Use of confocal laser scanning microscopy to locate Stenocarpella maydis in corn stalk (Zea mays)

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Short Report

Keywords: Stalk rot, Alexa488-WGA, Calcofluor White, Fluorochromes, Clarification protocol, Infectious process

Posted Date: July 8th, 2024

DOI: https://doi.org/10.21203/rs.3.rs-4566140/v1

Additional Declarations: No competing interests reported.

Version of Record: A version of this preprint was published at Phytoparasitica on October 24th, 2024.
See the published version at https://doi.org/10.1007/s12600-024-01213-y.

Abstract

Stenocarpella maydis causes high production losses in almost all countries where corn (*Zea mays*) is cultivated. The rot caused by *S. maydis* may occur on the stalks and ears of corn plants. *S. maydis* in corn poses a significant threat to human and animal nutrition, mainly due to mycotoxins such as diplodiatoxin. This study aimed to validate an efficient methodology for visualizing *S. maydis* colonization in corn using clarification protocol, fluorochromes, and Confocal Laser Scanning Microscopy (CLSM). Conidial suspensions were inoculated into the corn stalk at the V6 stage using a syringe. Corn stalk fragments of 1 cm^2 were collected 21 days after inoculation (dai) for CLSM analysis. The samples were fixed in Karnovsky's solution and clarified in KOH and chloral hydrate. The fungal structures were labeled with Alexa488-WGA at 1.0 mg mL^{-1} for 30 min under vacuum (excitation at 488 and emission at 510–540 μm). Thereafter, the corn plant tissues were labeled with Calcofluor White at 0.1 mg mL^{-1} (excitation at 405 and emission at 440–490 μm) for 30 min. The Laser Confocal LSM780 Zeiss Observer Z.1 microscope, LCI Plan-Neofluar 25×/0.8, and C-Apochromat 63×/1.20 objectives were used to acquire fluorescent images. At 21 dai, it was possible to observe the colonization and formation of pycnidia with bicellular conidia of the fungus *S. maydis* in corn stalk fragments. The fungus colonized parenchymatic tissues and vascular bundles of the corn stalk. In contrast, at 21 dai, colonization of the fungus *S. maydis* was not observed in the parenchymatic tissues and vascular bundles of the corn stalk from uninoculated control plants. Our study made it possible to validate a new methodology for studying the infectious process of *S. maydis* in corn stalk using clarification protocols, fluorochromes, and CLSM.

Introduction

Corn (*Zea mays* L.), also known as maize, is one of the most widely cultivated cereals in the world (Lobell et al. 2009). The largest global producers are the United States, China and Brazil. In 2022, Brazilian corn production was 109.4 million tons, occupying third place in the ranking of world production (FAOSTAT 2022). The ascomycete fungus *Stenocarpella maydis*, the causal agent of Diplodia ear and stalk rot, poses a significant threat to corn production and is present in nearly all countries where corn is cultivated. Serious threats to mycotoxin contamination in corn also extend to ear and stalk rot, mainly because corn is an essential cereal for human and animal nutrition, and mycotoxins as diplodiatoxin have been isolated from *S. maydis* infected cultures (Rossouw et al. 2009; Odriozola et al. 2005; Masango et al. 2015). The diplodiatoxin was found in *S. maydis* infected seeds and diseased stalk tissues (Wicklow et al. 2011). Diplodiatoxin is an unregulated neuromycotoxin affecting mainly cattle and sheep and has been associated with diplodiosis outbreaks in South Africa, Argentina, and Brazil (Kellerman et al. 1991; Riet-Correa et al. 2013).

Stenocarpella maydis is not capable of infecting and colonizing important cereal crops other than corn (Flett et al. 1991). The only other host reported for *S. maydis* was on *Arundinaria* sp. (Sutton and Waterston 1966). Over the years, in corn fields, ear and stalk rot outbreaks have become increasingly frequent and more severe resulting in a corn yield reduction. Corn plants exhibiting ear rot and stalk rot may have significant yield losses from the presence of light-weight, rotted ears with discolored, shriveled,

and non-viable seeds. Mueller et al. (2016) demonstrated that ear rot caused by *S. maydis* was responsible for significant yield losses, estimated at 3.7 million metric tons in 24 states of United States and the Canadian province of Ontario in 2016. The increase of *S. maydis* in corn fields is associated with the reduction of practices such as soil preparation and crop rotations (Flett et al. 1998; Flett et al. 2001). Another crucial aspect in the occurrence of this disease is the weather. *S. maydis* is well adapted to cooler regions because the conidia lose their viability when exposed to high temperatures and sunlight (McGee et al 1988; Bensch and van Staden 1992). In addition, milder temperatures may contribute to the increase in the virulence of *S. maydis* on corn cobs, especially during the grain-filling phase (Vincelli 2011).

