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Morphological and Cultural Characterization of Cercospora zeae-maydis Tehon and Daniels Isolates in Western Oromia Region, Ethiopia

Midekssa Dida[1]* Fikre Lemessa[2] Gezahegn Berecha[2]
1.Ethiopian Institute of Agricultural Research, Bako National Maize Research Center
2.Departments of Horticulture and Plant Sciences Jimma University P.O. Box 307 Jimma, Ethiopia

Abstract
Maize (Zea mays) is one of the most widely cultivated crops in the world. It occupies an important position in the world economy serving as food, feed, and industrial grain crop. Grey leaf spot (Cercospora zeae-maydis) is one of the major foliar diseases threatening maize production in Ethiopia. Most of the researchers estimated that losses as high as 100% occurred when the pathogen attacked before the flowering stage. Also, in Ethiopia the loss caused due to this disease reached 49.5%.Therefore, the objective of this paper was to study morphological and cultural characterization, of maize Grey leaf spot isolates. Morphological and cultural characterization studies of the 5 isolates were done at JUCAVM Plant Pathology lab and greenhouse, respectively. From 155 samples collected during the assessment, 52 isolates were re-cultured and grouped into 5 isolates. Accordingly Colony color showed (grey, light, brown, corn silk and white) on top, and (dark grey, brown, cornsilk3 and grey) on the reverse. Similarly, colony shape (round and irregular) Colony elevations were (Entire & Undulate) whereas Colony edges were Flat & Umbonate). Based on Conidial shape and number of septa isolates LD-G and DN-H were showed Slightly curved & isolates GS-O, IG-3, and LA-Ay were Straight, The highest number of septa was recorded on isolate LD-G with 6 and the minimum was recorded on DN-H with 4. Knowing the cultural and conidial characteristics of different isolates of C. zeae-mayydis is very important in the development of management option of the disease. Molecular diagnostics is very crucial to confirm their specific morphological differences and to design appropriate management options.
Keywords: morphological characterization Cercospora zeae-mayds, isolates
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Introduction

Maize (*Zea mays*) is one of the most widely cultivated crops in the world. It is one of the three most popular cereal crops next to wheat, and rice in the world (FAOSTAT, 2016). Maize occupies an important position in world economy as a food, feed and an industrial grain crop (Tolesa *et al*., 1993). It serves as a vital source of proteins, calories and some of the important vitamins and minerals to billions of people worldwide, particularly in Africa, South America and Asia, and has been considered a 'poor man's nutricereal' (Prasanna *et al*. 2001).
Maize is grown worldwide approximately on 226.94 million hectares (ha) annually with the production of 1291.94 million metric tons (FAOSTAT, 2018). In sub-Saharan Africa, maize is produced in an estimated area of about 26 million ha with an average of 460 million metric tons. In East Africa, maize occupies about 17 million of production area and 30.4 million tons of total production and with 1.8 tons per ha (FAOSTAT, 2016).
In Ethiopia, maize is the second largest food security crop after teff, (*Eragrostisteff* (Zucc.) Trotter. It is primarily produced and consumed by the small-scale farmers that comprise about 80% of Ethiopia's population (Alemu *et al.*, 2008).

Currently, about 2.13 million ha of land is covered by maize with an average production of 8.39 million tons, and the average national yield of maize is very low under small-scale farmers, which is 3.94 t/ha in the country (CSA 2018). However, it is very low as compared to the potential of maize (8 – 11 t ha-1) in the high rainfall and irrigated areas and also low as compared to world average productivity 5.78 ton ha-1 (USDA-FAS 2017).

A significant portion of this yield gap is attributable to the losses caused due to biotic and abiotic stresses. The major abiotic factors include nutrient deficiency and drought stress, which could be aggravated by land degradation. Among biotic constraints, foliar diseases play a major role in contributing to the reduction of maize production and productivity across the world (Berger *et al*., 2014; Masuka *et al*., 2017).

