# *Rhizopus oryzae* Associated with *Melanagromyza splendida* and Stem Disease of Sunflowers (*Helianthus annuus*) in California

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Accepted for publication 10 January 2015. Published 25 March 2015.

**ABSTRACT**

Mathew, F. M., Prasifka, J. R., Gaimari, S. D., Shi, L., Markell, S. G., and Gulya, T. J. 2015. *Rhizopus oryzae* associated with *Melanagromyza splendida* and stem disease of sunflowers (*Helianthus annuus*) in California. Plant Health Progress doi:10.1094/PHP-RS-14-0042.

In September 2012, a female parental line in a Yolo Co., California, sunflower seed-production field began displaying external stem symptoms that could not be attributed to any known disease. Symptoms appeared to be associated with tunneling caused by an unidentified insect. Stems were collected, and *Rhizopus oryzae* (causal agent of Rhizopus head rot) and a minute fly, *Melanagromyza splendida,* were identified as the causal agent and associated insect, respectively. Further, *R. oryzae* was isolated from intact fly puparia. All commercial hybrids evaluated in the greenhouse were susceptible to stem infection by *R. oryzae* isolates. Yield implications and geographic distribution of this novel stem disease are unknown. This is the first report of *R. oryzae* causing stem disease in sunflowers, and of its association with *M. splendida*.

## INTRODUCTION

Rhizopus head rot is a common disease of sunflower (*Helianthus annuus* L.) around the world, especially in hot, dry climates. Several *Rhizopus* species have been implicated as causal agents, including *Rhizopus microsporus* Tiegh., *Rhizopus stolonifer* (Ehrenb.) Vuill., and *Rhizopus oryzae* Went & Prins. Geerl. (Gulya et al. 1991). Among the three *Rhizopus* spp., *R. oryzae* is the most prevalent on sunflowers in U.S. production areas where flowering and seed maturation occur at high temperatures (such as California and the High Plains area of Texas, Kansas, and Nebraska) (Gulya et al. 1991; Yang et al. 1980). Rhizopus head rot can result in significant yield losses due to head loss, reduction in head size, seed weight, and oil content, and quality decreases due to increased free fatty acid content, resulting in a bitter taste (Berglund 2007). Head rot is the only disease currently observed on sunflower caused by *Rhizopus* species.

Sunflower heads are resistant to infection at the budding stage and become more susceptible to infection as they mature. *Rhizopus* spp. enters the sunflower head through wounds caused by hail, birds, and insects such as the sunflower moth (*Homoeosoma electellum* Hulst, Pyralidae) (Klisiewicz 1979). The disease typically appears first as dark spots on the back of ripening sunflower heads, followed by a watery, soft rot that later turns dark brown. As the disease progresses, heads dry prematurely and the tissue begins to shred. Thread-like mycelial strands are observed inside shredded tissues that are indicative of the fungus. Black reproductive structures (sporangia) appear on the mycelial strands that are filled with spores that are easily released and wind-blown to other plants.

In September 2012, pale brown lesions were observed on the lower stems of sunflower plants in a hybrid seed-production field in Yolo Co., California (Figs. 1 and 2). The lesions, ranging from approximately 10 to 30 cm in length, appeared to be associated with insect tunneling, and were either confined to one side of the stem or completely encircled the stem. The leaf petioles attached to the diseased portions of the sunflower stem became flaccid and died. When infected stems were split open, fluffy mycelial mats with black sporangiophores, typical of *Rhizopus* spp., were observed in the pith (Fig. 3). Infected female lines prematurely died (Fig. 4) and yield loss ranged from slightly reduced, as a result of the youngest seeds in the head not developing, to 100%. Neither external lesions nor internal symptoms were observed on sunflower heads. Insect damage, manifested as small external holes near the base of the stem, was widespread in the seed-production field on both male and female parents. However, stem lesions and leaf symptoms were only observed on the female line (approximately 15% of all plants). The objective of this study was to identify both the insect and the *Rhizopus* species involved, and to investigate the competency of *Rhizopus* species as a stem pathogen of sunflowers.

## INSECT ISOLATION AND IDENTIFICATION

To obtain insect specimens, ten stems each from the female line expressing external lesion symptoms, the female line without symptoms, and male parent without symptoms were collected from the seed-production field. All plants had external holes on the lower part of the stem, consistent with insect infestation. Stems were dissected and both empty fly puparia (i.e., exuviae) and intact puparia were noted and removed. The inside of each

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doi:10.1094/PHP-RS-14-0042

**FIGURE 1**
*Rhizopus* infection on the lower stems as observed on sunflowers in a sunflower seed-production field in Yolo Co., California on 5 September 2012.

