Groenewald *et al*, 2005). Airborne conidia of *C. beticola* play an important role in primary infection, secondary infection, progressive increase in disease development, and ultimately the spread of disease epidemics in a region. Given the key role played by airborne conidia in disease spread, knowledge of temporal dispersal of *C. beticola* inoculum during the Swiss chard growing season provides useful information that could be used to improve disease management (Khan *et al*, 2009).

In a controlled environment where Swiss chard is inoculated with *C. beticola* conidia, visible lesions appear on leaves at between 9 to 12 days, and 12 days is considered to be the approximate sporulation cycle under field conditions. An interesting alternative route to the invasion of the sugar beet plant by *C. beticola* has been proposed by Vereijssen *et al,* (2004) who reported that: sugar beet seedlings whose bare roots were exposed to fungal conidia succumbed to leaf spot disease several days after transplanting.

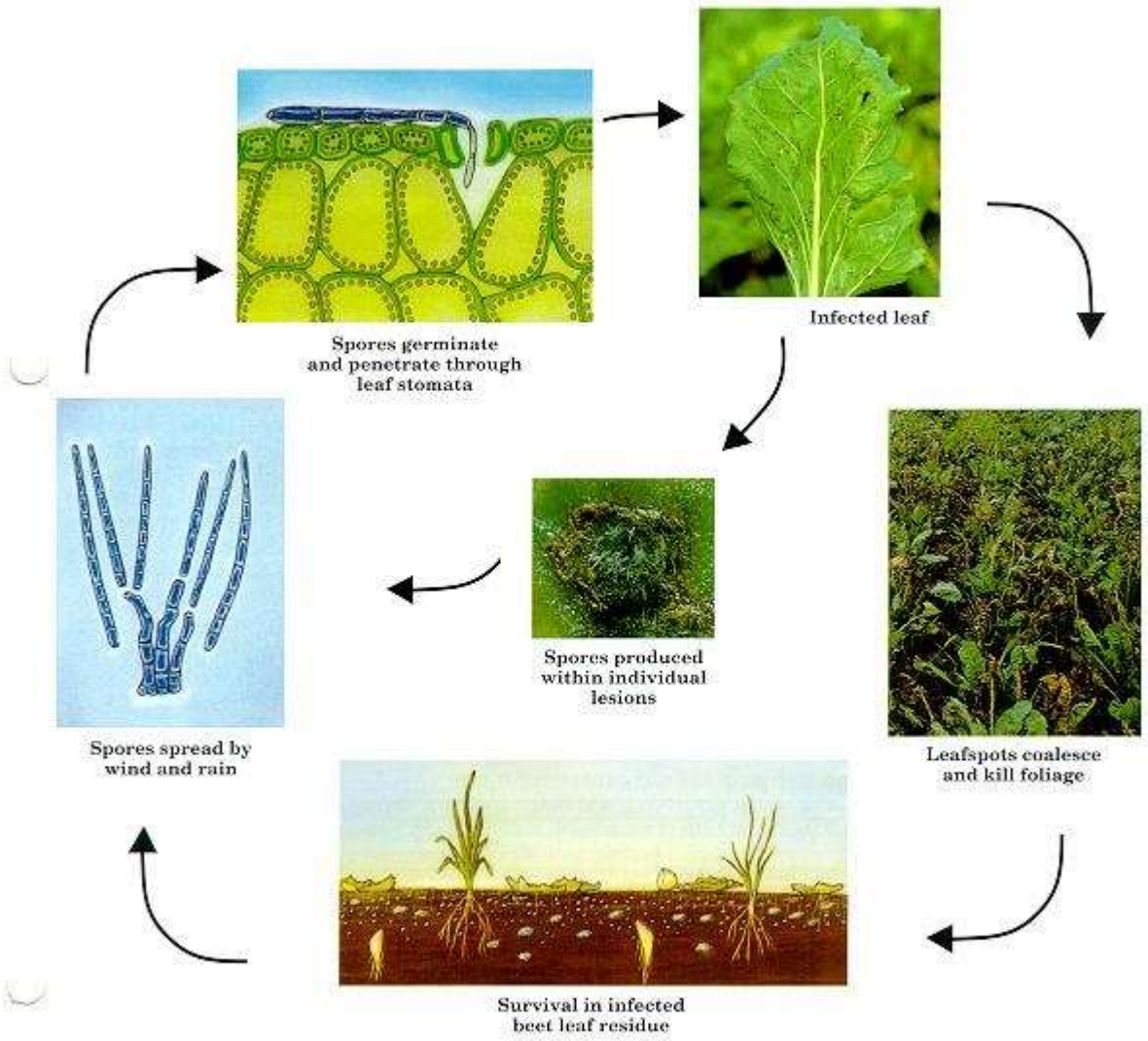

Figure 3: Shows the disease cycle of *C. beticola* (www.sbreb.org).

2.10 Collateral hosts

Besides Swiss chard, *C. beticola* produces leaf spots on most *Beta vulgaris* such as red garden beets, safflower, *Apium*, *Chrysanthemum*, *Limonium*, and *Spinacia* (Crous and Braun, 2003; Lartey *et al*, 2005; Groenewald *et al*, 2006) and thus exhibit leaf spot when inoculated

with the pathogen. Several weeds have been described by Rossi *et al*, (2000) as susceptible to *C. beticola.* This includes common lambsquarters *(Chenopodium album L.),* redroot pigweed *(Amaranthus retroflexus L.),* dwarf mallow *(Malva rotundifolia L.).* Broadleaf plantain *(Plantago major L.).* Great burdock *(Arctium lappa* L.) and lettuce *(Lactuca sativa L.)* (Rossi *et al*, 2000; Bakhshi *et al*, 2011). Although additional species have been reported as host to C. *beticola,* only a few are symptomatic (Ruppel, 1986). Indeed in recent years other common weeds such as field bindweed *(Convolvus arvensis L.),* (Windels *et al*, 1998), winged pigweed *(Cvcloloma atriplicifolium* (Spreng.) Coult.), wild buckwheat *(Polygonum convolvulus* L.) and devil's-claw *(Proboscidea lovisianica* (Mill). Thellung) (Jacobsen, 2000), other Beta hosts have been reported to be hosts and could serve as sources of inoculum. Clearly these host plants could be a serious reservoir of inoculum able to maintain the organism through long periods in the absence of Swiss chard however adequate cross inoculation tests and pathogen isolation are needed for verification of those reports (Racca and Jorg, 2007). The use of polymerase chain reaction protocols for sensitive detection of pathogens has revolutionized the assay of asymptomatic hosts for the presence and quantity of target pathogens. Such methods currently are being tested for determining alternative hosts and asymptomatic hosts of *C. beticola* (Lartey *et al*, 2003).

2.11 Fungicide control of *Cercospora beticola*

Different fungicidal chemicals are utilized worldwide for the control of *C beticola* (Ioannidis, 1994; Dexter *et al*, 1999; Dexter and Luecke, 2001; Karaoglanidis and Bardas, 2006; Nikou *et al*, 2009). The protectant fungicides used against the disease belong to the Dithiocarbamate class such as Maneb and Mancozeb, the nitrate class such as Chlorothalonil and the class of organotin compounds such as fentin acetate and fentin hydroxide. Systemic fungicides used against the disease belong to the benzimidazole class such as Benomyl and Carbendazim and

the sterol demethylation inhibiting (DMI) class such as flutriafol, difenoconazole, tetraconazole and propiconazole. However development of resistance to some of these fungicide classes in many countries worldwide has become a limitation to sustainable control of the disease (Ioannidis and Karaglanidis, 2000; Briere *et al*, 2001; Nikou *et al*, 2009).

The benzimidazole derivatives were the first systemic fungicides that became available for *C beticola* control in Greece. Application of Benomyl began in 1971 and within 2 years, fungal populations had developed resistance. This was reported in other several countries (Weiland and Halloin, 2001; Culbreath *et al*, 2002). After the emergence of Benzimidazole resistance in Greece, the use of Benomyl was discontinued. However in 1995, it was found that resistance frequency to Benomyl had greatly decreased since 1972. Since then Benomyl had been re-introduced into the spray program (Karaoglanidis and Bardas, 2006). As a result Benomyl has been used as one application early in the season in some areas in Greece (Karadimos *et al*, 2000).

Byford (1996) also mentioned a very important group of fungicides that are utilized worldwide, the Fentin fungicides. The Fentin derivatives became the only available fungicides that provided satisfactory control of *C. beticola* after the emergence of benomyl resistance. Unfortunately they too became ineffective as the pathogen developed resistance. Due to their unsatisfactory control of *C. beticola*, their use was restricted to 2-3 applications in mixture with maneb early in the season. The resistance of *C. beticola* to fentin fungicides has also been reported in the USA (Bugbee, 1995; Campbell *et al*, 1998).

Another group of fungicides that have been used for the control of *C. beticola* include the Sterol demethylation inhibiting fungicides (DMIs). These fungicides are used always in

mixture with a protectant fungicide, either maneb or chlorothalonil. After 1990, their performance declined. The reduction in performance was observed in field experiments for fungicidal evaluation on Sugar beet in Northern Greece and an extensive monitoring program showed that fungal populations had shifted towards decreased sensitivity (Karaoglanidis *et al,* 2000). A similar study was conducted in Serbia with similar results (Budakov *et al,* 2014; Trkulja *et al,* 2015).

Ioannidis *et al.* (2001) published a preliminary report on the determination of sensitivity of *C. beticola* populations to benomyl, fentin-acetate and flutriafol spray programs in four areas in Greece (Larissa, Imathia, Serres and Orestiada). These areas had a different history of fungicide use, thus isolates were collected from these areas to determine their sensitivity to fungicides. In Orestiada and Larissa, where benomyl had been used in one spray application per year since 1996- 2000 the resistance frequency was more than 50% compared with Imathia and Serres where benomyl has not been used since the detection of resistance in 1978. However resistance to fentin-acetate was significantly reduced, ranging from 2-3% in the Serres isolates and 8-11% in the Orestiada isolates. Larrisa and Orestiada isolates showed no decreased sensitivity to flutriafol but the Serres and Imathia isolates showed a decreased sensitivity which might be attributed to heavy treatment with Sterols demethylation Inhibiting fungicides (DMIs).

Most literature on fungicidal control of this disease is for sugar beet and very few studies have been carried out on Swiss chard and none in Botswana. Warm, humid growing regions are most acutely affected by Cercospora leaf spot and constitute greater than 30% of the area under sugar beet cultivation. Producers in such areas must diligently apply fungicides to varieties possessing moderate to high genetic resistance to the disease in order to bring the

crop to maturity (Windels *et al*, 1998; Meriggi *et al*, 2000; Wolf and Verreet, 2002). Without such measures, the leaf canopy of sugar beet fields can be destroyed by outbreaks of *C. beticola*, resulting in complete loss of the crop (Duffus and Ruppel, 1993; Rossi *et al*, 2000). When the disease was particularly serious, fungicides were applied routinely in affected areas as the principal method of control. Spray schedules were initiated following detection of symptoms with applications repeated at regular intervals (Gummert *et al*, 2015). Most fungicides registered for use on Cercospora leaf spot have provided satisfactory control. *Cercospora* leaf spot became a serious disease in some areas of the North Platte Valley of western Nebraska during the early 1980s. Growers adopted fungicide spray schedules similar to those used in eastern Nebraska as a preventive disease control program. Additional applications of fungicide were made assuming that the disease would increase in severity. In some years, disease did not progress as anticipated and the cost of fungicide application for leaf spot control was unnecessary. A system was needed for predicting the most efficient timing of fungicide application.