The major diseases include Turcicum leaf blight (*Exserohilum turcicum* (Pass) Leonard & Suggs), grey leaf spot (*Cercospora zeae-maydis* Tehon & Daniels), leaf rust (*Puccinia sorghi* Schr.), maize streak virus disease (Wegary *et al.*, 2001; Tilahun *et al.*, 2012). A foliar disease, particularly GLS caused by *Cercospora zeae-maydis* (Tehon and Daniels, 1925) is one of the necrotrophic and polycyclic foliar diseases of maize that poses a serious problem to tropical maize production (Renfro and Ullstrup, 1996). This pathogen causes intense water loss from the plant thereby leading to severe blighting of the leaves and reduced photosynthesis. This eventually leads to undersized ears, low grain yield and premature death of maize plants. Severe blighting of the upper eight or nine leaves that contribute 75 to 90% of the photosynthates for grain fill may lead to stalk weakening or even infectious stalk rot diseases leading to premature stalk death and lodging (Lipps *et al.,* 1996: Ward *et al.*. 1999).

In Ethiopia, a major epidemic occurred in the early 2000s and made considerable maize grain yield losses 36.9 % and 49.5% and there have been extensively disseminated through severe outbreaks every year, particularly in the warm and humid areas of the country (Tilahun *et al.*, 2012; Negash, 2013).

The potential threat of GLS to maize production was started and identified with a survey carried out during the year 1997-1998 to know the distribution and importance of the disease in most maize growing regions of Ethiopia (Wegary *et al.*, 2001). The severity of grey leaf spot was high in the warm humid maize belt areas of the country adversely affecting farmers who live with limited resources. Currently, GLS, caused by *Cercospora zeae-maydis*, is among the major maize foliar disease in South and Southwest Ethiopia (Nega *et al.*, 2016).

In Ethiopia, Wegary *et al.* (2004) reported that yield loss due to GLS on resistant, moderately resistant and susceptible varieties was between 0-14.9%, 13.7-18.3%, and 20.8-36.9% respectively during 2003/2004 cropping seasons at Bako areas. Similarly, the research carried out at South Ethiopia in the year 2004-2006 showed that the yield loss due to GLS is 29.5% (Tilahun *et al.*, 2012).

The survey study showed that the highest GLS prevalence, incidence and severity were 74%, 71.2% and 45.13% respectively in South and Southwest Ethiopia. Similarly, morphological characterization of GLS isolates were carried out and 10 different isolates were identified (Nega *et al*., 2016). Therefore, this study showed the importance of the pathogen distribution and diversity of the isolates in the country. Even though Western Oromia is a potential to produce maize crop, importance of the pathogen is not assessed and determined to take a measure.

Studies have been conducted on major foliar diseases of maize since late 1990's in the western part of the country (Wegary *et al*., 2001). However, there was little indicated specifically for cultural and morphological characterization of Grey leaf spot isolates in the study area. Therefore, the objective of this study was to identify and morphologically and culturally characterize *Cercospora zeae-maydis* isolates associated with Grey Leaf Spot of maize in Western Oromia

2. Materials and Method

2.1 Isolation, Identification, and Characterization

2.1.1 Isolation and Culture of *Cercosporra zeae-maydis* Isolates

A total of 155 leaf samples obtained from the study area were air dried and thereafter kept in a refrigerator at 4 °C. Three lesions were cut out from each leaf sample, surface sterilized using 5% sodium hypochlorite solution for 30 Sec and rinsed three times in sterile distilled water, placed on moist filter paper in a Petri dish and incubated for 5 days to stimulate sporulation (Asea *et al*., 2005). Conidia were dislodged from the lesions by adding sterilized distilled water and hand shook. Samples were placed on 3% Water Agar plates and incubated for 48 hours. Mono-conidial cultures of the isolates were then established by sub-culturing germinated distinct colony characteristics of *C. zeae-maydis to* fresh potato dextrose agar plates amended with 250mg/500ml chloramphenicol when the medium cool down to 50°C.Then the culture-dishes were sealed using parafilm and labeled and incubated at 25 °C for 7 daysunder alternative 12hrs dark and white light to allow the pathogen to grow and sporulate (Beckman and Payne, 1983). The isolates were sub-cultured from single conodium on sterilized PDA medium using hyphal tip transfer from distinct colonies with three replications using Completely Randomized Design (CRD). These hyphal tip purified isolates were used for examination of morphological and microscopic characterization.

