Reevaluation of *Phomopsis* species affecting sunflowers in the United States

Febina Mathew[1]*, Kholoud Alananbeh[2], Nikolay Balbyshev[3], Erik Heitkamp[1], Lisa Castlebury[4], Thomas Gulya[3], and Samuel Markell[1]

[1]North Dakota State University – Department of Plant Pathology, Walster Hall, Fargo, ND 58102, USA, febina.mathew@ndsu.edu [2]University of Medina, Saudi Arabia. kholoudenab@gmail.com [3]USDA-ARS Sunflower Research Unit, 1307 18th Street N, Fargo, ND 58102, USA, thomas.gulya@ars.usda.gov [4]USDA ARS Systematic Mycology and Microbiology Lab. Bldg. 010A, Rm. 225, BARC-West, 10300 Baltimore Ave. Beltsville, MD, 20705.

ABSTRACT

- Diaporthe stem canker (DSC) frequently causes serious economic damage on sunflowers in other countries, but in the United States (US), only a very low incidence and limited damage occurred before 2010. In 2010, DSC affected 8.7% of the US crop, and yield reductions occurred in isolated fields.   While *D. helianthi* was assumed to be the sole incitant of the disease in the United States, some researchers previously hypothesized more species could be involved in epidemics in other locations. The primary objective of this research is to identify and characterize aggressiveness of *Phomopsis* species in the US.

- An intensive survey of sunflower fields was conducted during the 2010-11 growing seasons. Approximately 1,500 sunflower stalks exhibiting wilt and/or stem disease symptoms were randomly sampled from sunflower production regions throughout the Northern Great Plains. Additionally, hierarchical sampling of selected fields in South Dakota, North Dakota, and Minnesota was completed in the summer of 2011. Stalks were dissected, surface-sterilized, and plated on potato dextrose agar. Cultures were scored for presence or absence of *Phomopsis*, hyphal tipped, and identified to species. DNA was extracted from the lyophilized mycelium of the individual isolates, the internal transcribed spacer region (ITS) was sequenced with primers ITS4 and ITS5, and analysis was performed using BLASTN via the NCBI database. Assessment of species collected using both sampling strategies is currently underway and will be presented.

- Preliminary analysis revealed at least four *Phomopsis* species – namely; *D. helianthi*, *D. stewartii*, *P. longicolla* and an unknown *Diaporthe* spp. *D. helianthi* has been associated with yield loss in the past, and preliminary data suggest the unknown *Diaporthe* sp. is similar to one identified on sunflowers in Australia in 2010, which was associated with unprecedented yield loss.

- The identification of three *Phomopsis* species on sunflowers, including *D. helianthi* and an unknown *Diaporthe spp.,* coinciding with dramatic yield losses in the United States for the first time since DSC was identified is cause for alarm. One hypothesis would suggest a recent exotic pathogen(s) introduction may have occurred in the U.S. One alternate hypothesis would be that the impact of other species may be occurring due to changes in cropping systems (no-til) or genetics. A final hypothesis could be the existence of diverse *Phomopsis* isolates were endemic, but the ability to distinguish species prior to molecular techniques was impossible and recent yield loss is coincidental.

- The identification of multiple species causing DSC in the U.S. is the first step to selecting resistant lines for breeding programs. Further, we provide a more precise taxonomic identification of the causal agent of DSC in the U.S.

INTRODUCTION

Phomopsis stem canker has emerged recently as a significant threat to sunflower production in the United States. Although the disease has caused significant yield loss in Europe (Maserivic and Gulya 1992), neither widespread nor severe epidemics have occurred in the United States until 2010. In a United States National Sunflower Association sponsored survey in 2010 (unpublished) the pathogen was found in 42% of surveyed fields. More remarkably however, was the severity of the disease in certain localized areas where wilted plants, premature death, and significant lodging made recovery of any yield questionable.

Historically, *Diaporthe helianthi* has been assumed to be the sole incitant of the disease in the United States through classical and morphological methodology. However, some researchers hypothesized a complex of more than one species or biotype of *Phomopsis* could be involved in epidemics in other locations (Maserivic and Gulya 1996, Thompson et al., 2010). Recently, Thompson et al. (2011) attributed an unknown *Diaporthe* speices to significant yield loss in Australia. The new causal agent of Phomopsis Stem Canker in Australia was later characterized and named *Diaporthe gulyae*. Given the recent and unprecedented epidemics of Phomopsis Stem Canker in North America and Australia and the identification of a new causal agent in Australia, identification of the species(s) causing the disease in North America is important to develop effective management tools the disease.

