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ARTICLE

Culture Media Options for Growth and Morphological Characterisation of *Cercospora coffeicola* Affecting Coffee in Zimbabwe

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ABSTRACT

Cercospora leaf spot is fast turning into a critically important disease in Zimbabwe. The disease is caused by *Cercospora coffeicola* which significantly reduces productivity and quality of coffee. Disturbingly, optimum sporulation of *Cercospora coffeicola* in culture remains a limiting factor for microbial analysis and quantitative studies of *Cercospora* leaf spot. Faced with this challenge, an in-vitro study was conducted at Coffee Research Institute, Manicaland, Zimbabwe to examine growth of *Cercospora coffeicola* in different nutrient media and to determine the best media for *Cercospora coffeicola* analysis. Six nutrient media were assessed (corn meal agar, oat meal agar, Czapek Dox agar, malt extract agar, yeast extract agar and potato dextrose agar) for the growth of *Cercospora coffeicola*. The laboratory-based experiment was duplicated, laid out in a Completely Randomized Design, replicated three times and based on *Cercospora coffeicola* nutrient inoculation. Data were collected on radial growth, colour and texture of mycelium at 3 and 6 days after inoculation. There were significant differences ($p < 0.05$) in the growth of *Cercospora coffeicola* in media after 3 and 6 days. Malt extract agar had the greatest radial growth (34 mm and 32 mm) of *Cercospora coffeicola* for trials 1 and 2 respectively, whilst the least growth was in the oat meal agar (14.2 mm and 15.7 mm) for trials 1 and 2 respectively. There were variations in colour and texture of mycelium with malt extract agar, potato dextrose agar and oat meal agar associated with darker colours and rough texture while smooth white mycelia were found in corn meal agar. After considering all nutrient media, malt extract agar was found to be the best media for the growth of *Cercospora coffeicola* in-vitro. On the basis of our findings, the authors recommend the use of malt extract agar as the primary media for identification and characterisation of *Cercospora coffeicola.*

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1. Introduction

Coffee (*Coffea arabica*) is currently one of the best incomes generating socio economic crops across the world for both the smallholder and large-scale commercial farmers. It is one of the most traded crop in the world and the most valuable primary agricultural product in international trade [1,2]. The production of coffee has significant positive reflection on economic growth of many developing and least developed countries in Sub-Saharan Africa, South East Asia, Central and Southern America [3]. It is estimated that there are about three billion coffee trees supplying the needs of the coffee industry worldwide [4,5]. Accordingly, the production of the crop has improved livelihoods of many smallholder farmers and farm workers who solely rely on the valuable commodity for their livelihoods. In addition, the crop due to its perennial nature is very important in climate change mitigation through the provision of carbon sinks across many agricultural landscapes [6]. It is also important in soil erosion management, offering environmental buffering capacity and general ecosystem services [7]. Zimbabwe exports well over 95% of its total production [8], which contributes significantly to the country's total gross domestic product (GDP).

Studies have demonstrated that production challenges in the coffee sector are increasingly becoming more pronounced, extended and severe because of climate change and variability [9,10]. Frequencies of droughts, floods, winds, and disease incidences have negatively impacted on coffee production hence threatening to wipe away the valuable source of livelihoods for many beneficiaries [11,12]. Notably, climate change has increased incidences and severity of pests and diseases, in addition to water stresses, nutrition limitations accelerated by low technology adoption to cope with the challenges.

Although Coffee Leaf Rust (*Hemileia vastatrix*) and Coffee Bark Disease (*Fusarium lateritium*) are among the major constrains to Coffee production in Zimbabwe, *Cercospora* leaf spot caused by *Cercospora coffeicola (C. Coffeicola)*have significantly contributed to reduced coffee yields in Zimbabwe [13]. The *Cercospora* leaf spot disease is one of the oldest and was first reported in 1881 in Jamaica. The disease affects coffee plant growth, cherry yield and also bean quality [14,15].