The infection process can begin through the *S. maydis* conidia deposition on the leaf attachments of the corn stalk (Durrel 1923). Furthermore, ear rot infections may occur via conidia invading the ear tips and the ear leaf shank at the base of the ear shank development (Koehler 1942). On corn stalk and leaf sheath, appressoria are formed on hyphal tips 72 h after inoculation, and the penetrating hyphae result in inter- and intracellular colonization (Bensch and van Staden, 1992). The infection and colonization modes of *S maydis* on corn tissues was also described by Bensch (1995); Bensch and van Staden (1992); Chambers (1988) and Xia (2011). The initial symptoms of ear infection appear as yellowing and drying ear bracts on green corn plants. Infected corn stalk shows the internodes with a dark brown color. Two weeks after corn silk appears, the infected cobs become entirely mummified and covered with a white to slightly gray-brown mycelium with light and without dull grains. Corn stalk rot compromises not only the translocation of water, but also the nutrients from the soil to the plant's aerial organs, reducing potential yield and grain quality (Denti and Reis 2003). To exacerbate the situation, plants with stalk rot may present premature death, lodging, and stalk breakage (White 1999; Reis et al. 2003).

Detailed studies of the infection and colonization of *S. maydis* on seeds' tissues have previously been reported using fluorescence microscopy and scanning electron microscopy. Isolates of *S. maydis* transformed with the gene expressing for GFP (green fluorescent protein) and DsRed have been observed with abundant mycelial growth and colonization of seed structures (Siqueira et al. 2014). Further, Siqueira et al. (2014) also observed green and red fluorescence in the cytoplasm of the hyphae and conidia of the fungus *S. maydis*, and the pycnidia retained its natural color. However, autofluorescent molecules are common in plant tissues and can be considered problematic in microscopy studies because they may interfere with the detection of fluorescent staining (Müller et al. 2013; García-Plazaola et al. 2015; Kodama 2016; Dumur et al. 2019). The mycelial growth and cytoplasm of conidia may also emit some natural autofluorescence (Wu and Warren 1984). Alternatively, the clarification protocol may help minimize the fluorescence signals originating from autofluorescent compounds and optimize the specific fluorescent label (Minker et al. 2016; Ursage et al. 2018; Warner et al. 2014). Thus, the objective of this study was to validate a new efficient methodology for visualizing the colonization of *S. maydis* on corn stalks using the clarification of samples in potassium hydroxide and chloral hydrate, fluorochromes, and confocal laser scanning microscopy (CLSM).

Materials and Methods

Plant material and inoculation

Plants of the corn cultivar DeKalb 230 were grown in 5 L plastic pot containing a 1:1 ratio of soil and substrate. Two plants were kept in each pot and irrigated daily. The plants were grown in a greenhouse under natural conditions until they reached the V6 corn growth stage to start the inoculations. Every 15 days, a 1-g dose of N-P_2O_5-K_2O (10-10-10) was applied to every pot. *S. maydis* CMLAPS 570 isolate was used throughout this study. The S. *maydis* isolate was deposited in the fungal collection from the Seed Pathology Laboratory at the Federal University of Lavras, Lavras, MG, Brazil.

For inoculum preparation, mycelial discs (7-mm diameter) of CMLAPS 570 isolate were transferred to plates containing (OA, 60 g of oatmeal flour, 12 g of agar) medium. Ten plates with OA medium were prepared and incubated for fifteen days at 28°C and a 12-h photoperiod. Conidial suspension was obtained by scraping the mycelia after adding 4 mL of distilled water with Tween 80 (10 µL. L^{-1}) to each plate. The concentration of conidial suspensions was determined using a Neubauer chamber and adjusted to 10^5 conidia mL^{-1}.

Corn plants were inoculated at the V6 corn growth stage in the second stalk. One milliliter of conidial suspension was injected into the tip of the stalk with a syringe. For the uninoculated control plants, one milliliter of sterile distilled water was also injected into the tip of the stalk with a syringe. For CLSM analysis, individual corn plants of each treatment were harvested, and corn stalk fragments of 1 cm^2 were collected 21 days after inoculation (dai). Each treatment contained eight plants. The experiments were repeated once.