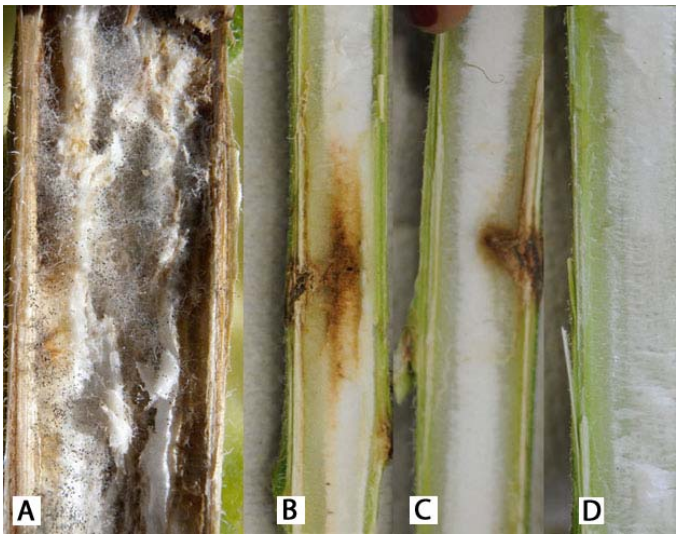


**FIGURE 3**
(**A**) *Rhizopus* infection on the lower stems as observed on sunflowers in a sunflower hybrid-production field in Yolo Co., California; (**B**) cv. Triumph767C caused by the *R. oryzae* isolate RO1, 14 days after inoculation in greenhouse ;(**C**) cv. 63A81 Pioneer caused by the *R. oryzae* isolate RO2, 14 days after inoculation in greenhouse; and (**D**) control sunflower plant, 14 days after inoculation in greenhouse.

**FIGURE 2**
External damage at the end of tunnels produced by *Melanagromyza splendida* larvae on the lower stems as observed in a sunflower seed-production field in Yolo Co., California, on 5 September 2012

**FIGURE 4**
Female sunflower lines prematurely dying as a result of *Rhizopus oryzae* infection in a sunflower seed-production field in Yolo Co., California, on 5 September 2012.

stem also was visually scored (+/-) for the presence of mycelia. Intact puparia were kept to allow for emergence of adult flies (needed for species-level identification) and to assess the possible presence of *Rhizopus* on or in the flies. After adult flies emerged, they were identified based upon external appearance and examination of the male genitalia (Spencer and Steyskal 1986).

External holes associated with fly infestation were found in all collected stems. A mean (± SE) of 6.0 (± 4.5) total exuviae and intact puparia were found per stem. Visual evidence of *Rhizopus* mycelia were found in all stems of female plants (with or without external symptoms) and four of ten stems from the asymptomatic male parent. Adult flies emerged from infested stems were identified as *Melanagromyza splendida* Frick (Diptera: Agromyzidae), which has a wide host range that includes cultivated sunflower. Specimens of similar flies emerged from sunflower stems in Minnesota and North Dakota were identified as *Melanagromyza veroniana* Steyskal, which had not been previously reported to attack sunflowers. A closely related seed-feeding fly, *Melanagromyza minimoides* Spencer, is also a serious pest of late-planted sunflowers in Argentina, and Uruguay (Zerbino 1991, Ves Losada and Figueruelo 2006), but has not been associated with any stem diseases.

Voucher specimens of *Melanagromyza splendida* and *M. veroniana*, identified by co-authors Gaimari and Shi, are housed in the California State Collection of Arthropods (Plant Pest Diagnostics Lab, California Department of Food and Agriculture, Sacramento, California).

## *RHIZOPUS* ISOLATION AND IDENTIFICATION

Infected stem samples were washed with tap water and 1-cm-long pieces were cut and surface-sterilized for 3 min in 0.5% NaOCl (sodium hypochlorite). After being rinsed twice in sterile distilled water, the pieces were plated on potato dextrose agar (PDA; Difco Laboratories, Detroit, MI) amended with 0.02% streptomycin sulfate. In addition, intact fly puparia were recovered from sunflower plants and surface-sterilized and plated intact on PDA. Within 3 to 7 days, mycelial colonies with abundant aerial growth and black sporangia emerged on PDA from both stem pieces and puparial cases. The fungus was identified morphologically as *R. oryzae* on the basis of the presence of pale brown sporangiospores with bluish stripes (Watanabe 2002) and mycelial growth at 36°C on PDA (Liou et al. 2007).