Speciation of *Diaporthe/Phomopsis* using classical and morphological methodology (included morphological characteristics such as color and colony appearance, size and shape of stromata, the ratio and size of α and β conidia, and the arrangement and shape of perithecia) has been notoriously difficult. Despite considerable effort in determining causal species, morphological characteristics are too variable (Zhang et al., 1998) and host association was often adopted to define species (Uecker, 1988). As such, *Diaporthe helianthi* was attributed as the causal agent of Phomopsis stem canker in sunflowers (Herr et al. 1983, Gulya, 1996, Meyer et al. 2009). A number of molecular tools have recently been developed that make it possible to assess the genetic diversity within Phomopsis species affecting sunflowers. A number of investigations have used similarity of the rDNA-ITS region to differentiate species of fungi and examine evolutionary relatedness among species including isolates of *Phomopsis* and *Diaporthe* (Zhang *et al.*, 1997, 1999; Rekab *et al.*, 2004; van Niekerk *et al.*, 2005). The same techniques could be used to characterize the pathogen(s) in sunflowers.

The objective of this paper is to identify species associated with Phomopsis Stem Canker in the United States.

MATERIALS AND METHODS

A survey was undertaken in Minnesota, North Dakota, and South Dakota in 2010 to determine identity stalk rot pathogens of sunflowers, including those inciting Phomopsis Stem Canker. A total of 1150 stalks were chopped, sterilized, and plated on potato dextrose agar (PDA) for 7-10 d. Plates were scored for presence or absence of different fungi, including *Phomopsis*. *Phomopsis* isolates were hyphal tipped and incubated on PDA. DNA was extracted from the lyophilized mycelium of the individual isolates and the rDNA-ITS region consisting of ITS1, 5.8S and ITS2 with primers ITS4 and ITS5 (White *et al*., 1990) was sequenced and analysis was performed using BLASTN via the NCBI database (www.ncbi.nlm.nih.gov). To infer species, reference sequences and the outgroup sequence (*Leucostoma niveum* and *Valsa ambiens*) from the study conducted by Thompson *et al.* (2011) were obtained from GenBank. Sequences of selected isolates from the 2010 collection and reference sequences were aligned using ClustalX, aligned using the Molecular Evolutionary Genetics Analysis (MEGA version 5) software, and analyzed using Maximum Likelihood with PAUP (v. 4.0b10).

RESULTS

A total of 91 *Phomopsis* isolates were recovered from infected sunflower stems collected from SD fields in 2010, and additional isolates were obtained from symptomatic sunflower samples collected from ND, TX and MN fields. The Maximum likelihood (ML) tree constructed on MEGA enabled characterization of *Phomopsis* isolates at the species level. Based on 50% bootstrap value (BS), the ML analysis inferred the identification of 26 South Dakota isolates to the cluster described as new Phomopsis species by Thompson *et al.* (2011), now called "*Diaporthe gulyae*". Two of the North Dakota isolates, two of the Texas isolates and four of the Minnesota isolates were closely related to *Diaporthe helianthi* supported by a BS 62%. One of the SD isolate is closely related to *Diaporthe*

stewartii, supported by a BS 62%. Results indicate that we have at least three distinct species causing Phomopsis in the Northern Great Plains.