Cercospora leaf spot is managed using an integrated program that includes the use of moderate resistant varieties, crop rotation with non-host crops, balanced soil fertility, weed and insect control, and proper crop culture, as well as the proper selection, timing, and method of applying fungicides (Wolf *et al*, 1995). Economical control depends on establishing an overall disease management system for the entire farm. Fungicides in the benzimidazole and triazole class as well as organotin derivatives and strobilurins have successfully been used to control *Cercospora* leaf spot (Carr and Hinkley, 1985). Elevated levels of tolerance in populations of *C. beticola* to some of the chemicals registered for control has been documented (Pfleiderer and Schaufele, 2000). Partial genetic resistance also is used to reduce leaf spot disease. Triazoles act as demethylation inhibiting fungicides

(DMIs) and their specific mode of action makes them susceptible to the risk of resistance development. In fact, a shift towards lower levels of sensitivity in populations of *C. beticola* in northern Greece areas has been recently observed, even though no significant lowering effects have been registered on bitertanol and flutriafol performance (Karaoglanidis *et al*, 2000).

Swiss chard variants resistant to *C. beticola* can still exhibit leaf spot if climatic conditions are favorable for the disease development. They exhibit reduced lesion size, reduced lesion number on infected leaves as well as reduced conidial production and are able to slow down disease progression (Rossi *et al*, 2000: Weiland and Koch, 2004). It is assumed that the generation of resistant varieties currently grown might hold the potential to reduce fungicide applications (Ossenkop *et al,* 2002; Kaiser *et al*, 2010) however this has not been scientifically proven so far (Gummert *et al*, 2015). For this reason, the timely application of fungicides in conjunction with forecasting models that predict the likelihood of *Cercospora* infection has become an important complement to genetic resistance in leaf spot control (Windels *et al*, 1998; Weiland *et al,* 2001; Wolf and Verreet, 2002). Compounding this issue is the well-documented occurrence of fungicide tolerance in *C. beticola* populations (Ioannidis and Karaoglanidis, 2000). As a consequence, the control of leaf spot disease necessitates the judicious rotation of fungicide chemistries as a means of preventing or forestalling the development or reducing their prevalence in the populations.

The current study will ensure that Botswana is also reaching world standards of proper identification of plant pathogenic microorganisms, in this case *C. beticola* through molecular methods (PCR). It will also enable farmers to manage CLS through the monitoring of temperature, humidity, inoculum concentration and fungicide use.

3.0. MATERIALS AND METHOD

3.1. Molecular identification and Analysis of genetic diversity of *Cercospora beticola* isolates from Swiss chard in Southern Botswana

3.1.1. Sample collection

Swiss chard leaf samples with infected CLS were collected and placed in sterile plastic bags (Lab-Loc® Specimen) from a farm in Glen Valley (24° 35' 55" S | 25° 57' 46" E) and a farm in Bokaa (24° 42’ 79’’ S | 26° 02’ 37’’ E). On the 16th of January 2017, forty diseased leaf samples each collected from a single plant were collected from each farm, transported to the Microbiology Laboratory (University of Botswana) in a cooler box containing ice packs and processed within 4h of collection.

3.1.2. Isolation and morphological identification of *Cercospora beticola*

Two discs (5mm x 5mm) containing lesions were excised from each Swiss chard leaf using a sterile rounded scalpel with marked measurements (5mm). The discs were surface sterilized using Sodium Hypochlorite for 15 minutes, rinsed three times using distilled water and mop dried between sterile paper towels. The discs were then aseptically plated on the surface of 2% Malt extract agar (Merck, Darmstadt, Germany), with the upper leaf surface touching the agar and incubated at 25°C for 5-7days in the light to induce sporulation. Each plate was labelled appropriately by location Gv for Glen valley and Bk for Bokaa. Both locations had plates numbered 1-80 (a leaf was plated onto two plates). The cultures were examined for typical *Cercospora beticola* morphological characteristics under a light compound microscope.

A dissecting microscope was used to locate individual spores growing on the agar and a marker used to encircle their location. The individual spore was picked together with the

piece of agar and transferred to fresh malt extract agar and then incubated at 25°C for 5-7 days. A total of 87 isolates where obtained.

3.1.3. DNA extraction and PCR amplification

Genomic DNA extraction from fungal mycelia of each isolate was done using the MasterPure yeast DNA purification kit (Epicetre Biotechnologies, Madison, WI, USA) according to instructions from the manufacturer. Colonies were harvested from solid media by scrapping mycelia with a scalpel and transferred into a micro-centrifuge tube. The tissue was rinsed with 1ml of 0.1M $MgCl_2$. The solution was discarded and sample centrifuged 2min at 5000rpm to remove any remaining wash solution. 300µl of yeast cell lysis solution was added to the sample tissue. Tissue was vortexed for 15 seconds and placed in a water bath for 1hour at 65°C. The tubes where spun down and placed on ice for 5min then spun down again. Protein precipitation reagent, MPC was added (150µl) and vortexed for 10 seconds. Cellular debris was pelleted by centrifugation for 10 minutes at 10 000rpm. The supernatant was transferred to a clean micro-centrifuge tube and 500µl isopropanol was added. The mixture was inverted 10 times to mix. DNA was pelleted by centrifugation at 10 000rpm for 10 minutes. The supernatant was removed by pipetting and discarding. When removing the supernatant care was taken not to disturb the pellet. The pellet containing DNA was washed with 300µl of 75% ethanol and centrifuged for 2 minutes to keep pellet on the bottom. This was repeated and the tubes were allowed to air dry for 20 minutes. DNA was suspended in 50µl of nuclease free distilled water and heated at 65°C for 2 minutes to dissolve, allowed to rest for 30minutes, briefly spun down and stored at -20°C. Afterwards the DNA (4µl) + 3µl of loading dye was run on agarose gel (1.2%) for 30 minutes at 80V.

PCR amplification utilized ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') primers (White *et al*, 1990) in a Techne thermocycler (Cole-Palmer, Staffordshire, UK). Each PCR reaction mixture consisted of 12.5µl of 2X Master Mix (New England Biolabs, Ipswich, MA, USA), 1µl each of reverse and forward primers, 2µl of genomic DNA and the mixture made up to 25µl with sterile nuclease-free water.

The PCR was run with the following thermocycling conditions: initial denaturation at 94°C for 8 min; followed by 35 cycles for 1 min at 94°C, 55°C for 1 min and 2 min at 72°C and final extension at 72°C for 12min. The PCR products were resolved on 1% agarose gel (Sigma Aldrich, Missouri, USA) for 1 hour at 80 volts. 5µl of molecular weight marker (New England Biolabs) was loaded alongside 4µl of PCR products. The gels were then visualized on a gel documentation system (Bio-Rad, California, USA).). The PCR products were cleaned with a Clean-Up Kit (Zymo Research, Irvine, CA, USA) following instructions from the manufacturer. After purification, the products were sequenced in both directions using the Big Dye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) in an automated ABI 3500XL sequencer (Applied Biosystems), following instructions from the manufacturer.

3.1.4. Phylogenetic analyses

Alignment of sequences was done using consistency-based algorithms implemented in MAFFT version 7.307 (Katoh and Standley, 2013) and assembly and editing of sequence data was performed using (BioEdit. [http://www.mbio.ncsu.edu/BioEdit/bioedit.html]). The phylogenetic tree of ITS rDNA was constructed using MEGA (Molecular Evolutionary Genetics Analysis program) version 6.06 (Tamura *et al*., 2013). The tree was inferred using

the Neighbour-Joining method based on the Kimura-2 model. Bootstrap analysis involved 1000 replicates for the tree. Furthermore, the genetic congruence among *C. beticola* isolates were analysed using pairwise distances based on the Jukes-Cantor model (Jukes and Cantor, 1969) implemented in MEGA6.

3.2. The effect of humidity, temperature and inoculums density on plant disease development under a controlled environment

3.2.1. Experimental location

A laboratory experiment was carried out at the Department of Agricultural Research, Sebele, Gaborone, plant pathology laboratory (Latitude 24 34'S, Longitude 25 57'E, Altitude 994 above sea level) from January 2017 to June 2017 in order to investigate the effects of varying humidity, temperature and inoculum density on the development of Cercospora leaf spot on Swiss chard under a controlled environment. A modified egg incubator was used as a growth chamber (Figure 4). The chamber was fitted with low intensity fluorescence lights (1100 lumens) with twelve-hour day period. Experimental trials on humidity and temperature were run in sequence for 28 days each.

Figure 4: The incubator used to harbour the experimental plants

3.2.2. Seedling growth and cultural practices

Seedlings of Fordhook giant, a variety of Swiss chard were raised in a greenhouse in two seedling trays each with a capacity of 200 plants per tray. The trays were watered twice daily using a watering can with a nozzle. The seeds emerged three to four days after sowing. Two weeks after emergence the plants were given Starke Ayres plant food 3:1:6(46). Ten gram was dissolved in 5 litres of water and each tray was watered with 500ml of the solution. The seedlings were transplanted four weeks after emergence into small flower pots (8cm). Petri plates were used as bases for the cups.

3.2.3. Isolation and identification of *C. beticola*

Swiss chad leaves (6kg) with circular brown spots containing ashen grey centres were collected from the same Glen Valley farm as above, (placed in a cooler box and transported to the laboratory where they were washed using distilled water to remove foreign debris such as soil particles and rinsed twice in 5litres of distilled water. Microscopic observation of the specimen was done to confirm the presence of the pathogen (pale brown sparingly septate conidiophores, hyaline multiseptate conidia). The leaves were then placed in a polythene plastic bag with holes to allow for air circulation and placed in a 25°C incubator for 3days. They were monitored for the development of conidiophores and spores. After three days, *C. beticola* conidia and conidiophore were checked on the leaf surface by scraping the leaf surface and viewing under a microscope. On confirmation, the leaves were placed in a clean polythene bag and conidia collected by shaking the bag with 100ml of distilled water to obtain a spore suspension. Serial dilutions were prepared up to 10^5 and the concentration of the conidia was determined using a hemocytometer per ml.

3.2.4. Determination of spore concentration using Hemocytometer readings

A hemocytometer was cleaned and dried with non linting tissue. The cover slip of the hemocytometer was centred and 10µl of spore suspension was drawn using a micropipette and pipetted onto one of the counting chambers. The sample was mixed before drawing of the sample. The chambers were filled slowly and steadily and counting proceeded. Spores were counted in each of the four $0.1mm^3$ corner squares labelled A through D. However, spores touching the boarders where not counted. The total spores counted in the four corner squares was computed using the equation;

Spores/ml= (n) x 10^{-4}, where n= the average cell count per square of the four corner squares counted. Spore concentrations of $0.1x10^4$, $1.2x10^4$, $2.3x10^5$ and $2.3x10^6$ spores/mL were used respectively.

3.2.5. Seedling inoculation

The plants were properly labelled and placed in a dark incubator at 95% relative humidity for 48 hours before being inoculated. The spore suspension was poured into a 500ml trigger hand spray bottle and sprayed onto the plants, both on the upper leaf surface and the underside and covered with aluminium foil for 12 hours in order to avoid cross contamination. Each plant was sprayed with 3ml of the spore suspension.

3.2.6. Effect of relative humidity and inoculum concentration on disease development over a 28-day period

3.2.6.1. Experimental design

A 3 x 5 x 4 factorial experiment with ten replicate plants per treatment arranged in a completely randomised design (CRD) was used with %RH (50, 70 and 90) as main plot (Factor A), Inoculum concentration ($0x10^4$ (Control), $0.1x10^4$, $1.2x10^4$, $2.3x10^5$ and $2.3x10^6$

spores/mL) as subplot (Factor B) and sampling time (7, 14, 21 and 28 days) as sub-sub plot (Factor C) and ten replications. There were a total of 15 treatments. Ten replicate plants inoculated with each of the spore concentrations and distilled water (control) were placed in the incubator at 25°C, with low intensity fluorescence lights (1100 lumens) with twelve-hour day period and RH set at 50% for 28 days. This was sequentially repeated with RH in the incubator set at 70 and 90%, respectively.

3.2.6.2. Data collection and analysis

Numbers of leaf spots per plant were recorded at seven day interval up to 28 days. Spot numbers were counted using a colony counter when necessary. The data were subjected to factorial analysis of variance (ANOVA) and if the f-values were significant ($p \leq 0.05$), the means were separated using least significance difference (LSD) test ($p \leq 0.05$) and, MSTAC statistical package (Michigan State University) was used.