To confirm the identification of the fungus molecularly, DNA of 20 isolates (10 from symptomatic stems and 10 from pupal cases) was extracted from lyophilized mycelium scraped from the surface of a 7-day PDA culture and resuspended in 50$\mu L^{-1}$ of rehydration solution (1% TE buffer) using a Wizard Genomic DNA Purification Kit (Promega, Madison, WI). A 5-µl aliquot of each DNA sample was run electrophoretically on a 1% agarose gel to confirm quality. DNA was also quantified with a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE). Two *Rhizopus* isolates were identified to species by amplifying and sequencing the internal transcribed spacer (ITS) regions using primers ITS1 and ITS2 (White et al. 1990). All DNA samples were sequenced (GenScript USA Inc., Piscataway, NJ) using the ITS primers. An approximate 600-bp region of the ITS was amplified from five *R. oryzae* isolates and used to query the GenBank nucleotide database (National Centre for Biotechnology Information, http://www.ncbi.nlm.nih.gov/) directly. A BLASTN search of GenBank showed the best match for the five isolates was *Rhizopus oryzae* strain CBS 387.34 (Accession # DQ641274) with identities = 598/598(100%) and gaps = 0/598(0%). DNA sequences generated in this study have been deposited in GenBank under accession numbers KP012564-KP012568.

## PATHOGENICITY AND AGGRESSIVENESS OF *RHIZOPUS ORYZAE* ISOLATES

To test the pathogenicity of *Rhizopus* isolates on sunflower stems, six commercial hybrids—'Pioneer 63A81' (Pioneer, Johnston, IA); 'CHS RH1122' (CHS Sunflower, Grandin, ND); 'Croplan 343' (Winfield solutions, Shoreview, MN); 'Mycogen 8D310' (Mycogen seeds, Indianapolis, IN); 'Panther DMR' (Nuseed, Alsip, IL); and 'Triumph 767C' (Triumph Seed Company, Inc., Ralls, TX) —were grown for three to four weeks in the greenhouse at 26°C with a 14-h photoperiod. The seeds of each hybrid were sown in planting mix (Sunshine Mix # 1, Sun Grow Horticulture Products, Belleview, WA) in 7.5-liter circular plastic pots and watered on alternate days.

To obtain inoculum, mycelial plugs (4 mm in diameter) were taken from the margin of 5-day PDA culture of two *R. oryzae* isolates (one from a symptomatic stem designated RO1 and one from a puparial case designated RO2). Stems of the six commercial hybrids between the V4 and V6 growth stages were wounded softly on the 2nd internode with a sterile toothpick and a *Rhizopus*-infested mycelial plug placed on the wound. A noninfested PDA plug was placed on the wound on control plants. All plugs were attached to the wounds with Parafilm to avoid rapid dehydration. After inoculation, plants were kept in the greenhouse for 14 days at 26°C with a 14-h photoperiod.

The trial was conducted in a completely randomized design with six plants evaluated per treatment (hybrid/*Rhizopus* isolate) and the experiment was repeated twice. The experimental unit was the single plant in each pot and each treatment was replicated six times (six plants per hybrid). Data from the two experiments were combined after a test for homogeneity of variance within and between experiments. Disease severity was calculated as the percentage of host tissues covered by external lesion or damaged internally by the disease. Internode length and lesion length measured 14 days after inoculation was used to calculate the lesion length as a percentage of the internode length. The extent of vascular discoloration was measured length-wise 14 days after inoculation and calculated as a percentage of the internode length. Analysis of variance (ANOVA), with main effects of *R. oryzae* isolates, was performed using the general linear models procedure (PROC GLM) in Statistical Analysis System (SAS v9.3; SAS Institute Inc., Cary, NC). Mean comparisons for the lesion length and extent of vascular discoloration were based on least significant difference (LSD) at $P \leq 0.05$.

For the greenhouse experiments, test statistics indicated no significant effect ($P > 0.05$) of *Rhizopus* isolates or sunflower hybrids or interactions between *Rhizopus* isolates and sunflower hybrids in the overall development of Rhizopus stem disease. No significant differences in lesion length and vascular discoloration ($P > 0.05$) were observed among six sunflower hybrids for the main effect of the two *R. oryzae* isolates, RO1 and RO2 (Table 1). Overall, all evaluated commercial hybrids were susceptible to stem infection by *R. oryzae* isolates. Thirty-six control plants (six plants per hybrid) similarly treated with sterile PDA discs did not display symptoms in each of the experiments.