2.1.2 Characterization of Cercospora zeae-maydis Isolates

Characterization of *Cercospora zeae-maydis* isolates was done by grouping the culture isolates, after carefully observing the colony features (growth, a color of the top and reverse side) of the culture plates and comparing with the color illustrated on RGB chart (Anonymous, 2013) (Figure 1). The colony shape and elevation were also characterized, and isolate grouping was done and colonial growth diameter was tested on both PDA and Malt Extract Agar (MEA). Similarly, specimen from cultures plate was taken and diluted with sterile water and drops of the suspension and placed on slides and put under a microscope for further identification of the conidia. Finally, identification of macro and micro-conidial features of *Cercospora zeae-maydis* such as conidial shape, and septa per conidia were morphologically examined at 400x magnification using a compound microscope. The purified isolates were kept at 5°C and sub-cultured at some intervals onto fresh PDA medium to maintain fungal viability (Kinyua *et al.*, 2010).

2.1.3 Data collection and Analysis

Data on colony/mycelial growth such as color (pigmentation) shape (form), elevation and margin were recorded, and the colony diameters of every culture of the isolates were recorded. Similarly, conidial shapes/forms, and a number of septa were recorded following Carson, M.L., & Goodman, M.M. 2006 manual on species of *Cercospora zeae-maydis* was used.Analysis of variance (ANOVA) was performed using SAS V 9.2 statistical

SAS (2008).Means were separated using LSD t-test at significance levels of 0.05.

Results and Discussion

Morphological and cultural characteristics of Cercospora zeae-maydis

Of 155 Grey Leaf Spot samples collected from 31 maize fields across Western Oromia, a total of fifty two purified isolates were recovered. Based on the similarity of colony appearance on PDA and conidial characters, all the isolates were grouped into 5 C. zeae-maydis isolates.

Colonial Characteristics

The isolates were then assigned tentative identities by examining their cultural and morphological features such as growth habits, color, elevation, and edge of the fungal colonies were studied. Accordingly, 5 isolates were showed different colony appearance on PDA both at upper and the reverse sides.

Therefore, the upper colors of the two isolates (LD-G, LA-AY) were recorded as Grey color which consists of 38.71% of the total isolates, and the rest three isolates (GS-O, IG-3 and DN-H) were brown, Cornsilk and white gross color which comprises 19.35%, 25.81% and 16.13% of the total isolates, respectively. Similarly, the reverse sides of the two isolates (LD-G, LA-AY) colony colors were recorded as Cornsilk which comprises 41.94% of the total isolates, and the rest three isolates (GS-O, IG-3 DN-H) were recorded as Dark Grey, Cornsilk3 and White smoke which comprised 25.81%, 16.13%, 16.83%, respectively (Table 1). Overall, the study of cultural characteristics showed the existence of variation among the five isolates of *Cercospora zeae-maydis* in colony growth, and colony color. The reason for the variation in morphology could be related to genetic and /or environmental factors. Similarly Colonial gross color appeared cottony and grey in color, with a grayish white cast on colony surface was the result reported by (Kinyua *et al*., 2010). This result also supported by Latterell and Rossi, (1983) who reported that the cultural performances of *Cercospora zeae-maydis* grow on agar media with the dense, sluggish growing colony type characteristics of the genus. Growth character ranges from black, densely sporulating cushion like colonies to white, cottony mycelial growth. Intermediate types include gray, moderately sporulating colonies often with pink, red, or purple pigment, depending on the substrate, due to the formation of cercosporin crystals. Similarly, Lyimo *et al. (*2013) reported that most isolates he identified were grey to light grey colony color.

Table 1. Colony color of C. zeae-maydis isolates collected from western Oromia in 2017

Isolate code	Colonial color on PDA		
	Front view	Reverse view	proportion of isolates (%)
LD-G, LA-AY	Grey	Dark grey	41.94
GS-O	Light brown	Brown	25.81
IG-3	Cornsilk	Cornsilk3	16.13
DN-H	white	Light grey	16.83

LD-G=Leka-Dulecha_ Gudina GS-O= Gobu-Seyo _Ongobo
IG-3=Ilu-Gelan DN-H= Dano_Haro LA-AY=LaLo-Asabi__Yesus

Colony Shape, Edge, and elevation were also used as a means of cultural characteristics of the Isolates. Accordingly, based on colony shape LD-G, LAAY, IG-3 isolates were categorized as round which comprises 61.29% and DN-H, GS-O isolates were having 38.71% of the total isolates had an irregular shape. Similarly, Colony Edge and Elevation were used as means of characterizing the isolates as flat with LD-G, LAAY, IG-3, GSO isolates comprising 80.65% flat and one isolate was Unbonate with 19.5% of the total isolates based on colony elevation and four isolates (83.87%) entire and one isolate (16.13%) undulate based on colony edge (Table 2). Even though, the isolates are with some differences in colonial morphology, they are known to produce the same symptoms on maize. The colonies were compact (hard), dome-like, well raised from the medium surface and dark grey to black in color. Some sectoring was exhibited in culture, whereby whitish-grey mycelial patches developed from the typical grey black colonies (Kinyua *et al*., 2010).