3.2.7. Effect of temperature and inoculum concentration on disease development over a 28-day period

3.2.7.1. Experimental design

A 4 x 5 x 4 factorial experiment with ten replicate plants per treatment arranged in a completely randomised design (CRD) was used with temperature (15, 20, 25 and 30°C) as main plot (Factor A), Inoculum concentration ($0x10^4$ (Control), $0.1x10^4$, $1.2x10^4$, $2.3x10^5$ and $2.3x10^6$ spores/mL) as subplot (Factor B) and sampling time (7, 14, 21 and 28 days) as sub-sub plot (Factor C) and ten replications. There were a total of 20 treatments. Ten replicate plants inoculated with each of the spore concentrations and distilled water (control) were placed in the incubator at 90% RH with low intensity fluorescence lights (1100 lumens) with

twelve-hour day period and temperatures set at 15°C for 28 days. This was sequentially repeated with temperatures in the incubator set at 20, 25 and 30°C, respectively.

3.2.7.2. Data collection and analysis

Numbers of leaf spots per plant were recorded at seven day interval up to 28 days. Spot numbers were counted using a colony counter when necessary. The data were subjected to factorial analysis of variance (ANOVA) and if the f-values were significant ($p \leq 0.05$), the means were separated using least significance difference (LSD) test ($p \leq 0.05$) and, MSTAC statistical package (Michigan State University) was used.

3.3. Assessing the effectiveness of locally available fungicides in managing Cercospora leaf spot of Swiss chard under field conditions

3.3.1. Experimental location

A field experiment was carried out at the Botswana University of Agriculture and Natural Resources, Sebele, Gaborone (Latitude 24 34'S, Longitude 25 57'E, Altitude 994 above sea level) from August 2013 to April 2014 in order to assess the effectiveness of three selected locally available fungicides, namely Benomyl, Mancozeb and Copper oxychloride.

3.3.2. Land preparation and cultural practices

A field where Swiss chard was previously grown was ploughed by a tractor and partitioned into 24 plots with an area of 10 m^2 (5mx2m) each and a spacing of 1m between the plots. The plots were split into 3 blocks each with eight plots. Seedlings were raised in a net shade in three seedling trays, each with a capacity of 200 plants per tray. The trays were watered twice daily using a watering can with a nozzle. Two weeks after emergence (WAE) the plants were

given Starke Ayres plant food 3:1:6(46). One gram was dissolved in 1litre of water and each tray was watered with 1litre of the solution.

The seedlings were transplanted into the field 6 weeks after emergence at a spacing of 40cm between the rows and 30cm between the plants. Each plot had a population of 45 plants. The plots were weeded as the weeds emerged. Watering was carried out twice a day in the morning and afternoon during the 1st month after transplanting; thereafter watering was done once in the afternoon. Lime ammonium nitrate (LAN) fertilizer was added at a rate of 140g per plot at 21 day intervals.

3.3.3. Treatments and experimental design

Control of Cercospora leaf spot of Swiss chard using fungicides commenced once the first symptoms were observed within the plots. The treatments were as follows: 1. No fungicide application (Control); 2. copper oxychloride; 3. benomyl alternating with mancozeb; 4. mancozeb alternating with copper oxychloride; 5. benomyl; 6. benomyl alternating with copper oxychloride; 7. mancozeb; 8. benomyl alternating with mancozeb and copper oxychloride. Fungicides were applied at 14 day intervals using a 5-litre Knapsack sprayer. A plastic cage was made around each plot during spraying in order to avoid drift of the fungicide to other plots using plastic and wooden stakes. Benomyl was applied at a rate of 50g/100l of water, copper oxychloride at 40g/ 100l of water and mancozeb at 200g/100l of water as per manufacturer's instructions.

The experimental design was a randomized complete block design (RCBD) with fungicide treatments as the main plot (Factor A) and sampling period as a subplot (Factor B).

3.3.4. Data collection

Data collected were the average number of leaf spots per leaf. For each plot a total of 21 experimental plants were numbered for use after excluding the border plants. At each sampling time ten plants were randomly chosen by placing numbers 1 to 21 in a bag and picking 10 numbers for sampling. The first sample was collected 2 weeks after the first fungicide spray and subsequently every 2 weeks just before fungicidal application for the weeks that followed. One leaf with most spots was taken per plant. Spot count was done under a magnifier using an inoculating needle. Temperature and rainfall data for the duration of the study were collected from the Department of Agricultural Research Meteorological Station.

3.3.5. Statistical analyses

Data were subjected to one-way analysis of variance (ANOVA-1) and if the f-value was significant ($p \leq 0.05$), means were separated using Least Significant Difference (LSD) test. The MSTATC statistical package (Michigan State University) was used to analyze the data.

4.0. RESULTS

4.1. Molecular identification and Analysis of genetic diversity of *Cercospora beticola* isolates from Swiss chard in Southern Botswana

Cultural and morphological characteristics of isolates from infected Swiss chard leaves

A total of 87 isolates of *C. beticola* were isolated from the Swiss chard samples. Only 48.2% and 51.7% of the cultured leaf samples from Glen Valley and Bokaa yielded *C. beticola* isolates. Isolates were tentatively identified as *C. beticola* using morphological characteristics under the microscopic as described by Groenewald *et al*, (2013). Colonies on MEA were grey with patches of dirty white (Figure 5). Conidia (20-200µm x 2.5-4µm) were smooth, straight walled to slightly curved and gradually attenuated from the truncate base with 3-14 septa (Figure 6). The pseudostromata formed in clusters, with conidiophores measuring 10-100µm x 3.5- 5 µm and brown becoming paler towards the apex (Figure 7).

Figure 5: Cultures of *C. beticola* from symptomatic Swiss chard leaves growing on Malt extract agar.

Figure 6: Filliform conidium of *C. beticola*, short and conically truncate at the tip viewed at 1000X.

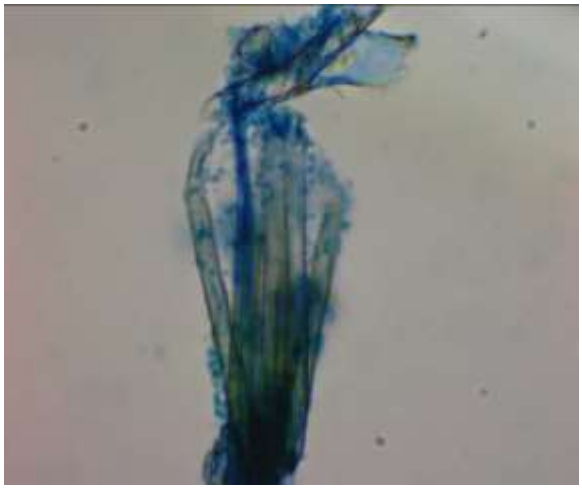

Figure 7: *Cercospora beticola* stroma viewed at 400X

Molecular identification and analysis of genetic diversity of *Cercospora beticola* isolates

The isolates were further identified by amplification of the internal transcribed spacer (ITS) region of rDNA utilizing ITS1 and ITS4 primers. All the 87 isolates that were amplified yielded bands that were between 500 and 600 base pairs (Figure 8). *Beta vulgaris* from Glen valley farms (Lanes 1-7),). *Beta vulgaris* from Bokaa farms (Lanes 8-14).

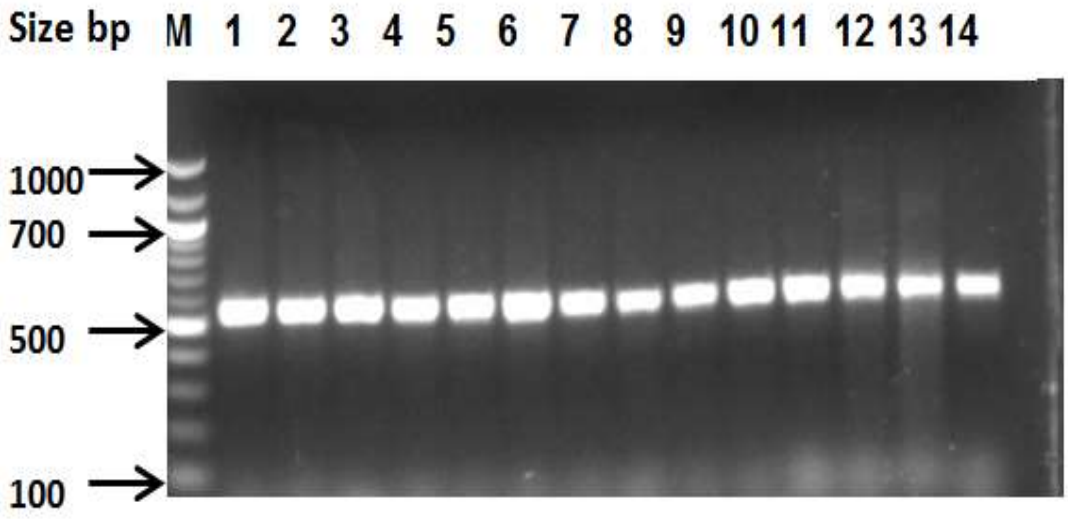

Figure 8: Gel electrophoresis of the amplified rDNA internal transcribed sequence (ITS) region of *C. beticola* isolates with ITS1 and ITS4 primers pairs. (M) 100 bp DNA ladder (New England Biolabs).

The phylogenetic tree of the 18 randomly selected isolates (9 from each farm) which were PCR sequenced (Figure 9) shows four distinguishable clusters. In cluster 2, isolate Gv24 and Bk7 dispersed from the other four isolates within the cluster and can therefore be considered a sub-population within a cluster. This phylogenetic tree was a representative of Swiss chard isolates within the four clusters.

The level of pairwise nucleotide variation between individual haplotypes of ITS gene were determined to be 0.000 to 0.004 (Table 2), indicating high genetic diversity among *C. beticola* isolates. Comparatively *B. vulgaris* isolates showed low pairwise distances amongst themselves with 0.004 being the highest recorded among and between isolates from both farms.

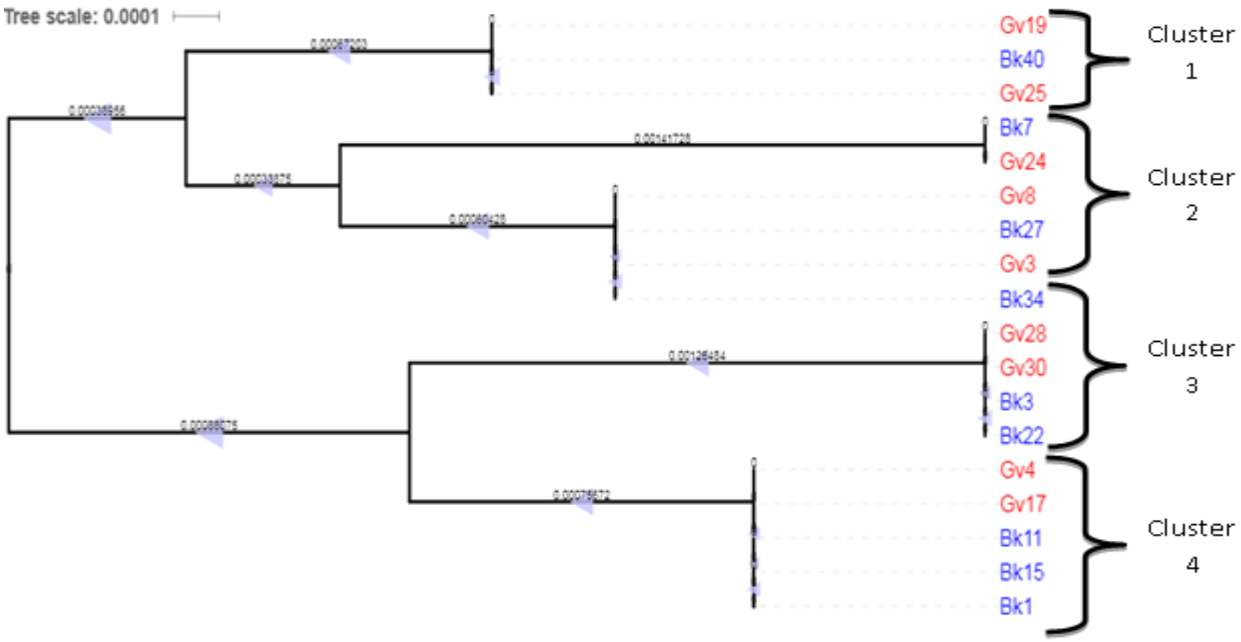

Figure 9: Evolutionary relationships among *C. beticola* isolates of Swiss chard from Bokaa (denoted in blue) and Glen valley (in red)