The symptoms on leaves are circular spots with grey, or white centres and lesions on berries and leaves which are initially brown in colour, sunken, oval in shape, with ashy centres [14,39,15]. *Cercospora* leaf spot and Berry blotch are also referred to as two phases of the same disease. Damage to leaves will lead to defoliation, reduced photosynthetic leaf area and loss of plant vigour [16]. Stressful environment predisposes the coffee trees to attack by *C. Coffeicola* [17]. The bean quality is spoiled by a discolouration symptom which deteriorates the quality of the bean. Under field conditions, Cercospora leaf spot is managed by routine copper based fungicide sprays; a contact fungicide which requires routine sprays which may lead to copper toxicity [18-20]. Several management tactics have been employed to manage the disease which include growing coffee under shade, fertilisation of coffee [21], green manuring in combination with urea treatment [22], were also found to be important in reducing the effects of the disease.

Historically, *Cercospora* of coffee has been considered a minor disease owing to its sporadic nature, confinement to nurseries and low severity ratings recorded in coffee plantations. The occurrence of this disease has also been associated with low management practices such as poor nutrition, water stress and high pest infestation levels [21]. However, in recent years, the incidences and severity of the disease have increased dramatically in all the coffee production zones, mainly in the eastern parts of Zimbabwe [23]. The disease is becoming even more severe with climate change; warm humid summers and the perennial nature of coffee, which can keep inoculum in the field for more than 30 years as a monoculture. Significant losses have been observed within the smallholder communities of Zimbabwe due to the disease [23]. According to Bernardo [24], significant damages caused by *Cercospora* of coffee can be reflected in losses ranging from 15% to 30% in plantations, implying the devastating nature of the pathogen.

Since the disease is assuming importance, the need for early and proper identification is key to avoid significant economic losses. The identification of the pathogen is at times confounded with nutrient deficiency and or water stress [21]. This is also happening because there are no developed ready standards for rapid *Cercospora* identification under laboratory conditions. Limited information is available concerning the biology of the pathogen. It is therefore very important to understand the behaviour of the pathogen under laboratory conditions for proper and early diagnosis.

Conventional pathogen culturing methods have been used for a long period and are regarded as the gold standard procedures in pathogen identification due to their simplicity, low cost, efficiency, sensitivity and reliability over a range of applications with no need for high throughput equipment [25]. This makes them an important step for detection and enumeration of pathogens for various phenotypic and genotypic predictions and analysis. The methods

utilize selective media using traditional methodologies under aseptic techniques [26].

There are no specific protocols and suitable media documented for laboratory identification of the *C. coffeicola* on synthetic media. This is the first study which evaluated the response of *C. coffeicola* under laboratory conditions. Potato Dextrose agar has been used to culture *Cercospora* of coffee under laboratory conditions. However, slow mycelia growth has generally compromised the epidemiology process. In addition, difficulty in isolation, obtaining abundant sporulation in the culturing of many species of *Cercospora* remains a limiting factor for quantitative studies of these diseases [27]. It is therefore important to develop standard identification protocols for the pathogen, which will enhance the understanding of its etiology, biology and management options. The study therefore seeks to determine the most suitable media that enhance *Cercospora* mycelia growth under laboratory conditions.

2. Materials and Methods

2.1 Study Sites

The study was carried out at Coffee Research Institute (CoRI), Chipinge, Zimbabwe (latitude 20°12' south and longitude 32°37' east at an altitude of 1100 m above sea level) in the Plant Pathology laboratory. The mean maximum temperature is 20 °C and mean minimum temperature is 14 °C.

2.2 Experimental Design

Two laboratory experiments were laid out in a Completely Randomised Design (CRD) with six media as treatments. The six media treatments were: (i) Corn meal agar (CMA), (ii) Oat meal agar (OMA), (iii) Czapek Dox agar (CDA), (iv) Malt extract agar (MEA), (v) Yeast extract agar (YEA) and (vi) Potato dextrose agar (PDA) which was the standard. The treatments were replicated three (3) times, with three (3) Petri dishes used as a single plot per treatment.