In general, dark brown discoloration developed at the inoculation site on inoculated stems and expanded internally, and pale brown sporangia was observed within 14 days after inoculation (Fig. 3). *Rhizopus oryzae* was re-isolated from the

**TABLE 1**
**Mean percent lesion and discoloration caused by *Rhizopus oryzae* isolates (RO1 and RO2) at 14 days after inoculation averaged over six replicates per sunflower hybrid[a].**

| | *R. oryzae* isolate RO1 | | *R. oryzae* isolate RO2 | |
|---|---|---|---|---|
| Sunflower cv. | % lesion[b] | % discoloration[b] | % lesion[b] | % discoloration[b] |
| 63A81 Pioneer | 7.6 a | 17.2 a | 6.2 a | 18.0 a |
| CHS RH1122 | 7.2 a | 15.7 a | 5.8 a | 14.1 a |
| Croplan 343 | 5.8 a | 16.1 a | 5.9 a | 15.5 a |
| Mycogen 8D310 | 5.3 a | 16.5 a | 5.3 a | 16.0 a |
| Panther DMR | 6.9 a | 14.8 a | 4.8 a | 16.6 a |
| Triumph 767C | 6.5 a | 17.9 a | 7.1 a | 16.9 a |
| LSD ($P \leq 0.05$)[b] | 3.34 | 4.39 | 2.28 | 4.16 |
| *P* value | > 0.05 | > 0.05 | > 0.05 | > 0.05 |

[a] Control plants representing six commercial hybrids (n = 36, six plants per hybrid) had an average % lesion = 0.0 and % discoloration = 0.0; there were no significant differences ($P \leq 0.05$) among the hybrids.

[b] Means followed by the same lowercase letter are not significantly different based on Fisher's protected least significant difference ($\alpha = 0.05$).

[c] Because there were no significant interactions among the main effects, LSD values are given only for the main effects.

inoculated sunflower plants according to the isolation techniques previously described. The identity of the pathogen was confirmed via sequencing of the ITS regions using primers ITS1 and ITS2 (White et al. 1990). *Rhizopus oryzae* was not isolated from the control plants.

## IMPORTANCE AND MANAGEMENT RECOMMENDATIONS

To the best of our knowledge, this is the first report demonstrating that *R. oryzae*, which commonly causes Rhizopus head rot, is capable of causing a previously undescribed stem disease on sunflower plants. Similarly, this is the first report suggesting *R. oryzae* infection in stems may be associated with the stem-mining fly, *M. splendida*. While the co-occurrence of *M. splendida* and *R. oryzae* (and evidence of *R. oryzae* inside fly puparia) suggests the association of the insect and pathogen, this is not conclusive evidence that the fly vectors *Rhizopus* spp. in sunflower stems, and we were unable to generate enough adult flies to conduct such experiments. If *M. splendida* does contribute to *R. oryzae* stem infection, it is likely that the major U.S. sunflower-production areas (e.g., North Dakota, South Dakota) could also be impacted by stem infection with *R. oryzae*. *Rhizopus oryzae, M splendida* and the congener *M. veroniana*, which appears to feed in a similar manner to *M. splendida,* all have wide geographic ranges and are thought to be present in the region (Berglund 2007; Spencer 1973). The external lesions are not very distinctive and are likely to be overlooked or mistakenly ascribed to other pathogens. Consequently, Rhizopus stem infection may exist in other areas of the United States or in other countries.

The effects of *Rhizopus* stem infection on sunflower yield is unknown. In the field in Yolo Co., California, premature plant death caused by Rhizopus stem infection resulted in no seed yield on some infected female lines, suggesting that yield loss on commercial hybrids is at least possible. Additionally, Rhizopus head rot, which is caused by the same pathogen, and can also be introduced to heads by insect wounding, frequently causes significant yield loss in hot and dry environments. In the Yolo Co. seed-production field, only one of the male parental line was visibly affected by *R. oryzae*, suggesting that resistance to infection may be present in sunflower germplasm. However, no differences were detected among the six hybrids used in this experiment. Another possible explanation for lack of infection in the male parental line may be related to differences in plant structure. Male sunflower lines typically have much denser pith than females lines or commercial hybrids (as was the case in the Yolo Co. seed-production field), presenting the possibility that a physical difference could limit or prevent infection. No management strategies for Rhizopus stem infection are recommended at this time, but agricultural professionals working with sunflowers should be cognizant of this "new" stem disease.

## ACKNOWLEDGEMENTS

This research was supported by the National Sunflower Association (Mandan, ND, USA). We thank Bob Harveson (University of Nebraska, Scottsbluff) for manuscript review and Mark Mancl (DuPont Pioneer, California) for providing plant samples and photos. We also thank Scott Meyer, James Jordahl, Theresa Gross, Solji Choi, Austin Streifel, Christian Steffen, and Seunghyun Choi for their technical assistance in this study.

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