Table 2. Colonial shape, edge, and elevation of isolates of Cercospora. zeae-maydis in 2017

Isolates	Based on Colony	Morphology	proportion of isolates (%)
LD-G, LAAY, IG-3	Shape	Round	61.29
DN-H, GS-O		Irregular	38.71
LD-G, LAAY, IG-3, GSO	Edge	Entire	83.87
DN-H		Undulate	16.13
LD-G, LA-AY, IG-3, GS-O	Elevation	Flat	80.65
DN-H		Umbonate	19.5

The highest colony growth was recorded on LD-G isolate on PDA and MEA having 55.67 mm and 54mm, respectively, followed by DN-H with 54.67mm and 52.33mm on PDA and MEA respectively, whereas the minimum colony diameter growth was recorded on IG-3 and GS-O isolates with 51.67mm and 51.33mm on

PDA and 51mm and 49.67mm on MEA respectively (Table 3). There were statistical differences ($p<0.01$) among the isolates on both media. Overall, the result revealed that there was significant variation between growth on the two media investigated with the highest vegetative growth on PDA and the lowest growth occurred on MEA and also we observed an effect of medium on colony growth. This result is supported by Latterell and Rossi (1974) and Nega et al. (2016) who reported that various media supported different types or degrees of development of erect, or submerged stromata and of sub-spherical bodies containing either macro or micro spermatia of Cercospora zeae-maydis.

Table 3. Effect of PDA and MEA on mycelial growth diameter (mm) of C. zeae-maydis isolates

Isolate	PDA	MEA
DN-H	54.67^{ab}	52.33^{ab}
IG-3	51.67^{ab}	51.00^{ab}
GS –O	51.33^{bc}	49.67^{b}
LD- G	55.67^{a}	54.00^{a}
LA- AY	50.00^{c}	50.67^{b}
CV (%)	4.09	3.17
LSD (0.05)	4.12	3.27

Mean Values in the same letter within a column are not significantly different at 5% Probability level. LSD= Least significant difference.

Conidial Characteristics

Conidial shape and number of septa of the conidia were used as a means of characterizing GLS isolates. Accordingly, three isolates showed straight conidial shape whereas two isolates showed a slightly curved conidial shape. The maximum mean number of septa was 6 and the minimum was 4.7 (Table 4). The mean variation in the shape of conidia and number of septum reveal the presence of variation in the conidial morphological characteristic among 5 isolates of *Cercospora zeae-maydis*. Although the isolates are with some differences in conidial morphology, they are known to produce the same symptoms on maize. Similar to the current study, the result reported by Kinyua *et al*. (2010); Nega *et al*. (2016) indicated, significant variation in the mean number of septa of conidia which ranges (3-10).

The conidial shapes of IG-3, GS-O, LA-Ay isolates were showed straight, whereas LD-G, DN-H isolates were identified to be a slightly curved in shape. Straight and slightly curved, hyaline, subcylindrical in shape, with gradual tapering shape, were observed on IG-3, GS-O, LA-Ay and LD-G, DN-H, respectively (Table 4 and Figure 1). The variation in colony color also backed by variation of the conidia shapes. The widest part of the conidia was around the one-third position along the spore length from the base. This variation might be due to the difference in the agro-ecology from where the pathogen came and/or inherent variation among the isolates. This study is supported by Ward and Nowell, (1997), who reported that the ecological conditions have a consequence on the development of grey leaf spot disease on maize. In addition, there was a comparable difference in conidial shape and number of septa of the *C. zeae-maydis* isolates. Similarly, Donahue *et al.* (1991), also reported that conidial shape and septa can vary among different isolates of maize GLS.