Table 2: Pairwise distances among *Cercospora. beticola* isolates of Swiss chard based on nucleotide sequences of the ITS gene

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1. Gv8																		
2. Gv17	0.004																	
3. Bk7	0.002	0.004																
4. Gv19	0.002	0.002	0.002															
5. Bk11	0.004	0.000	0.004	0.002														
6. Bk40	0.002	0.002	0.002	0.000	0.002													
7. Bk27	0.000	0.004	0.002	0.002	0.004	0.002												
8. Bk15	0.004	0.000	0.004	0.002	0.000	0.002	0.004											
9. Gv28	0.002	0.002	0.004	0.004	0.002	0.004	0.002	0.002										
10. Gv24	0.002	0.004	0.000	0.002	0.004	0.002	0.002	0.004	0.004									
11. Gv30	0.002	0.002	0.004	0.004	0.002	0.004	0.002	0.002	0.000	0.004								
12. Bk3	0.002	0.002	0.004	0.004	0.002	0.004	0.002	0.002	0.000	0.004	0.000							
13. Bk22	0.002	0.002	0.004	0.004	0.002	0.004	0.002	0.002	0.000	0.004	0.000	0.000						
14. Bk1	0.004	0.000	0.004	0.002	0.000	0.002	0.004	0.000	0.002	0.004	0.002	0.002	0.002					
15. Gv3	0.000	0.004	0.002	0.002	0.004	0.002	0.000	0.004	0.002	0.002	0.002	0.002	0.002	0.004				
16. Gv4	0.004	0.000	0.004	0.002	0.000	0.002	0.004	0.000	0.002	0.004	0.002	0.002	0.002	0.000	0.004			
17. Bk34	0.000	0.004	0.002	0.002	0.004	0.002	0.000	0.004	0.002	0.002	0.002	0.002	0.002	0.004	0.000	0.004		
18. Gv21	0.002	0.002	0.002	0.000	0.002	0.000	0.002	0.002	0.004	0.002	0.004	0.004	0.004	0.002	0.002	0.002	0.002	

4.2. The effect of humidity, temperature and inoculums density on CLS disease development under controlled environment

Averaged across inoculum concentrations, the mean number of spots per leaf on plants incubated at 50%, (0.795), 70% (1.855 and 90% (2.865) RH were significantly different ($p \leq 0.5$), and similarly number of spots/leaf increased when plants were inoculated with increasing inoculum concentration from 0 spores/mL (1.150) to $2.3x10^6$ spores/mL(4.35) (Table 3). The highest number of spots/leaf was observed on plants inoculated with $2.3x10^6$ spores/mL (2.850) and the lowest was for the control at 50% RH (0.400). For each inoculum concentration the trend was that disease increased as the RH increased and similar trend was observed for each RH where disease increased as the inoculum increased (Table 3).

Averaged across inoculum concentrations, number of spots per leaf recorded after 7 (0.64), 14 (1.747), 21 (2.34) and 28 (2.62) days significantly increased (Table 4). The highest numbers of spots/leaf were recorded on plants incubated for 28 days at 90% RH (3.96) and lowest on those incubated for 7 days at 50%RH (0.08).

Table 3: The effect of humidity and inoculum concentration on the development of Cercospora leaf spot of Swiss chard under controlled environment

% Relative Humidity	Mean number of spot/leaf on plants inoculated with various inoculum concentrations ($x10^4$ per ml)					Overall humidity means
	0	0.1	1.2	23	230	
50	0.400 k[1]	O.575k	0.775ijk	0.975hij	1.250ghi	0.795C[2]
70	1.325gh	1.525fg	1.625efg	1.850ef	2.950c	1.855B
90	1.725efg	2.050de	2.525cd	3.675b	4.350a	2.865A
LSD	0.4902					0.2192
Overall Means for inoculum concentration	1.150D[3]	1.383CD	1.642C	2.167B	2.850A	
LSD	0.2830					

[1]Means for the interaction of % Relative humidity and inoculum concentration in a column and in a row followed by the same letter are not significantly different, LSD test($P<0.05$).

[2]Overall means in a column followed by the same letter are not significantly different, LSD test ($P<0.05$).

[3]Overall means in a row followed by the same letter are not significantly different, LSD test ($P<0.05$).

Table 4: The effect of humidity on the development of Cercospora leaf spot of Swiss chard during a 28-day period under controlled environment

Sampling day after inoculation	Mean number of spots/leaf recorded on plants incubated at various %Relative humidity			Overall Means for sampling time
	50	70	90	
7	0.080g[1]	0.400g	1.460de	0.647D[2]
14	0.860f	1.780d	2.600c	1.747C
21	1.100ef	2.480c	3.440b	2.340B
28	1.140ef	2.760c	3.960a	2.620A
LSD	0.4384			0.2531

[1]Means for the interaction of sampling time and humidity in a column and in a row followed by the same letter are not significantly different, LSD test ($P \leq 0.05$).

[2]Overall means in a column followed by the same letter are not significantly different, LSD test ($P \leq 0.05$).

Averaged across concentrations, the mean number of spots/leaf on plants incubated at 90%RH were 0.000, 1.925, 2.835, and 2.485 when exposed to 15, 20, 25 and 30°C, respectively and differences were significant ($p \leq 0.05$) (Table 5). At 15°C, the disease did not develop at all concentrations while maximum number of spots/leaf were recorded on plants incubated at 25°C while at 30°C the disease slightly declined. The highest number of spots was recorded on plants inoculated with 2.3×10^6 spores/mL and incubated at 25°C. For each temperature, disease generally increased with increase in inoculum concentration and for each inoculum concentration, the disease increased with increase in temperature from 15°C up to 25°C and then declined at 30°C (Table 5).

Table 5: The effect of temperature and inoculum concentration on the development of Cercospora leaf spot of Swiss chard under controlled environment

Temperature (°C)	Mean number spots/leaf on plants inoculated with various inoculum concentrations (x10^4/mL)					Overall temperature means
	0	0.1	1.2	23	230	
15	0.000j[1]	0.000j	0.000j	0.000j	0.000j	0.000D[2]
20	1.025i	1.500gh	1.825fg	2.475de	2.800d	1.925C
25	1.725fgh	2.050ef	02.450de	3.600bc	4.350a	2.835A
30	1.375hi	1.750fgh	2.100ef	3.375c	3.925ab	2.485B
LSD	0.4317					01931
Overall inoculum concentration means[3]	1.031E	1.325D	1.594C	2.338B	2.769A	
LSD	0.4317					

[1]Means for the interaction of temperature and inoculum concentration in a column and in a row followed by the same letter are not significantly different, LSD test ($p \leq 0.05$).

[2]Overall means in a column followed by the same letter are not significantly different, LSD test ($p \leq 0.05$).

[3]Overall means in a row followed by the same letter are not significantly different, LSD test ($p \leq 0.05$).

Averaged across the inoculum concentration the mean number of spots/per plant recorded 7(0.76), 141.68), 21 2.22) and 28 (2.58) days after inoculation significantly increased with time ($p \leq 0.05$) (Table 6). The highest number of spots/leaf was recorded on plants incubated at 25°C for 28 days (3.96) followed by those at 30°C (3.46), 20°C (2.9) and 15°C (0.0)

Table 6: The effect of temperature on the development of Cercospora leaf spot on Swiss chard during a 28-day period under controlled environment

Days after inoculation	Mean number of spots/leaf on plants incubated at various temperatures (°C)				Overall sampling time means
	15	20	25	30	
7	0.000j[1]	0.600i	1.340h	1.100h	0,760D[2]
14	0.000j	1.800g	2.600ef	2.320f	1.680C
21	0.000j	2.400f	3.440bc	3.060cd	2.225B
28	0.000j	2.900de	3.960a	3.460b	2.580A
LSD	0.1931				0.3862

[1]Means for the interaction of sampling time (measured at 7 day intervals) and temperature in a column and in a row followed by the same letter are not significantly different, LSD test($P<0.05$).

[2]Overall means in a column followed by the same letter are not significantly different, LSD test ($P<0.05$).

Averaged across temperatures the mean number of spots/plant after 28 days were highest on plants inoculated with $2.3x10^6$ spores/mL (3.2) and lowest on control plants (0.15) (Table 7) In general for each inoculum concentration the number of spots increased with time.

Table 7: The effect of inoculum concentration on the development of Cercospora leaf spot of Swiss chard under a controlled environment

Days after inoculation	Mean number of spots/leaf on plants inoculated with various inoculum concentrations ($x10^4$ per ml)				
	0	0.1	1.2	23	230
7	0.150l[1]	0.400hi	0.700hi	1.050ig	1.500de
14	1.050ij	1.300gh	1.425fg	2.075cd	2.550bc
21	1.425hi	1.650fg	2.025ef	2.825ab	3.200a
28	1.500kl	1.950jk	2.225ij	3.400h	3.825a
LSD	0.4317				

[1]Means for the interaction of sampling time (measured at 7 day intervals) and inoculum concentration in a column and in a row followed by the same letter are not significantly different, LSD test($P<0.05$).

4.3. Assessing the effectiveness of locally available fungicides in managing Cercospora leaf spot of Swiss chard under field conditions

Meteorological data

Total monthly rainfall received during the study period (August 2013 to April 2014) ranged from 0 mm in August to 137.7 mm in December 2013 with a cumulative total of 427.7 mm (Figure 10). Rainfall distribution was good from mid-November to the end of January with rain showers almost every week creating ideal conditions for development of CLS. The season was also characterized by relatively high temperature condition with the average maximum temperatures ranging from 25.5°C in April 2014 to 32°C in November 2013 and minimums ranging from 6.2°C in September 2013 to 19.4°C in March 2014.

Fungicide application significantly reduced disease development compared to the non-treated control but among the fungicides benomyl application alone or alternated with mancozeb, and copper oxychloride was the most effective resulting in 83-88% disease reduction compared to the control (Figure 11, Table 8). Copper oxychloride and mancozeb alone achieved 79% and 72%, respectively while their combination achieved 71% disease reduction compared to the control. From the time of initiation of spraying after disease was first noticed, disease slightly increased in plants treated with copper oxychloride, mancozeb or their combinations before leveling off and eventually decline while benomyl alone or in combination with the other two caused immediate disease decline. There were no significant differences in disease intensity among the fungicide-treated plants 84 days after disease onset while the disease in untreated plants was significantly higher than in the treated plants (Table 8).