2.3 Culture Media Preparation

In the preparation of the different culture media, the following procedures were followed.

2.3.1 Oatmeal Agar

Seventy-two grams (72 g) of oatmeal agar was measured and suspended in 1000 mL distilled water. The mixture was heated and stirred until the agar was evenly distributed in the distilled water and then autoclaved at 121 °C for 15 minutes.

2.3.2 Cornmeal Agar

Seventeen grams (17 g) of cornmeal agar powder was suspended in 1000 mL of distilled water. The medium was heated and stirred to dissolve the powder completely. Then, the medium was autoclaved at 121 °C for 15 minutes.

2.3.3 Czapek Dox Agar

Forty-nine grams (49 g) of Czapek Dox agar powder was suspended in 1000 mL of distilled water. The medium was heated and stirred to dissolve the powder completely. Then, the medium was autoclaved at 121 °C for 15 minutes.

2.3.4 Malt Extract Agar

Fifty grams (50 g) of malt extract agar powder was suspended in 1000 mL of distilled water. The medium was heated and stirred to dissolve the powder completely. Then, the medium was autoclaved at 121 °C for 15 minutes.

2.3.5 Yeast Extract Agar

Twenty-three grams (23 g) of yeast extract agar powder was suspended in 1000 mL of distilled water. The medium was heated and stirred to dissolve the powder completely. Then, the medium was autoclaved at 121 °C for 15 minutes.

2.3.6 Potato Dextrose Agar

Thirty-nine grams (39 g) of Potato Dextrose agar was suspended in 1000 mL of distilled water, dissolved in water and autoclaved at 121 °C for 15 minutes.

2.4 Pathogen Isolation

C. coffeicola is a coffee pathogen which exists in the plantation throughout the whole season. Cercospora infected leaf samples were collected at Coffee Research Institute farm during the months of March when the highest infection levels are experienced in Zimbabwe. The infected leaf samples were packed in paper packages and transferred to the laboratory. In the laboratory, one centimeter sections containing half diseased and half health leaf tissue were cut and surface sterilized in 30% sodium hypochlorite solution for about 30 seconds before rinsing three times in distilled water to remove some opportunistic pathogens. The sample was dried on damp filter paper chamber on the laminar airflow. The

diseased portions were then transferred to 3 (three) Petri dishes containing PDA. The inoculated Petri dishes were incubated for about 48 hours at a temperature of 25 °C in the constant temperature room, and transferred to the laboratory benches after two days. Cercosporacolonies that developed were characterized, selected and isolated after 4-5 days. The procedure of isolation from PDA, incubation and re-isolation was repeated three times until a pure culture of Cercospora remains in the Petri dishes. On inoculation into the different media treatments, a piece of agar block, approximately 5 × 3 mm in diameter, was cut from a 7-day old PDA culture and transferred into each individual treatment agar (Corn meal agar, Oat meal agar, Czapek Dox agar, Malt extract agar, Yeast extract agar and Potato dextrose agar). The plates were closed tightly and sealed with parafilm.

2.5 Data Collection and Analysis

Data were collected on radial growth estimated by measuring the radius of each colony with a ruler from the centre of the Petri dish along two perpendicular axes (four measurements per dish) at the intervals of 24 hours. Data were collected until the fastest growing treatment reached the perimeter of the Petri dishes. This data was used to calculate the daily rate of growth (mm/day). Data were also recorded on mycelia colour, and texture using a Likert scale where 1 = smooth, 2 = medium and 3 = rough. The data were subjected to the analysis of variance using Genstat 18th edition. The means were separated using the LSD test. Graphs on the growth of Cercospora in different media was plotted using Microsoft Excel.