Figure 1. ML tree generated from the internal transcribed spacer (ITS, ITS1-5.8S-ITS2) regions of the nuclear ribosomal DNA (rDNA) of reference *Phomopsis* sequences of Thompson *et* al. (2011) and select isolates collected in 2010. Reference sequences are indicated with GenBank accession numbers followed by the species.
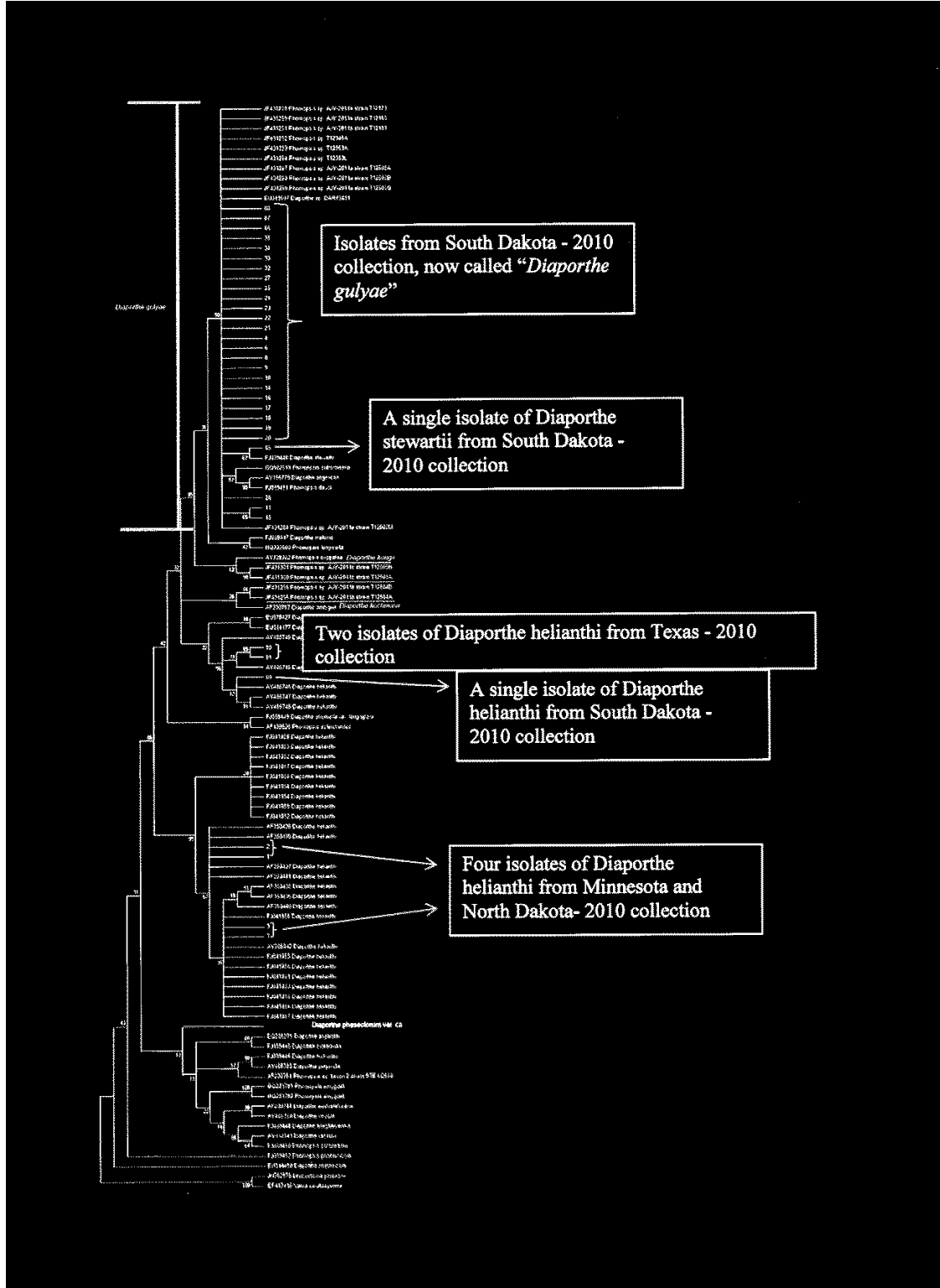
DISCUSSION

The identification of two pathogens known to cause yield loss, *D. gulyae* and *D. helianthi*, and one pathogen for which little is known on sunflower, *D. stewartii*, in the United States may be cause for concern. However, it is unclear if the recent epidemics in the United States and the identification of multiple species is related. Multiple species may have existed for many years, but modern tools for identification were not available, and similarly, there was limited incentive to reevaluate species identity prior to yield loss in 2010. Similarly, multiple species have existed in the United States for many years, but the agronomic or environmental factors favorable for an epidemic did not occur until recently. However, it is impossible to rule out an introduction of an exotic pathogen(s) into the U.S.

Although the results of this preliminary work have shown that several species can incite disease in the United States, future work is needed. In 2011, exhaustive sampling through a hierarchical strategy was done in the Northern Great plains region of the United States. Isolates derived from these samples are being speciated to further resolve the causal agents in the United States. Similarly, aggressiveness tests on different species and evaluation of germplasm is planned for the future.

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