Table 4. Mean number of septum and conidial shape of 5 isolates of C. zeae-maydis collected in 2017

Isolates	Conidial shape and No. of Septum		
	shape	Septum range	Mean septum
DN-H	Slightly curved	3-9	4.7
IG-3	Straight	3-7	5.2
GS –O	Straight	3-8	5.8
LD- G	Slightly curved	3-9	6.0
LA- Ay	Straight	3-7	5.2

Figure1 LD-G Isolate

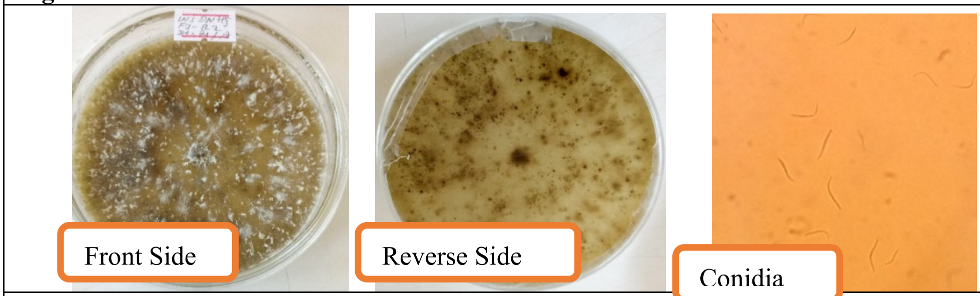

Figure2. DN-H Isolate

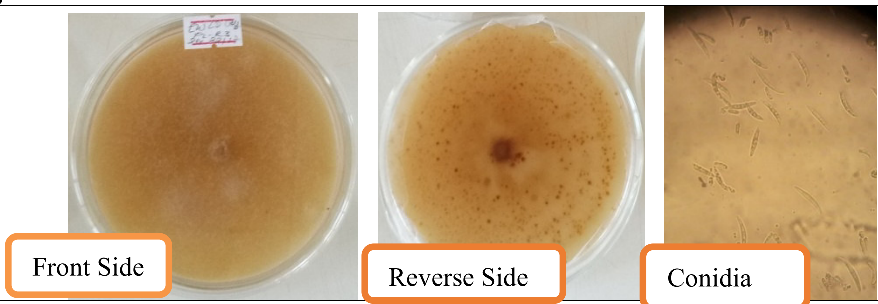

Figure3. IG-3 Isolate

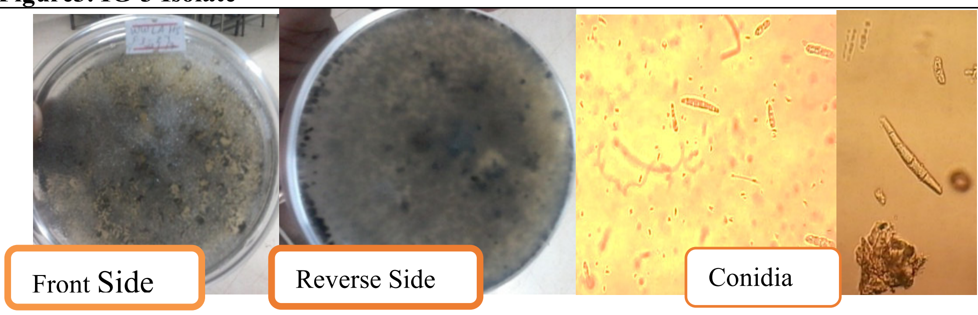

Figure 4. LA-AYIsolate

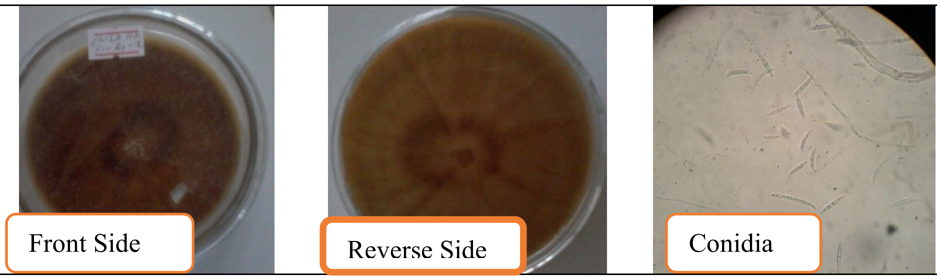

Figure 5. Isolte GS-O

Figure 1. Pictorial illustration of 5 Isolates of C. zeae-maydis collected from western Oromia in 2017.