3. Results

3.1 Growth Characteristics of *C. coffeicola* at 3 and 6 Days after Inoculation (DAI)

The effect of different culture media on radial growth of *C. coffeicola* after 3 and 6 days of incubation are summarised in Table 1. There were significant differences ($p < 0.001$) in mycelia growth due to the effect of different media. Malt extract agar gave the best pathogen growth which had 34.33 mm and 32.00 mm for experiments 1 and 2 respectively, and was significantly different from all the other treatments. Second best medium was Corn meal agar with 28.17 mm in experiment 1 and was not significantly from Czapek Dox and Potato Dextrose agar. In the second experiment 2, PDA gave the second-best growth with 29.17 mm. The least growth of the pathogen was observed in the Oat meal agar for both experiment 1 and 2. In terms of texture Corn Meal Agar showed smooth mycelium while Yeast Extract agar and Czapek Dox Agar had moderate mycelium and Potato Dextrose Agar, Oat Meal Agar and Malt Extract Agar had rougher textured mycelium in appearance in the Petri dishes. In terms of colour deviations, mycelium appears whitish in Corn Meal Agar, light brown in Yeast Extract agar and Czapek Dox Agar. Darker brown colour appears in Potato Dextrose Agar, Oat Meal Agar and Malt. These results are summarised in Table 1.

Table 1. Media options on the growth characteristics of *Cercospora coffeicola*

Treatment	Radial growth				Texture	colour
	DAI 3 (mm)		DAI 6 (mm)			
	Trial 1	Trial 2	Trial 1	Trial 2	Trials 1 and 2	Trials 1 and 2
Corn meal agar	14.5bc	18.3c	27.8b	30.5c	Smooth	White
Oat meal agar	10.0d	11.5f	13.7d	17.8e	Course	Dark brown
Czapek Dox agar	13.0c	17.0d	27.7b	31.5c	Medium	Light brown
Malt extract agar	17.7a	21.3a	34.0a	37.2a	Course	Dark brown
Yeast extract agar	12.8c	15.7e	20.7c	20.8d	Medium	Light brown
Potato dextrose agar	15.2b	19.5b	25.3b	33.8b	Course	Dark brown
P. value	< 0.001	< 0.001	< 0.001	< 0.001	-	-
Grand mean	13.9	17.2	24.9	28.6	-	-
C.V (%)	10.8	5.60	15.3	4.70	-	-
LSD	1.77	1.14	4.50	1.60	-	-

*Means in the same column with different letters are different according to the LSD technique

3.2 Mean Mycelia Growth of Cercospora for Trials 1 and 2

Two experiments were conducted to validate the effect of different media on growth of coffee *Cercospora*. Overall malt extract agar had the highest mean radial growth which differed significantly from the other media treatments ($p < 0.0001$). The least growth was obtained in oat meal agar for both experiments (Figure 1). Mean radial growth for trials 1 and 2 were not significantly ($p < 0.05$) in all nutrient media.

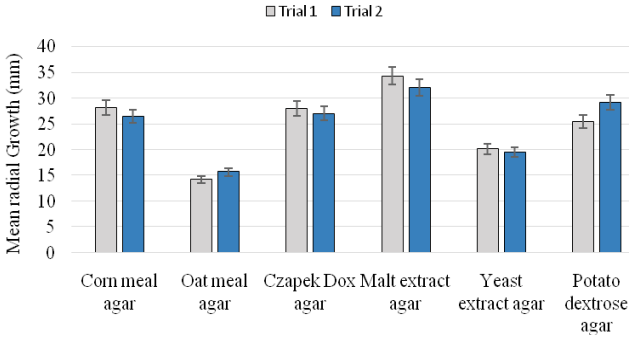

Figure 1. Mean radial growth in different media for the two experiments.

3.3 Percentage Differences in Growth between the Media Treatments against PDA (Standard)

Figure 2 is showing the percentage differences for the different media in relation to the standard media, PDA. Malt extract agar had the greatest positive difference which was 35.5% and 9.4% for trials 1 and 2 respectively. On the other hand, oat meal agar had negative percentage differences of 44% and 46% for trials 1 and 2 respectively.