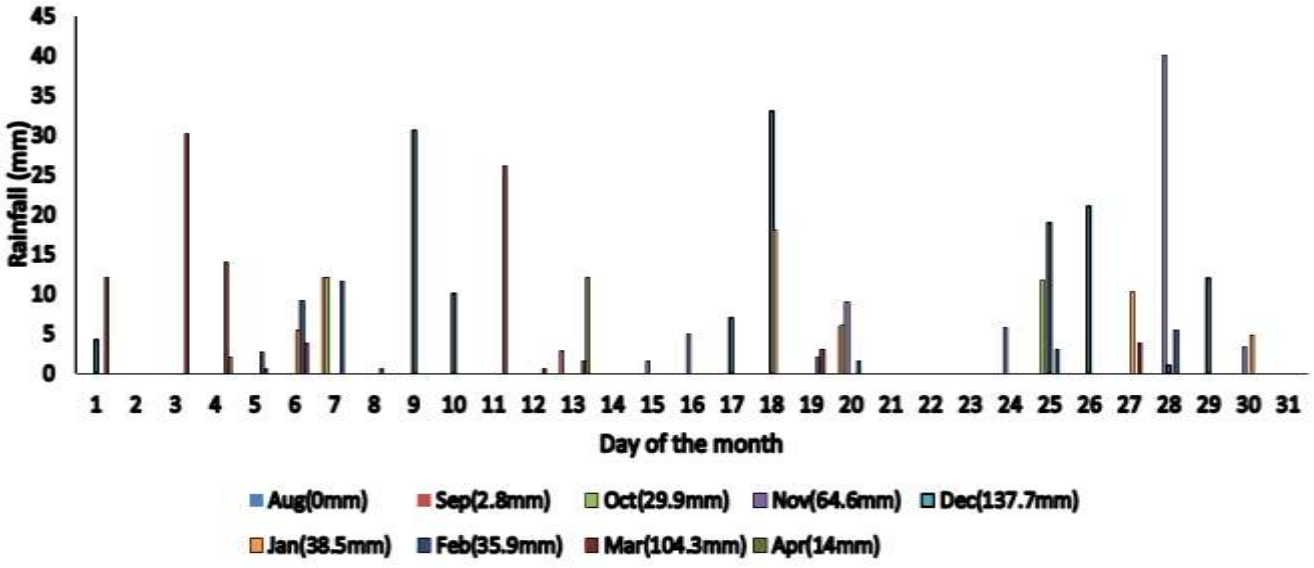

Figure 10: Daily total rainfall recorded at the Department of Agricultural Research station from August 2013 to April 2014.

(Figures in parenthesis in the legends are monthly total rainfall).

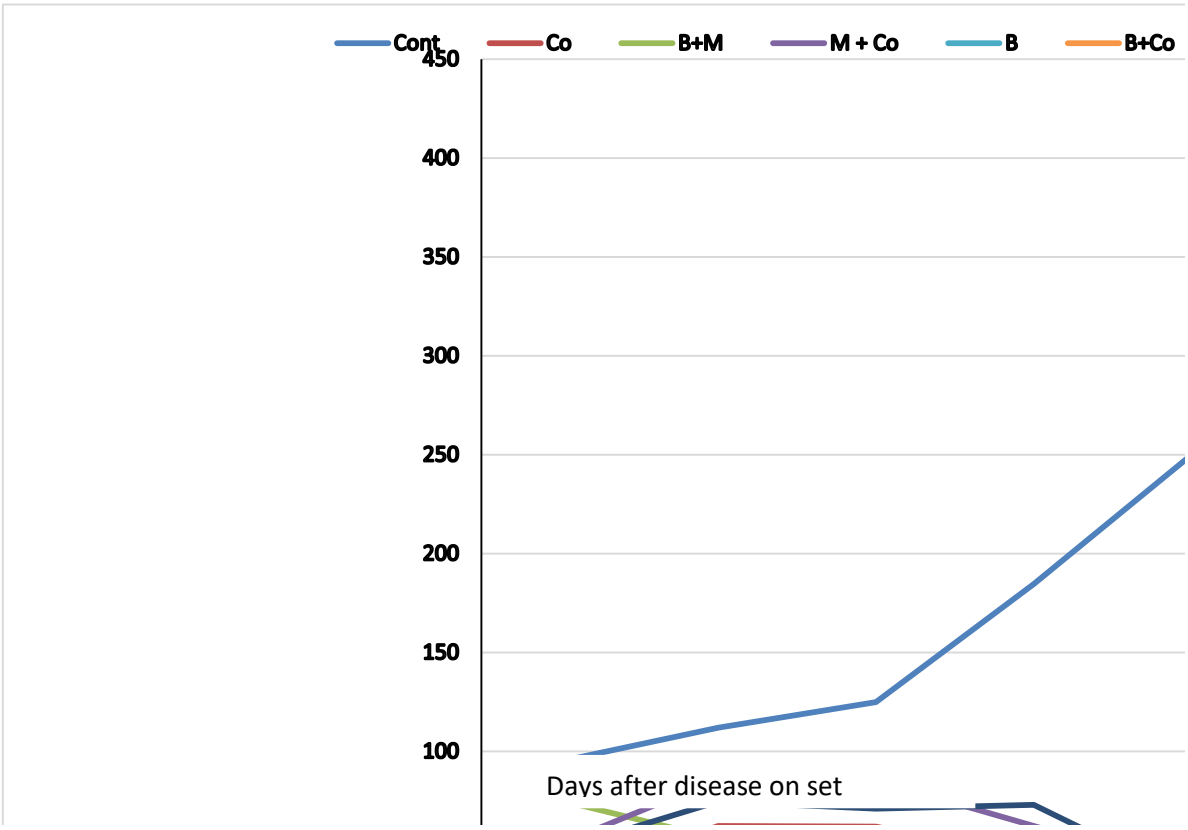

Figure 11: Effect of fungicide treatments on CLS disease progress curve.

Table 8: The effect of locally available fungicides on the severity of Cercospora leaf spot of Swiss chard under field conditions in Botswana.

Fungicide Treatment [1]	**Mean number of Swiss chard Cercospora leaf spots per leaf recorded at 14-day interval from first fungicide application**						**Overall Fungicide Means**	**Percentage disease reduction**
	14	28	42	56	70	84		
Control	95.00def[2]	112.70de	125.00d	184.7c	250.00b	414.00a	196.9a[3]	0
Copper oxychloride;	34.33hijklmno	62.33fghijk	62.00fghijk	45.67ghijklmn	27.67jklmno	16.00mno	41.33bc	79
Benomyl+ Mancozeb;	75.33efgh	55.67fghijkl	38.67hijklmno	28.00jklmno	3.00no	5.00no	34.28c	83
Mancozeb + Copper oxychloride;	53.33fghijkl	86.00defg	85.33defg	62.33fghijk	39.33hijklmno	16.00 lmno	57.06b	71
Benomyl	59.67fghijkl	38.33hijklmno	36.00 hijklmno	23.67 klmno	16.33lmno	6.330 mno	30.06c	85
Benomyl + Copper oxychloride;	30.67ijklmno	40.33hijklmno	33.67hijklmno	18.67 klmno	18.33 klmno	5.330no	24.50 c	88
Mancozeb;	50.33ghijklm	74.67efghi	71.00efghij	73.00efghi	34.00hijklmno	22.00klmno	54.17b	72
Benomyl + Mancozeb + Copper oxychloride.	53.67 fghijkl	40.33hijklmno	55.67fghijkl	16.33lmno	1.00o	0.00o	27.83c	86
LSD	78.6					18.05		
Overall Sampling Means	56.542	63.792	63.417	56.542	48.708	60.583	58.264	
LSD	NS							

[1] Where more than one fungicide was used, the fungicides were applied alternately every two weeks and leaf samples were collected just before spraying

[2] Means for the fungicide and sampling interactions at 2 weeks interval in a column and in a row respectively followed by the same letter(s) are not significantly different, LSD test (p=0.05)

[3.] The overall means in a column followed by the same letter are not significantly different, LSD test (p=0.05

5.0. DISCUSSION

5.1. Molecular identification and analysis of genetic diversity of *Cercospora beticola* isolates from Swiss chard from Southern Botswana

Molecular characterization of pathogens of agricultural importance is a crucial factor in understanding epidemiology and control of these pathogens. Cattanach, (1999) and Khan and Smith, (2005) reported that *Cercospora beticola* caused losses estimated at US$45 million to the American Sugar Company, owing to fungicide application costs and sugar beet yield losses. Several genomic loci such as parts of the histone, actin and calmodulin genes have been employed for the molecular identification of *Cercospora* species (Groenewald *et al*, 2013). In the present study, we employed the amplification of the internal transcribed spacer regions (ITS) and intervening 5.8S nrRNA genes of *C. beticola* isolates from two farms in Southern Botswana. Amplification resulted in fragments ranging between 500-600 base pairs, a finding that was consistent with previous studies (Crous, 1998; Lartey *et al*, 2003; Groenewald *et al*, 2005; Groenewald *et al*, 2005). These workers got an amplicon of about 600 base pairs when establishing a PCR protocol for the detection of *C. beticola*. Therefore these results imply that, the amplified DNA fragments were indeed of *C. beticola*.

In this study, *Cercospora beticola* was isolated from diseased leaves of Swiss chard at high frequencies from both Bokaa (51.7%) and Glen Valley (48.2%). Genetic diversity of the isolates was assessed by randomly pooling 18 isolates from the 87 strains that were putatively identified as *Cercospora beticola* by PCR for sequencing. Phylogenetic analysis revealed that the 22 strains clustered into four major clusters, including a distinct sub-cluster within cluster 2. This indicates a high degree of genetic diversity of *C. beticola* isolates in the present study. Notably, clustering of the isolates could not be distinguished by their geographic origin (Bokaa or Glen Valley farms). Moretti *et al,* (2004) found great intraspecific variability

within a small population of *Cercospora beticola* infecting sugar beet. Groenewald *et al*, (2008) also found high genetic diversity within populations of *C. beticola*.

Furthermore, the genetic diversity of Swiss chard populations of *C. beticola* assessed using pairwise distances revealed high genetic diversity of *C. beticola* isolates ranging from 0.000 to 0.004. Similar to results obtained utilizing phylogenetic analysis, populations of *C. beticola* could not be differentiated based on place of origin. These results are consistent with a recent study (Vaghefi *et al*, 2017) who found genetically diverse *C. beticola* isolates obtained from Swiss chard and table beet that could not be discriminated based on sampling location or host in five fields in New York and Hawaii. Contrary to the present results, Rousset, (1997), postulated that genetic diversity can be attributed to fungal populations with long distance dispersal of ascospores where a pattern of isolation by distance is detected resulting from gradual spread of the disease from its original source hence resulting in genetic differentiation as distance increases. The discrepancy between the present study and Rousset's study can be attributed to the potential survival of *C. beticola* on alternative hosts thereby promoting genetic diversity through the reduced impact of genetic drift thus increasing CLS population size. Indeed, Vaghefi *et al*, (2017) found that if a weed is the source of primary infection, genetic structuring of C. *beticola* populations would be effected, thus resulting in more diversity within a sampling location. The results of the present study imply that indeed diversity among *C. beticola* exists and is not affected by sampling location or distance among the sampling locations, however the presence of other hosts (weeds or other plant hosts) results in more diversity among *C. beticola* isolates. This study has demonstrated the genetic diversity *Cercospora beticola* from Swiss chard at two farms in southern Botswana. The high genetic diversity is rather surprising because the pathogen is asexual, with no known sexual phase. However, Groenewald *et al*, (2006) studied mating

type distribution of *Cercospora* species and found evidence that suggested a cryptic sexual cycle in *C. beticola*. In light of the high levels of genetic diversity associated with isolates of the present study, we propose for further study the assessment of the frequency of mating type genes in *C. beticola*. To ascertain this however, cross-inoculation tests are needed for verification (Ruppel, 1986).

Knowledge of genetic diversity is an important tool in optimizing control of crop pathogens (Moretti *et al* 2006). A recent study by Utlwang *et al*, 2017 found resistance to some pesticide by *C. beticola* causing CLS in Swiss chard in Botswana, thus suggesting that chemical control may not prove a viable option. It remains a distinct possibility that some resistance may be linked to the genetic diversity of *C. beticola* isolates in Botswana. It thus remains important in pathogen disease cycles, to study the hosts as well as the genetic diversity of the pathogen for enhanced efficacy of control methods that prevent yield losses in important crops such as Swiss chard.