Clarification protocol and confocal laser scanning microscopy analysis

The samples of each treatment were placed in a Karnovsky fixative solution [2.5% glutaraldehyde, 2.0% paraformaldehyde in 0.05M sodium cacodylate buffer, $CaCl_2$ 0.001M, pH 7.2] and stored at 4°C for 24 h. After fixation, the samples were washed with phosphate buffer solution (PBS) three times and clarified in potassium hydroxide (KOH) solution 10% (p/v) with Tween 20 0.5% (v/v) for 24h. Thereafter, the samples were washed with PBS three times and clarified in chloral hydrate ($C_2H_3Cl_3O_2$) with distilled water (2.5:1) (p/v) for 24h and washed again with PBS three times (Warner et al. 2014; Minker et al. 2016). After that, the fungal structures were labeled with AlexaFluor 488® Conjugate (Alexa488-WGA) (ThermoFischer, CAT-W11261) at 1.0 mg mL^{-1} for 30 min under vacuum (excitation at 488 and emission at 510–540 µm) (Moreira et al., 2022). Thereafter, the fluorochrome Calcofluor White (Fluorescent brightener 28, Sigma, CAS-4404-43-7) at 0.1 mg mL^{-1} (excitation at 405 and emission at 440–490 µm) was used to label corn plant tissues for 30 min. The Laser Confocal LSM780 Zeiss Observer Z.1 microscope from the Laboratory of Electron Microscopy and Ultrastructural Analysis (LME)/UFLA, LCI Plan-Neofluar 25×/0.8, and C-Apochromat 63×/1.20 objectives were used to obtain fluorescent corn stalk images. For cellulose marking, the Ch1 detector, Beam splitter MBS 405 µm, pinhole 51.5 µm, laser 6%, Master Gain 390, Digital Gain 1, and Emission Filter at 435 to 490 µm were used. The ZEN Microscopy Software (Carl Zeiss®)

was used in the image acquiring and edition. The marked fungus *S. maydis* was localized using the ChS1 detector, Beam splitter MBS 488 □m, pinhole 57.5 µm, laser 60%, Master Gain 530, Digital Gain 1, and Emission Filter at 508 to 536 □m. Multiple focal planes were captured (z-stack) to overlap information from different depths of field with the Maximum Intensity Projection tool, creating a final 2D image with depth of field. Images captured from different focal planes were also used to generate 3D images. The final diagramation of the figures was done using CorelDraw® software.

Results and discussion

To shed light on the colonization process of *S. maydis* on corn stalks, we injected a conidial suspension of *S. maydis* into the stalk's tip with a syringe. At 21 dai, we harvested individual corn plants, split the corn stalk fragments, clarified them in 10% KOH and chloral hydrate, labeled them with Calcofluor White and Alexa488-WGA, and observed the *S. maydis* colonization of stalk tissue in detail by confocal laser scanning microscopy. In fungi-plant interaction studies, Alexa488-WGA and Calcofluor White may be used together. In this method, fungi are dyed with Alexa488-WGA and plant tissues are marked with Calcofluor White (Moreira at al., 2022). By 21 dai, the formation of pycnidia with bicellular conidia of *S. maydis* and colonization of plant tissues were observed by marking the stalk fragments with Alexa488-WGA (Fig. 1A and 1C). Similarly, as conducted to observe the colonization of *S. maydis* on corn stalk, Ha et al. (2016) also used Alexa488-WGA to detect the colonization of wheat tissues by *Pyricularia oryzae Triticum.* For uninoculated control plants, the samples marked at 21 dai with Calcofluor White and Alexa488-WGA allowed only the observation of parenchymatic tissues and vascular bundles of the corn stalk without colonization of *S. maydis* and formation of pycnidia associated with vascular bundles (Fig. 1G, 1H, 1I, 1J, 1K, and 1L).

The Figs. 1B and 1E show that at 21 dai, plant tissues and vascular bundles were found after corn stalk fragments with *S. maydis* were labeled with the fluorochrome Calcofluor White. The parenchymatic tissues and vascular bundles were found colonized by the fungus *S. maydis* (Fig. 1D and 1F). This result is in line with a previous report of *Stenocarpella macrospora* on corn, where fungal growth was noticed in the parenchyma cells, vessel elements and bundle sheath cells at 20 dai (Bermudez-Cardona 2016). Siqueira et al. (2014) also observed that transformed isolates of *S. maydis* containing the gene expressing for GFP and DsRed show abundant mycelial growth in the colonized seed structures. In addition, green and red fluorescence were detected in the cytoplasm of the hyphae and conidia of these isolates, and the pycnidia retained their natural color (Siqueira et al. 2014). Zhang et al. (2016) also stated that conidial germination and intense colonization of corn stalk tissues infected by *Fusarium graminearum* occurred 12 h after inoculation.