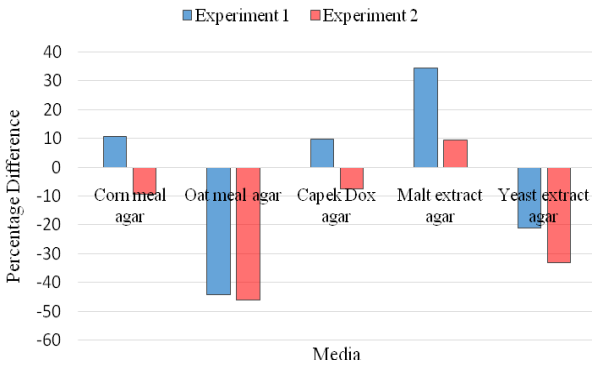

Figure 2. Percentage difference in radial growth as compared with the standard (PDA)

3.4 Nutrient Media Options and Growth Rate of *C. coffeicola*

In trials 1 and 2, there were significant differences in mycelia growth rate due to the effect of different media ($p < 0.001$). Malt extract agar gave the best growth rate of the pathogen (*C. coffeicola*) for both trials (Table 2) and was significantly different ($p < 0.05$) from all other nutrient media. The second-best medium was Corn meal agar (4.64 mm) at 5 days after inoculation and CDA after seven days (4.86 mm) in the first trial. In the second trial, PDA gave the second-best growth 6 and 7 days after inoculation with 5.64 mm and 5.83 mm respectively. The least growth of the pathogen was observed in the Oat meal agar for both experiments throughout the experimental periods.

Table 2. Nutrient media options and growth rate of *C. coffeicola*

Treatment	Mycelium growth rate (mm/day)			
	5 DAI	6 DA1	7 DAI	
	Trial 1	Trial 2	Trial 1	Trial 2
Corn meal agar	4.64b	5.083c	4.62b	5.214d
Oat meal agar	2.28d	2.972e	2.29d	2.905f
Czapek Dox agar	4.61b	5.250c	4.86b	5.571c
Malt meal agar	5.68a	6.194a	5.93a	6.262a
Yeast extract agar	3.21c	3.472d	3.44c	3.476e
Potato dextrose agar	4.12b	5.639b	4.22b	5.833b
P. value	< 0.001	< 0.001	< 0.001	< 0.001
Grand mean	4.14	4.77	4.18	4.88
C.V (%)	15.3	4.7	16.60	3.5
LSD	0.75	0.27	0.82	0.20

*Means with different letters in same column are different according to the LSD technique

3.5 Area under Disease Progress Curves for Experiments 1 and 2

According to the area under disease progress curve (Figures 3 and 4), Malt extract agar had the highest growth for *Cercospora.* Second best was the Czapek Dox agar while least growth was observed from Oatmeal agar for both trials throughout the experimental periods. Malt extract agar supported the best growth of the pathogen in the second experiment and PDA was second best media.

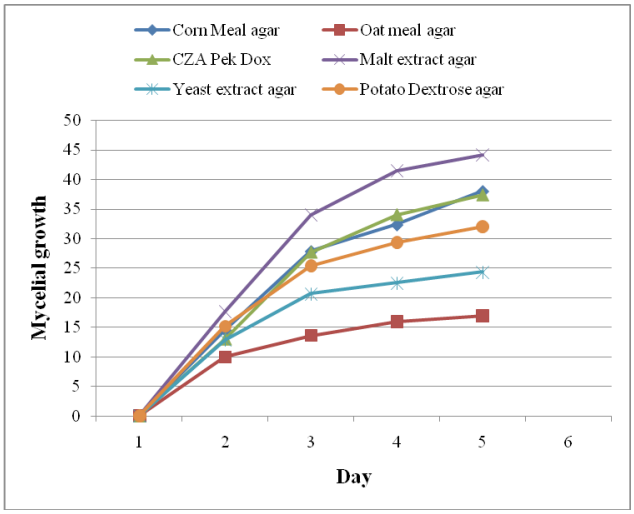

Figure 3. Area Under Disease Progress Curve of the experiment 1.