5.2. The effect of humidity, temperature and inoculum density on plant disease development under a controlled environment

The success of disease management practices frequently depends on knowledge of the growth determinants of a pathogen in Agricultural ecosystem such as optimum temperature, humidity and presence of inoculum respectively (Robbertse and Crous, 2000; Robbertse *et al*, 2000; Robbertse *et al*, 2001). The significant increase of numbers of spots/leaf in inoculated plants incubated at increasing humidity across all sampling times compared well with literature (Lapaire and Dunkle, 2003; Jacobsen *et al*, 2004; Khan *et al*, 2009;). These workers found significant increase in conidial numbers with increase in humidity, thus used conidial numbers to estimate disease severity. Their optimum humidity was in the ranges 87- 90%,

with conidia being readily produced at RH of greater than 60%. The study by Jacobsen *et al,* (2004) optimised humidity at 100% in his controlled environment growth chamber experiments. Artificial infection experiment further established that air relative humidity >95% or leaf wetness was required for infection and subsequent lesion development. Under field conditions the probability of leaf wetness was estimated at 75% at relative humidity >90%. The extent of the damage depends on time of initiation and severity of the epidemic (Cooke and Scott, 1993). The difference in the optimum humidity concentrations can be attributed to the different methods that were employed to quantify disease severity. The present study quantified disease (CLS) by the development of leaf spots while previous studies mainly focused on conidial numbers. However Khan *et al,* (2009) explained that conditions may be possible for sporulation and ultimately conidial production but may not be conducive for disease development. The results of the present study imply that from 70% RH, as humidity increases, CLS also increase with 90% RH resulting in optimal CLS development.

The epidemic onset of the disease is dependent on canopy development, level of cultivar resistance, inoculum potential and weather conditions. Canopy development has to be considered an important factor due to subsequent changes of microclimate. In particular, after row closure, leaf wetness duration is prolonged and relative humidity is higher within the canopy favouring epidemic progress (Wolf *et al,* 2001, Wolf and Verret, 2005). This supports the present study in that as sampling time increased, the plants grew bigger therefore closer to one another which enabled cross contamination as moisture was more preserved.

The current study revealed that 25°C was optimal for CLS development, with numbers of spots/leaf decreasing at 30°C. Cercospora leaf spot did not develop at 15°C; however, a lower number of spots developed at 20°C. These results are in line with literature that the optimum

temperature for CLS development is 25°C (Lapaire and Dunkle, 2003; Poornima and Hedge, 2014). These authors also reported an optimum temperature range of 20- 25°C. Conidia of *C. beticola* are readily produced at temperatures from 15-23°C and relative humidity greater than 60%. However they do not form at temperatures less than 10°C or above 38°C (Khan *et al,* 2009). However Paul and Munkvold, (2005) when studying effects of RH on lesion expansion, reported that temperatures of 25-30°C were not significantly different at $P \leq 0.05$, beyond that lesion expansion decreased and minimal development of CLS was observed after periods of high temperatures of 30°C or above, although most authors have predicted a moderate to high CLS development. Previous studies have shown that extended exposure to temperatures above the optimal for disease development can inhibit *C. beticola* and reduce severity. Conidia may appear as early as 48 hours after inoculation on a plant exposed to favourable temperatures 25-30°C and high relative humidity >90%. Conidia are carried by air current or rain splash and cause secondary infections throughout the growing season when environmental conditions are favourable or suitable control measures are not employed. These results imply that for optimum disease development temperatures should be set at 25°C; however temperatures can be reduced to 15°C in order to prevent disease development, most applicable under controlled environments such as greenhouses. In Botswana, where summers are hot and humid, CLS epidemics would hinder production compared to the cool and dry winters which pose a conducive setting for Swiss chard production.

The results of the present study showed that the $2.30x10^6$ inoculum concentration resulted in the highest CLS across all weeks followed by the $2.3x10^5$, $1.2x10^4$, $0.1x10^4$, and the results agree with literature that $1.2x10^4$ infectious units per ml resulted in CLS development. Kaizer and Varrelmann, (2009) used artificial inoculation to evaluate resistance and yield effects in sugar beet cultivars against *C. beticola*. They found an optimum inoculum concentration

range between $1x10^4$ and $2x10^4$ infectious units per ml. Some of the outcomes of this study included the fact that post inoculation climatic conditions are vital on epidemic progress and remain unpredictable. He went on to state that initial infection does not guarantee for successful disease spread and rapid disease progress but the use of optimal inoculum densities will render Cercospora field resistance tests more reliable and reproducible under variable climatic conditions. The results in this study imply that for optimum disease development, there should at least be $1.2x10^4$ spores/mL). However at least 1000 ($0.1x10^4$ concentration) infectious units should be present for CLS to develop, which was the lowest inoculum density that resulted in disease development in this study.

The infection process depends on availability of inoculum produced by sporulating lesions. Once these conditions are met the progress of leaf spot disease is characteristic of a typical polycyclic disease with more than one infection cycle during the growing season, (Paul and Munkvold, 2005). Inoculum potential is a major factor affecting Cercospora leaf spot epidemics especially in the context of cultural practices used. The fungus is able to persist on infected leaf residues as stromata preferably on the soil surface whereas in the soil survival is reduced to 2 years. Therefore cultural practices such as tillage to turn under leaf debris are of major importance as is the distance to beet fields of the previous year. Although a crop rotation with a minimal of 2 years with a non-host crop is recommended as a practical standard beet cropping. Overall cropping may vary even within the same production region leading to large field to field variation in inoculum potential (Wolf and Verret, 2005).

From the 21st to 28th day after inoculation, conidia were primarily dispersed through air circulation causing secondary infections, thus increase in CLS numbers. It is important to note that production of secondary inoculum by plant pathogenic fungi such as *C. beticola* is significant only if there is a mechanism for liberation and dispersal of secondary conidia.

Conidia of Cercospora species are borne on erect conidiophores the upper and lower the leaf surface (Lapaire and Dunkle, 2003). Conidia may be raised above the lamiar boundary layer of non-turbulent air at the leaf surface and into the layer of turbulent air above the surface. It is of outmost importance to note that this fungus is polycyclic within a growing season, with each sporulation cycle taking 12days depending on environmental conditions (Khan *et al*, 2009).

This research has shown a significant relationship between *C. beticola* conidial suspensions with temperature and humidity. In this study interaction of temperature humidity and inoculum concentration contributed to development of cercospora leaf spot of Swiss chard seedlings. However as humidity and temperature increased there was a steady increase in the number of leaf spots. It was apparent that at 15°C there was no stomatal penetration/ vegetative growth of the pathogen. The results are similar to those of Lapaire and Dunkle, (2003) who reported relative humidity greater than 60% as favourable for higher conidial production.

5.3. Assessing the effectiveness of locally available fungicides in managing Cercospora leaf spot of Swiss chard under field conditions

Cercospora leaf spot disease outbreak from natural infection was dependent on the onset of favourable weather conditions, mainly rainfall and temperature in December 2013 and January 2014 with a cumulative total rainfall of 162.2 mm and this continued up to the end of the rain season in April 2014. Khan *et al*. (2009) confirmed what the result showed by stating that favourable environmental conditions for the development of Cercospora leaf spot are day temperatures of 25-35°C, night temperatures of 16°C, and prolonged periods of relative humidity of 90-95% or free moisture on the leaves after which conidia are dispersed from

their source of inoculum by wind or water splash, running water and insects onto the Swiss chard leaves. Larson. (2004) correlated a higher humidity in the stomata opening with infection through the stomata, which do not need to open to facilitate fungal entry.

The most effective fungicide against *C. beticola* during the study was benomyl alternated with copper oxychloride, with a disease reduction of 88% compared to the untreated control. Copper oxychloride alone achieved 79% reduction which was significantly higher than Mancozeb (72%) or mancozeb alternated with copper oxychloride (71%). Benomyl is a systemic fungicide whose mode of action is inhibition of mitosis and cell division in target fungi by polymerization of tubulin into microtubules (MacCarroll *et al*, 2002; Gupta *et al*, 2004; Davidson *et al*, 2006; Koo *et al*, 2009;). That is why the benomyl treated plants generally had declining numbers of leaf spots after the initial spray compared to the non-systemic fungicides where the disease increased. Harvesting of Swiss chard leaves commenced 3 days after spraying with all the fungicides. The 3 day waiting period rendered the leaves safe for human consumption as per manufacturer's instructions.

The second most effective fungicide was copper-oxychloride which is non-systemic contact fungicide with limited therapeutic activity. Effective control of disease by copper oxychloride depends on complete coverage of plant surfaces and minimal weathering factors such as rain soon after application. When copper oxychloride was used alone the reduction in spot counts was not satisfactory between second and fourth week, however effective control was observed through the fifth to sixth week. This is because copper oxychloride action as a fungicide is due to the release of small quantities of copper (Cu++) ions when in contact with water. The mode of action of copper oxychloride is the nonspecific disruption of cellular proteins when the toxic copper ion is absorbed by germinating fungal spores, hence for best

results copper must be reapplied as plants grow to maintain coverage and prevent disease establishment (Husak, 2015). However copper oxychloride being an insoluble compound generally left unattractive residues on the leaves rendering them to be unmarketable as noted by Schwatz and Gent (2007).

Mancozeb was the least effective compared to benomyl and copper oxychloride but was much better than the control (Table 8), mancozeb is also a contact fungicide with preventive activity. Its mode of action is the inhibition of enzyme activity of fungi, by forming a complex with metal-containing enzymes including those involved in the production of adenosine triphosphates (ATP). Calviello *et al,* (2006) suggested that mancozeb caused post apoptotic cell membrane integrity due to its oxidative effect. Mancozeb is a typical multisite protectant fungicide and for this reason use rates and frequency of application need to be relatively high in order to counteract the effect of weathering and plant growth, which can rapidly diminish the protection afforded by the product. It must also be present on the leaf prior to the arrival of fungal spores if it is to be effective and hence it is not as flexible as curative or eradicant fungicides. Mancozeb was first applied after the appearance of spots therefore; an increase in spots was observed on the weeks that followed however a significant decline in the number of spots was observed on the fifth and sixth week because this is foliage that underwent preventative spraying. Although preventive spraying can eliminate or reduce disease losses, it may result in use of more sprays than are necessary.

In Botswana the Ministry of Agricultural Development and Food Security has a number of fungicides registered for control of fungal pathogens in certain crops. These include among others metalaxyl, flusilazole, terbuconazole, benomyl, mancozeb, copper oxychloride and pyraclostrobin (Plant Protection, 2016). One of the greatest challenges faced by horticultural

farmers in Botswana in the control of *C. beticola* is to determine the best fungicide and time for fungicide application in order to minimize losses. Disease severity is directly related to favourable environmental conditions characterized by extended rainfall (Khan *et al*, 2009) as was the case during the study. For effective control of the disease, monitoring of environmental factors such as rainfall, relative humidity and duration of leaf wetness and scouting for disease is critical. Trkulja. (2015) stressed that accurate data on the presence and prevalence of resistant populations can help to enable chemical treatment programs to be adjusted to improve fungicide efficiency so that anti resistance strategies may be applied in the future.

A number of authors have reported the development of resistance in the Benzimidazole class of fungicides; however these provided significantly better results in the control of CLS (Dexter and Luecke, 1999; Karaoglanidis *et al*, 2000; Weiland and Halloin, 2001). Although resistance to benomyl by *C beticola* developed, with resistance frequency reaching 80-90%, previous sensitivity monitoring revealed a decrease in values to 20-25% resistance frequency (Smith, 1988; Karadimos *et al*, 2000).

Karaoglanidis *et al*, (2003) noted that farmers who are faced with problems arising from resistance tend to continue to use the same fungicides as previously, only in higher dosages or more frequently which can negatively affect the success of disease management (Ossenkop *et al* 2004; Ishii, 2006). Therefore monitoring of *C beticola* population in terms of resistance to fungicides in the field is of vital importance for the development of CLS management strategies. It is important to note that breeders have not yet succeeded in combining *Cercospora beticola* resistance gene with the yield potential of susceptible varieties (Gummer *et al*, 2015). Less susceptible / resistant varieties have been reported to produce low yield

compared with the high yielding susceptible varieties (Miller *et al,* 1994; Mechelke, 2000; Gummer *et al,* 2015) presumably making them less acceptable for cultivation on a large scale in commercial farms.