Besides the fluorescent markers, other elements of sample can be excited and emit light, being captured by the photomultiplier, generating a fluorescence that does not correspond to the desired one. Autofluorescence may be used to detect the formation of secondary metabolites of fungi and endogenous fluorophores that are used in fluorescence microscopy (Knaus et al. 2013). In our present study, the autofluorescence of corn stalk plant tissues may be green in color, in contrast to the blue

coloration of corn stalk plant tissues (Fig. 1G, 1I, 1J and 1L). This is in line with a previous report on *Hemileia vastatrix* on coffee, where the autofluorescence of fungal pustules on coffee leaves was observed at the green emission spectra (Moreira et al., 2022). Autofluorescence occurs naturally in yeast and plant cells. Red chlorophyll fluorescence spectrum occurs with emission maxima near 690 and 735 m (Stober and Lichtenthaler 1993). In addition to these autofluorescence compounds mentioned, some reagents, such as glutaraldehyde fixative, also allow fluorescence imaging of fungal mycelium (Donaldson 2020). However, this fixator may be useful for imaging fungal hyphae which are difficult to label with fluorescent stains (Donaldson 2020). On the other hand, the clarification protocol may help minimize the fluorescence signals originating from autofluorescent compounds and optimize the specific fluorescent label (Minker et al. 2016; Ursage et al. 2018; Warner et al. 2014).

In conclusion, we validated the new methodology for visualizing the presence of the fungus *S. maydis* in corn stalk using confocal laser scanning microscopy. Further, structures such as pycnidia and the colonization of vascular bundles through the presence of hyphae colonizing the plant tissues were also observed. Our results provide new options for using clarification protocols, fluorochromes, and confocal laser scanning microscopy in plant fungal pathogens and offer new insights into the detection of *S. maydis* on corn stalk.

Declarations

Compliance with ethical standards

Conflict of interest: The authors declare no conflict of interest.

Research involving human participants and/or animals

This study does not include experiments with either human participants or animals.

Author Contribution

E.A, S.I.M and A.F.D design of the study; A.F.D, E.A and S.I.M wrote the main manuscript text and A.F.D prepared figure 1. All authors developed the study and reviewed the manuscript.

Acknowledgements

Thanks are due to Dr. José da Cruz Machado, Federal University of Lavras, who generously shared his *Stenocarpella maydis* isolate with us. Adriano Francis Dorigan and Aline Vieira Barros were supported by Brazilian Coordination for the Improvement of Higher Education Personnel (CAPES, finance code 001). Silvino Intra Moreira was supported by PDJ/CNPq Postdoc Scholarship (152074/2022-8). Eduardo Alves is supported by the National Council for Scientific and Technological Development (CNPq 305482/2017-

3, 313825/2018-1, 432445/2018-8, and 306133/2021-0), and The Laboratory of Electron Microscopy and Ultra structural Analysis (LME)/UFLA, is supported by FINEP.

Data Availability statement:

The data that supports the findings of this study are available upon request.