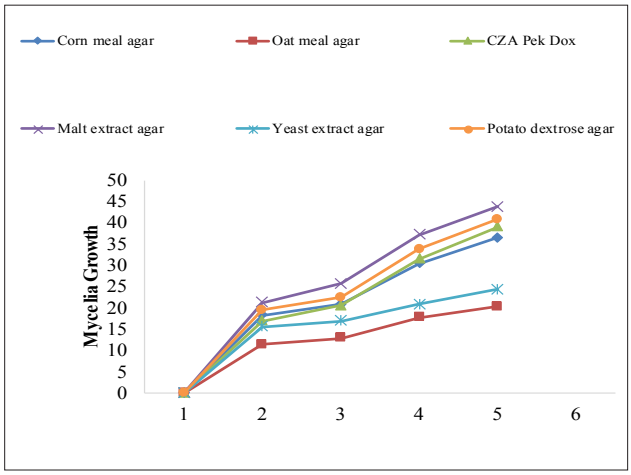

Figure 4. Area Under Disease Progress Curve of the experiment 2.

4. Discussion

Malt extract agar (MEA) had the highest growth of *Cercospora coffeicola* and its performance was significantly different from the rest of the other media treatments, suggesting an excellent support media for early identification of *Cercospora* under laboratory conditions. Early identification of *Cercospora* in the field and the laboratory is key for the implementation of control and regulatory procedures for plant pathogens before crop damage [28,29]. Differences in growth of the pathogen in the different media can be attributed to the variations in the nutritional profiles of the different media [30]. Based on experiments performed, it is the nature and concentration of nitrogen and carbon source and the ratio of C/N that influence fungal growth and sporulation [31,32]. Malt Extract Agar (MEA) contains a high concentration of maltose which makes it suitable for the growth of fungi and molds. Generally, MEA is used as a general-purpose growth media to isolate and cultivate yeasts and molds from a wide range of environmental sources. It contains carbon, protein and nutrient sources essential for fungi growth [31]. Additionally, MEA contains digests of animal tissues (peptones) which provide significant quantities of amino acids and nitrogenous compounds for the growth of *Cercospora coffeicola*. The vegetative growth of a fungus e.g. *Cercospora coffeicola*, lies in its ability to utilize and exploit nitrate, ammonium, and organic sources of nitrogen.

It was interesting to note that MEA had a fastest growth of mycelium with a 34% higher growth than PDA and the findings further indicates that MEA provides conditions allows for faster growth (0.5 to 2 days quicker) in growth than the rest of the other media. This allows timeous crop pathogens identification. On the contrary, oatmeal agar had the slowest growth taking at least 2 days to achieve the same radial growth as MEA. A similar study was conducted by Surendra [16], who observed that *C. arachidicola* of groundnuts takes a maximum of 7 to 10 days to achieve maximum growth in artificial media. This is in agreement with our observations in which MEA reached maximum growth in 7 days after inoculation. In a study by Poornima and Yashoda [33], twelve solid media were evaluated and maximum growth of *C. beticola* was observed on PDA (89.66 mm) and Oat meal agar (81.67 mm), with MEA following behind (79.67 mm). These results are contrary to the findings of this current study, where MEA was the best followed by PDA, with oat meal agar being the least in *Cercospora coffeicola* growth. In another experiment to evaluate the effect of media and light exposure on sporulation of *Cercospora zea maydis*, it was observed that more conidia were produced in V8 agar media when compared with Potato Dextrose agar, tomato juice, coconut water, oat, maize leaf extract [34]. Various *Cercospora* species have different growth performances in different media. However, it is therefore central to understand how each species grows in different media for selection of an optimum growth media.