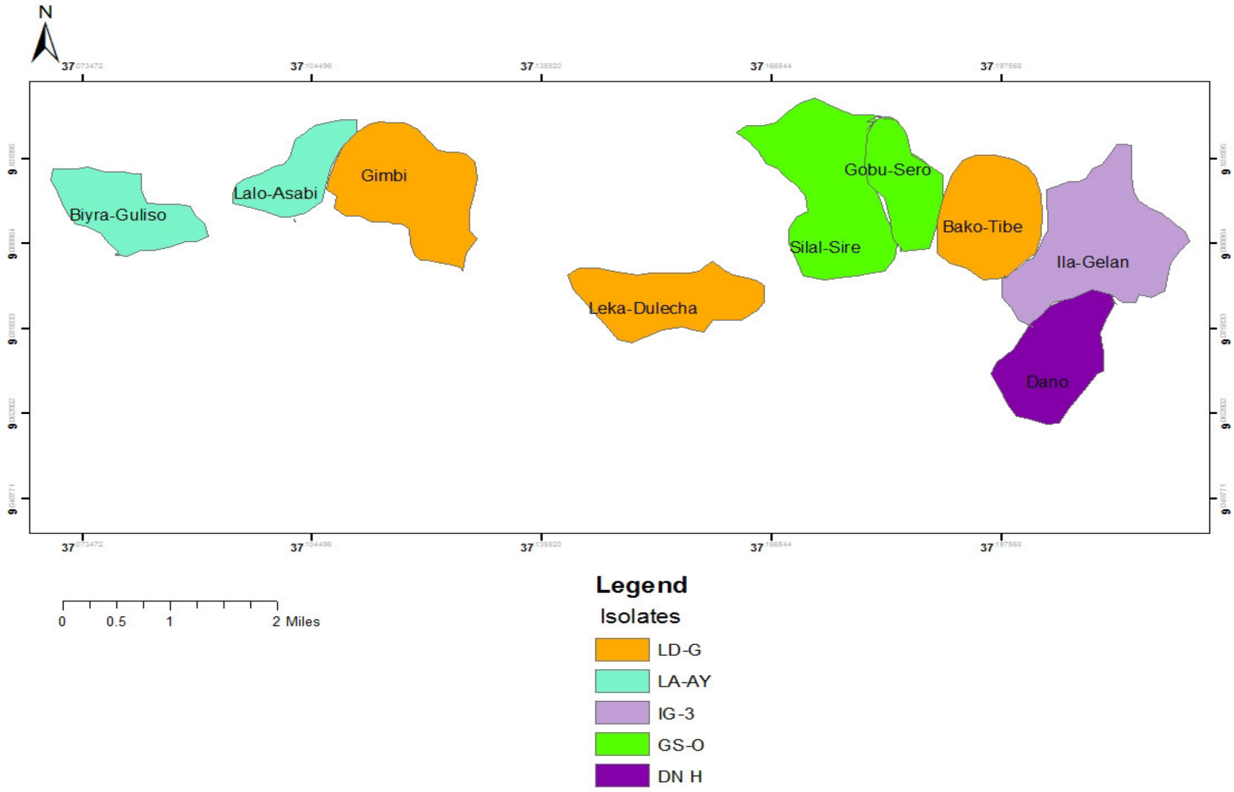

Figure 2. Distribution of maize GLS isolates across surveyed districts in 2017

Conclusion and Recommendation

Laboratory results of morphological characterization showed that, from 155 samples collected during the assessment, fifty two isolates have been re-cultured and grouped into five isolates and the identity was confirmed as DN-H, IG-3 GS-O, and LD-G, LA-AY. Based on the colony color the upper colors of the two isolates (LD-G, LA-AY) were recorded as Grey, whereas isolates GS-O, IG-3 DN-H were showed brown, Cornsilk and white, respectively. There was also a variation among the isolates based on colony shape as round and irregular for (LD-G, LAAY, IG-3) and (DN-H, GS-O) respectively. Additionally, straight conidial shape for (IG-3, GS-O, LA-Ay) isolates and slightly curved for LD-G, DN-H isolates. The result from morphological characterization showed that there are different colonial and conidial growth features among *C. zeae maydis* isolates. Therefore, there is a need to develop effective, affordable and sustainable management strategies to reduce the effect of GLS on maize production in the study area. Molecular diagnostics is very crucial to confirm their specific morphological differences and to design appropriate management options.

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