6.0. CONCLUSION AND RECOMMENDATIONS

Cercospora beticola was isolated from diseased Swiss chard leaves from both Bokaa and Glen valley farms and identified using morphological and molecular methods. Phylogenetic analysis revealed that *C. beticola* could not be distinguished by geographic location in Southern Botswana therefore showing a high genetic diversity, which may be associated with development of fungicide resistant genes. Development of CLS can be controlled by controlling the growth determinants being; humidity, temperature and inoculum concentration. Ninety percent RH resulted in optimum conditions for CLS development, however as RH decreased to 70% and 50% respectively, CLS also reduced. An optimum temperature of 25°C was necessary for disease development and temperatures below (20°C) or above (30°C) did not favour the disease. No disease developed at 15°C supporting the low incidence of CLS disease in Swiss chard grown during the winter months in Botswana Another important aspect of CLS development was the presence of viable inoculum. As inoculum increased, CLS severity also increased. Benomyl altenated with copper oxychloride, was much more effective in managing CLS than mancozeb or copper oxychloride used alone or alternated.

Horticultural farmers in Botswana can be recommended to produce Swiss chard during winter as it is cool and dry (April to July, temperatures are below 20°C and the air is dry, with little chance of rainfall). However those attempting to produce Swiss chard during the summer can be advised to resort to fungicide control, which includes alternating Benomyl

with copper oxychloride. Though the current study was only limited to Southern Botswana, more work requires to be done in terms of studying *C beticola* diversity across all districts in Botswana in order to understand the pathogen progression with respect to the establishment of fungicide resistance genes.

In Botswana there is no surveillance programme in place that monitors plant pathogens especially now when the horticultural sector is growing. It may be relevant to design a database on the research into this important disease so that information may be readily available to researchers as well as for collaborative purposes with other researchers in other countries.

7.0. REFERENCES

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8.0. APPENDIX

8.1. Published journal from this study

ASSESSING THE EFFECTIVENESS OF LOCALLY AVAILABLE FUNGICIDES IN MANAGING CERCOSPORA LEAF SPOT OF SWISS CHARD UNDER FIELD CONDITIONS IN BOTSWANA

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ABSTRACT

Cercospora leaf-spot (CLS), caused by *Cercospora beticola*, is an important foliar disease of Swiss chard in Botswana. The regularity of outbreaks of the disease in Botswana is a major cause of concern and thus have necessitated the use of fungicides under field conditions in order to control the disease and bring the crop to maturity. The objective of the study was to assess the most effective, locally available fungicides in the control of CLS of Swiss chard under field conditions. The experiment was carried out in Sebele, where a tractor-ploughed field was partitioned into 24 plots, which were split into three blocks. Seedlings were transplanted into the plots, with each plot containing a maximum of 45 plants which were left for natural infection. Disease control commenced once symptoms were observed, using Copper oxychloride; Benomyl + Mancozeb; Mancozeb + Copper oxychloride; Benomyl; Benomyl + Copper oxychloride; Mancozeb; Benomyl + Mancozeb + Copper oxychloride applied at recommended rates. Where more than one fungicide was used, the fungicides were applied at alternating intervals. Control plants were sprayed with water only. The treatments were applied for a period of twelve weeks at two week intervals. The average number of leaf spots per leaf was recorded two weeks after each fungicide application and samples were collected prior to spraying. Data were subjected to analysis of variance and means separated using LSD test at p=0.05. Cercospora leaf spot severity was significantly lower in all treatments involving Benomyl than in those treatments involving Mancozeb and Copper oxychloride used alone or in combination. This study confirms that when Benomyl is alternated with Copper oxychloride, it is much more effective in managing Cercospora Leaf spot than Mancozeb or Copper oxychloride used alone or alternated.

Key words: *Cercospora leaf spot, Swiss chard, fungicides, control, Botswana*

INTRODUCTION

Swiss chard, [*Beta vulgaris* subsp. *cicla* (L.) W.D.J. Koch] also known as spinach beet belongs to the Chenopodiaceae or goosefoot family (Tindall, 1988). A study carried out by (Madisa *et al.*, 2010) revealed that Swiss chard is grown in most regions of Botswana and farmers that were involved in the study within Gantsi, Southern and Kgalagadi regions produced Swiss chard. This correlated with results obtained by Obopile *et al*, (2008) which showed that the most commonly grown vegetable in Gaborone region (Kgatleng) and western

region (Gantsi and Kweneng) was Swiss chard. According to farmers' ranking of the important vegetable crops in Botswana, brassicas, Swiss chard and tomato are the most popular vegetable crops (Bok *et al.* 2006, Munthali *et al.* 2004). These crops (except Swiss chard) are significantly associated with major pests of vegetable crops.

The Horticultural sector in Botswana is still at its infancy stage, with a lot of government programs mainly targeting this sector due to its potential to reduce the rates of poverty, unemployment and imports of vegetables. This therefore, necessitates information sharing with farmers who are already in production and those that are upcoming. Swiss chard being a potentially worthwhile vegetable to produce in Botswana needs not only to be promoted among farmers in Botswana, but information should be shared with farmers on its production limitations especially the disease known as Cercospora leaf spot (CLS), caused by *Cercospora beticola* Sacc. Losses caused by this disease may initially be underestimated, however intense pressure from this pathogen results in increased lesions which would ultimately lead to necrosis of whole tissue (Windels *et al.*, 1998). Most literature on fungicidal control of this disease is on sugar beet and very little studies have been carried out on Swiss chard and none in Botswana. Producers in affected areas must thoroughly apply fungicides in order to bring the crop to maturity (Windels *et al*., 1998; Meriggi *et al*., 2000). Without such measures, the leaf canopy can be destroyed by outbreaks of *C. beticola,* resulting in complete loss of the crop (Duffus and Ruppel, 1993; Rossi *et al.*, 2000). The objective of this study was to assess the most effective, locally available fungicides in the control of Cercospora Leaf Spot of Swiss chard (CLS) under field conditions through the monitoring of leaf spot numbers.

MATERIALS AND METHODS

Experimental location

A field experiment was carried out at the Botswana University of Agriculture and Natural Resources, Sebele, Gaborone (Latitude 24 34'S, Longitude 25 57'E, Altitude 994 above sea level) from August 2013 to April 2014 in order to assess the effectiveness of three selected locally available fungicides, namely Benomyl, Mancozeb and Copper oxychloride.

Land preparation and cultural practices

A field where Swiss chard was previously grown was ploughed by a tractor and partitioned into 24 plots with an area of 10 m^2 (5mx2m) each and a spacing of 1m between the plots. The plots were split into 3 blocks each with eight plots. Seedlings were raised in a net shade in three seedling trays, each with a capacity of 200 plants per tray. The trays were watered twice daily using a watering can with a nozzle. Two weeks after emergence (WAE) the plants were given Starke Ayres plant food 3:1:6(46). One gram was dissolved in 1litre of water and each tray was watered with 1litre of the solution.

The seedlings were transplanted into the field 6 weeks after emergence at a spacing of 40 cm between the rows and 30 cm between the plants. Each plot had a population of 45 plants. The plots were weeded as the weeds emerged. Watering was carried out twice a day in the morning and afternoon during the 1st month after transplanting, thereafter watering was done once in the afternoon. Lime ammonium nitrate (LAN) fertilizer was added at a rate of 140 g per plot at 21 day intervals.

Treatments and experimental design

Control of Cercospora leaf spot of Swiss chard using fungicides commenced once the first symptoms were observed within the plots. The treatments were as follows: 1. No fungicide

application (Control); 2. Copper oxychloride; 3. Benomyl alternating with Mancozeb; 4. Mancozeb alternating with Copper oxychloride; 5. Benomyl; 6. Benomyl alternating with Copper oxychloride; 7. Mancozeb; 8. Benomyl alternating with Mancozeb and Copper oxychloride. Fungicides were applied at 14 day intervals using a 5-litre Knapsack sprayer. A plastic cage was made around each plot during spraying in order to avoid drift of the fungicide to other plots using plastic and wooden stakes. Benomyl was applied at a rate of 50g/100l of water, copper oxychloride at 40g/ 100l of water and mancozeb at 200g/100l of water as per manufacturer's instructions.

The experimental design was a randomized complete block design (RCBD) with fungicide treatments as the main factor (Factor A) and sampling period as a subplot (Factor B).

Data collection

Data collected were the average number of leaf spots per leaf. For each plot a total of 21 experimental plants were numbered for use after excluding the border plants. At each sampling time ten plants were randomly chosen by placing numbers 1 to 21 in a bag and picking 10 number for sampling. The first sample was collected 2 weeks after the first fungicide spray and subsequently every 2 weeks just before fungicidal application for the weeks that followed. One leaf with most spots was taken per plant. Spot count was done under a magnifier using an inoculating needle. Temperature and rainfall data for the duration of the study were collected from the Department of Agricultural Research Meteorological Station.

Data analysis

Data were subjected to one-way analysis of variance (ANOVA-1) and if the f-value was significant (p=0.05), means were separated using Least Standard Deviation (LSD) test. The MSTATC statistical package (Michigan State University) was used to analyze the data.

RESULTS AND DISCUSSION

Meteorological data

Total monthly rainfall received during the study period (August 2013 to April 2014) ranged from 0 mm in August to 137.7 mm in December 2013 with a cumulative total of 427.7 mm (Figure 1). Rainfall distribution was good from mid-November to the end of January with rain showers almost every week creating ideal conditions for development of CLS. The season was also characterized by relatively high temperature condition with the average maximum temperatures ranging from 25.5^{o}C in April 2014 to 32^{o}C in November 2013 and minimums ranging from 6.2ºC in September 2013 to 19.4ºC in March 2014.

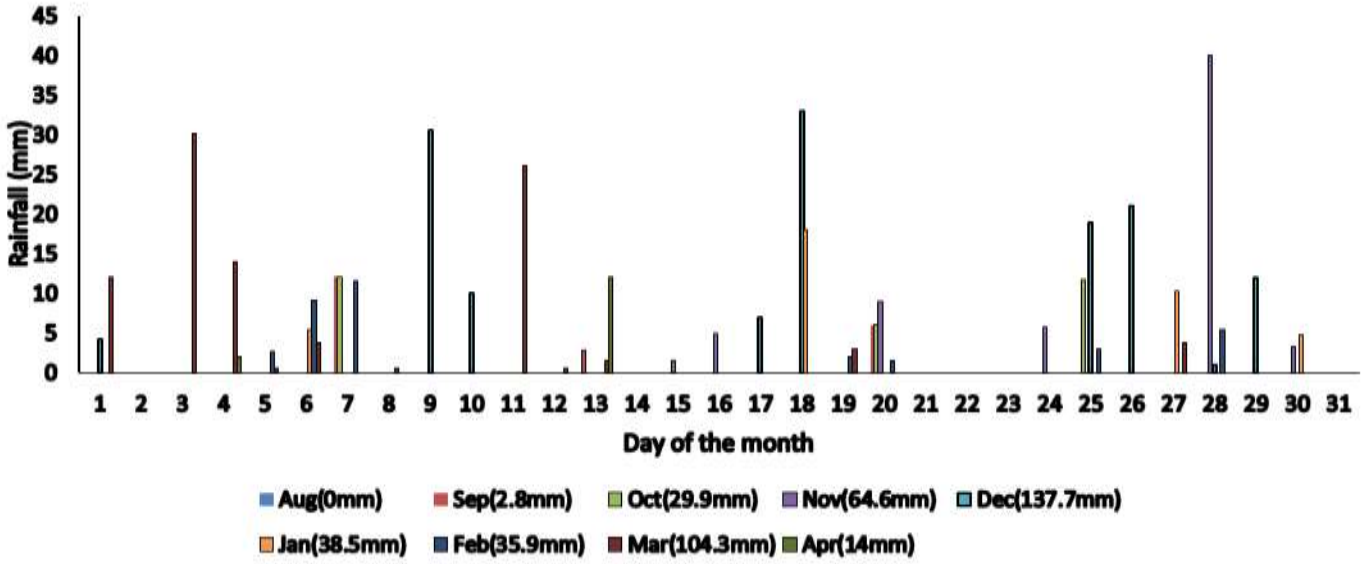

Figure 1. Daily total rainfall recorded at the Department of Agricultural Research station from August 2013 to April 2014 (Figures in parenthesis in the legends are monthly total rainfall).