Corn meal and PDA were the second-best media and this observation was in contradiction with the norm where PDA is generally known as the default culture media for fungi cultures. Studies have shown that growth media for fungi should contain enough sources of carbon (C) and nitrogen (N) required for growth and reproduction [31]. Potato Dextrose Agar (PDA) is composed of dehydrated potato infusion and dextrose that encourage luxuriant fungal growth, with agar as the solidifying agent.

Surendra [16] observed maximum growth of *Cercospora* of groundnuts in Potato dextrose agar better than in oat meal agar. This corroborates with the findings of this study since potato-based media performed better than oatmeal agar. Corn meal agar was second best in experiment 2, performing equally the same with PDA and Czapek Dox agar. Notably, fungal species have different patterns and properties in various probable culture media. *Fusarium oxysporum* grew best in Czapek Dox agar and PDA [35], while *Fusarium adun* also grew best in PDA when compared with Potato sucrose agar, Oatmeal agar, V8-juice agar, Leaf extract agar, Carrot juice agar and Peanut hull extract agar [36]. *Alternaria solani* was found to grow best in Potato Dextrose agar and oat meal agar when tested among different solid and liquid media [37]. Potato Dextrose agar and Potato Dextrose Broth were found to be more favourable to *Fusarium moniliforme* when compared to some solid and liquid media respectively [38]. In one study, growth performances of 30 fungal isolates were examined on different growth media and largest number of isolates significantly grow on malt yeast extract [41]. Here growth was supported by nutrients such as vitamins B1, and B12 which normally support mycelia development.This could be the reasons for growth patterns of *Cercospora coffeicola in culture media.*

Different media produced different colony colours and texture, which ranged from smooth to rough while colours ranged from while generated by corn meal agar to dark brown from MEA and PDA. Malt extract agar is therefore recommended for effective culturing of *Cercospora coffeicola* under laboratory conditions amongst the studied media. This is the first report on studying the growth of *Cercospora* under laboratory conditions. This has implications for the epidemiology of the disease going ahead, forming the basis for morphological and molecular identification of the pathogen to understand.

The trend of the area under disease progress curves showed that Malt extract agar supported maximum growth for the entire experimental periods for both experiments 1 and 2. Czapek Dox agar was second in the experiment 1 while in experiment 2, PDA was the second best after Malt extract agar. The present study results indicated that Malt extract agar proved to be the most suitable media for optimum growth of the pathogen under laboratory experiments. The results of this study contradicts the findings by Surendra [16], who reported that PDA performed the least among other seven media in the growth of *Cercospora arachidicola* of groundnuts. This implies different pathogens in different genera prefer different media for optimum growth.

5. Conclusions and Recommendations

The method used in this study is very simple, low cost, efficient considering resources and reliability over a long period of time [25]. However, it is important to understand that the methods are generally basic, and may need to be complimented by more robust and high throughput technologies. Biochemical and molecular identification methods can be used to fully characterize the pathogen. These methods may be quicker and more reliable as compared to the conventional culture media identification methods [29]. The results of the current study revealed that culture media differed in influencing growth and colony characters of *Cercospora coffeicola*. Out of the six-culture media tested in this study, Malt extract agar was found to be the best suitable media in radial growth of *Cercospora* of coffee. However, potato dextrose agar, corn meal agar and Czapek Dox Agar may be used conservatively for routine cultural and morphological characterisation of *Cercospora coffeicola*. In resource constrained settings we recommend the use of MEA culture-based methods for rapid identification of *Cercospora* of coffee.

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Author Contributions

This work was carried out in collaboration between all authors. Authors NM and CM designed the study, wrote the protocol and wrote the first draft of the manuscript. Authors PC and ZM managed the literature searches and analysis of the study. Author DK managed the experimental process. All authors read and approved the final manuscript.

Conflict of Interest

The authors report no conflict of interest.

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