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Figures

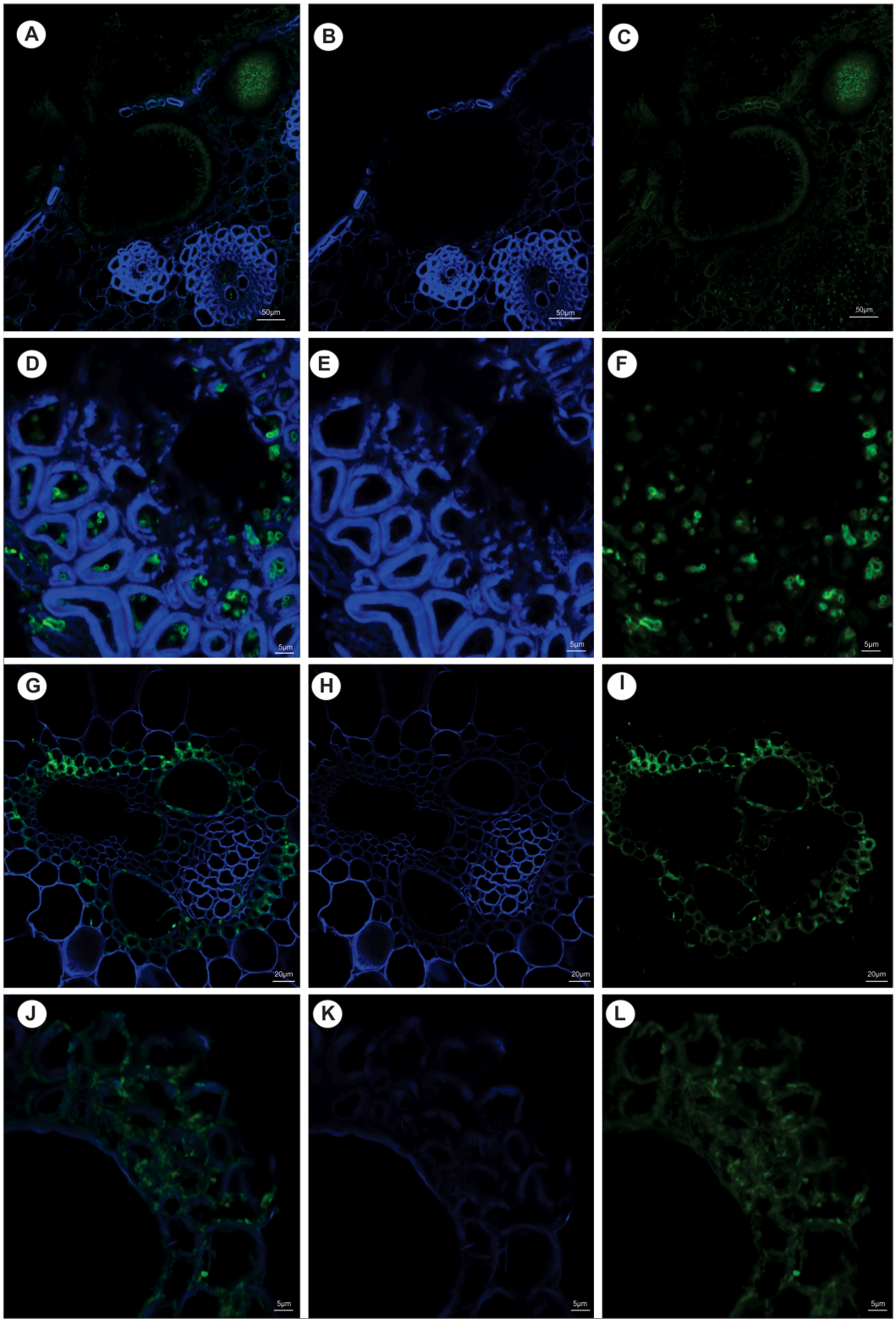

Figure 1

Confocal Laser Scanning Microscope images (CLSM) on corn stalk. A) Calcofluor White dyeing the plant tissues and vascular bundles of the corn stalk, and Alexa488-WGA marking colonization and pycnidia formation by the fungus *S. maydis* on corn stalk at 21 days after inoculation (21 dai); B) Calcofluor White dyeing plant tissues and vascular bundles of corn stalk at 21 dai; C) Alexa488-WGA marking colonization and pycnidia formation of the fungus *S. maydis* on corn stalk at 21 dai, D) Parenchymatic tissues and

vascular bundles of corn stalk colonized and marked with Calcofluor White and Alexa488-WGA at 21 dai; E) Calcofluor White dyeing parenchymatic tissues and vascular bundles of corn stalk at 21 dai; F) Alexa488-WGA dyeing colonization of *S. maydis* in the parenchymatic tissues and vascular bundles of corn stalk at 21 dai; G) Calcofluor White dyeing the plant tissues and vascular bundles of the corn stalk, presence of natural autofluorescence of the plant tissues, and absence of the fungus *S. maydis* in the uninoculated control plants; H) Calcofluor White marking the plant tissues and vascular bundles of the corn stalk from the uninoculated control plants; I) Natural autofluorescence of plant tissues from the uninoculated control plants; J) Calcofluor White marking the plant tissues and vascular bundles of the corn stalk, the presence of natural autofluorescence of the plant tissues, and the absence of the fungus *S. maydis* on uninoculated control plants; K) Calcofluor White dyeing the plant tissues and vascular bundles of the corn stalk from the uninoculated control plants; L) Natural autofluorescence of plant tissues from the uninoculated control plants. The Laser Confocal LSM780 Zeiss Observer Z.1 microscope, LCI Plan-Neofluar 25×/0.8, and C-Apochromat 63×/1.20 objectives were used to obtain fluorescent corn stalk images. The magnification of figures A, B, C, G, H, and I was 150×, and that of figures D, E, F, J, K, and L was 630×.