Effects of fungicide application on CLS disease development

Fungicide application significantly reduced disease development compared to the non-treated control but among the fungicides benomyl application alone or alternated with mancozeb, and copper oxychloride was the most effective resulting in 83-88% disease reduction compared to the control (Figure 2, Table1). Copper oxychloride and mancozeb alone achieved 79% and 72%, respectively while their combination achieved 71% disease reduction compared to the control. From the time of initiation of spraying after disease was first noticed, disease slightly increased in plants treated with copper oxychloride, mancozeb or their combinations before leveling off and eventually decline while benomyl alone or in combination with the other two caused immediate disease decline. There were no significant differences in disease intensity among the fungicide-treated plants 84 days after disease onset while the disease in untreated plants was significantly higher than in the treated plants (Table 1).

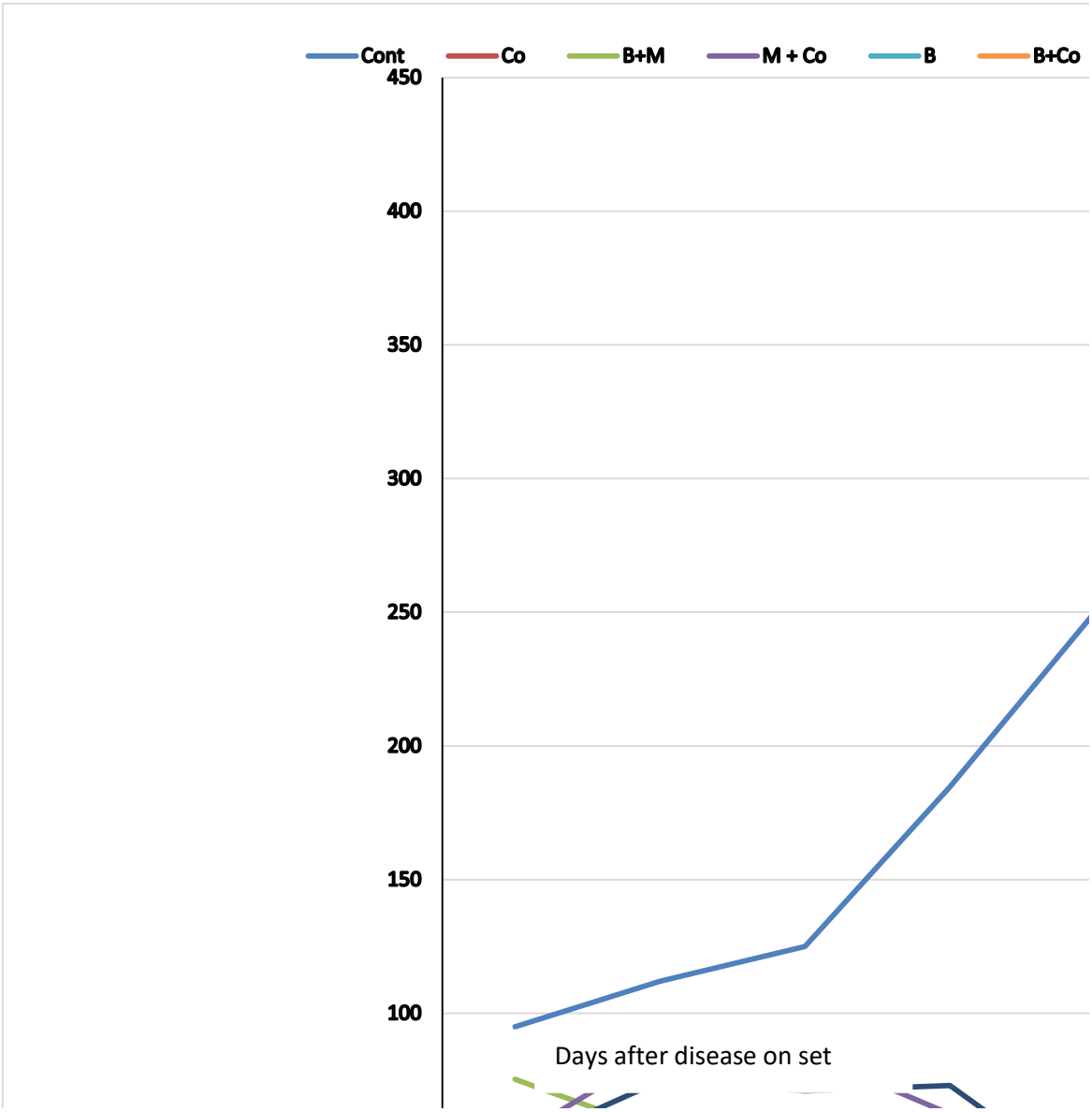

Figure 2. Effect of fungicide treatments on CLS disease progress curve (Cont= Control, Co = copper oxychloride, B = benomyl, M= mancozeb).

Table 1. The effect of locally available fungicides on the severity of Cercospora leaf spot of Swiss chard under field conditions in Botswana.

Fungicide Treatment [1]	**Mean number of Swiss chard Cercospora leaf spots per leaf recorded at 14-day interval from first fungicide application**						**Overall Fungicide Means**	**Percentage disease reduction**
	14	28	42	56	70	84		
Control	95.00def[2]	112.70de	125.00d	184.7c	250.00b	414.00a	196.9a[3]	0
Copper oxychloride;	34.33hijklmno	62.33fghijk	62.00fghijk	45.67ghijklmn	27.67jklmno	16.00mno	41.33bc	79
Benomyl+ Mancozeb;	75.33efgh	55.67fghijkl	38.67hijklmno	28.00jklmno	3.00no	5.00no	34.28c	83
Mancozeb + Copper oxychloride;	53.33fghijkl	86.00defg	85.33defg	62.33fghijk	39.33hijklmno	16.00 lmno	57.06b	71
Benomyl	59.67fghijkl	38.33hijklmno	36.00 hijklmno	23.67 klmno	16.33lmno	6.330 mno	30.06c	85
Benomyl + Copper oxychloride;	30.67ijklmno	40.33hijklmno	33.67hijklmno	18.67 klmno	18.33 klmno	5.330no	24.50 c	88
Mancozeb;	50.33ghijklm	74.67efghi	71.00efghij	73.00efghi	34.00hijklmno	22.00klmno	54.17b	72
Benomyl + Mancozeb + Copper oxychloride.	53.67 fghijkl	40.33hijklmno	55.67fghijkl	16.33lmno	1.00o	0.00o	27.83c	86
LSD	78.6					18.05		
Overall Sampling Means	56.542	63.792	63.417	56.542	48.708	60.583	58.264	
LSD	NS							

[1] Where more than one fungicide was used, the fungicides were applied alternately every two weeks and leaf samples were collected just before spraying

[2] Means for the fungicide and sampling interactions at 2 weeks interval in a column and in a row respectively followed by the same letter(s) are not significantly different, LSD test (p=0.05)

[3.] The overall means in a column followed by the same letter are not significantly different, LSD test (p=0.05)

Cercospora leaf spot disease outbreak from natural infection was dependent on the onset of favorable weather conditions, mainly rainfall and temperature in December 2013 and January 2014 with a cumulative total rainfall of 162.2 mm and this continued up to the end of the rain season in April 2014. Khan *et al* (2009) confirmed what the result showed by stating that favorable environmental conditions for the development of Cercospora leaf spot are day temperatures of 25-35ºC, night temperatures of 16ºC, and prolonged periods of relative humidity of 90-95% or free moisture on the leaves after which conidia are dispersed from their source of inoculum by wind or water splash, running water and insects onto the Swiss chard leaves. Larson (2004) correlated a higher humidity in the stomata opening with infection through the stomata, which do not need to open to facilitate fungal entry.

The most effective fungicide against *C. beticola* during the study was benomyl alternated with copper oxychloride, with a disease reduction of 88% compared to the untreated control. Copper oxychloride alone achieved 79% reduction which was significantly higher than Mancozeb (72%) or Mancozeb alternated with Copper oxychloride (71%). Benomyl is a systemic fungicide whose mode of action is inhibition of mitosis and cell division in target fungi by polymerization of tubulin into microtubules (Koo *et al* 2009 MacCarroll *et al*, 2002 ; Gupta *et al* 2004). That is why the benomyl treated plants generally had declining numbers of leaf spots after the initial spray compared to the non-systemic fungicides where the disease increased. Harvesting of Swiss chard leaves commenced 3 days after spraying with all the fungicides. The 3 day waiting period rendered the leaves safe for human consumption as per manufacturer's instructions.

The second most effective fungicide was Copper-oxychloride which is non-systemic contact fungicide with limited therapeutic activity. Effective control of disease by copper oxychloride depends on complete coverage of plant surfaces and minimal weathering factors such as rain soon after application. When Copper oxychloride was used alone the reduction in spot counts was not satisfactory between second and fourth week, however effective control was observed through the fifth to sixth week. This is because Copper oxychloride's action as a fungicide is due to the release of small quantities of copper (Cu++) ions when in contact with water. The mode of action of Copper oxychloride is the nonspecific disruption of cellular proteins when the toxic copper ion is absorbed by germinating fungal spores, hence for best results copper must be reapplied as plants grow to maintain coverage and prevent disease establishment (Husak, 2015). However Copper oxychloride being an insoluble compound generally left unattractive residues on the leaves rendering them to be unmarketable as noted by Swartz and Gent (2007).

Mancozeb was the least effective compared to Benomyl and Coper oxychloride but was much better than the control (Table 1), Mancozeb is also a contact fungicide with preventive activity. Its mode of action is the inhibition of enzyme activity of fungi, by forming a complex with metal-containing enzymes including those involved in the production of adenosine triphosphates (ATP). Calviello *et al* (2006) suggested that Mancozeb caused post apoptotic cell membrane integrity due to its oxidative effect. Mancozeb is a typical multisite protectant fungicide and for this reason use rates and frequency of application need to be relatively high in order to counteract the effect of weathering and plant growth, which can rapidly diminish the protection afforded by the product. It must also be present on the leaf prior to the arrival of fungal spores if it is to be effective and hence it is not as flexible as curative or eradicant fungicides. Mancozeb was first applied after the appearance of spots

therefore, an increase in spots is observed on the weeks that follow however a significant decline in the number of spots was observed on the fifth and sixth week because this is foliage that underwent preventative spraying. Although preventive spraying can eliminate or reduce disease losses, it may result in use of more sprays than are necessary.

In Botswana the Ministry of Agricultural Development and Food Security has a number of fungicides registered for control of fungal pathogens in certain crops. These include among others Metalaxyl, Flusilazole, Terbuconazole, Benomyl, Mancozeb, Copper oxychloride and Pyraclostrobin (Plant Protection, 2016). One of the greatest challenges faced by horticultural farmers in Botswana in the control of *C. beticola* is to determine the best fungicide and time for fungicide application in order to minimize losses. Disease severity is directly related to favorable environmental conditions characterized by extended rainfall (Khan *et al*, 2009) as was the case during the study. For effective control of the disease, monitoring of environmental factors such as rainfall, relative humidity and duration of leaf wetness and scouting for disease is critical. A number of authors have reported the development of resistance in the Benzimidazole class of fungicides, however these provided significantly better results in the control of Cercospora leaf spot (Dexter and Luecke 1999; Karaoglanidis *et al* 2000; Weiland and Halloin, 2001).

CONCLUSION AND RECOMMENDATIONS

The purpose of applying fungicides against fungal pathogens at the recommended dose is to ensure production of large quantities of high quality Swiss chard yield using minimal amounts of active ingredients. It can thus be concluded that Benomyl alternated with Copper oxychloride not only it is significantly effective but it counteracts the development of resistant strains of the *C beticola* fungus which may become problematic in controlling also noting that Copper oxychloride or Mancozeb alone or both lack the post infection activity thus only act as protectant fungicides, killing pathogenic cells on plant surfaces. As a recommendation to farmers, in order to reduce chemical residues on produce/ Swiss chard leaves, potential environmental contamination and the occurrence of fungicide tolerant strains, biocontrol could be researched and used as an alternative.

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