

**Molecular detection of pathogenic *Fusarium* species in roots and stalks of maize plants
with or without transgenic resistance to corn rootworm**

by

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A thesis submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Major: Interdisciplinary Graduate Studies (Biological and Physical Sciences)

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2009

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Acknowledgements

I would like to thank my major professor, Dr. Gary Munkvold, for his kind support and for his able guidance, valuable discussions and unflinching encouragement throughout the course of my research work. It gives me great pleasure to express my deep sense of gratitude and indebtedness to Dr. Gary Munkvold. I would also like to thank my collaborators Dr. Lance Meinke for providing samples from Nebraska and Dr. Anania Fessehaie for his guidance in the PCR assay.

I would like to thank the members of committee, Dr. Madan Kumar Bhattacharyya and Dr. Patricia Murphy for their valuable time, suggestions, support and guidance.

I wish to express my thanks to Michele Beattie, for extending a helping hand in dilution plating and whenever required during my research work. I also appreciate the cooperation and assistance of my colleagues from my lab group Mercedes, Erin, Pablo, Marcos and Yiqing. I would like to thank Amy, Alex and Marcio for processing the plant material.

I express my deep sense of gratitude to my mother Rama Devi, who came all through from India to take care of my daughter Nikhita and son Sreekrishna. I wish to thank my husband, Dr. Ch. Venkat Reddy for his constant support and encouragement. I would like thank my father M. Venkat Reddy and brothers Shravan and Raghavendra for their moral support. I would like to thank my friends Pavana, Tejeswi, Veerendra, and Vijay for their help.

Abstract

Fusarium species are among most common fungal pathogens of maize, causing root rot, stalk rot, stalk lodging and ear rot. *Fusarium verticillioides* and *F. graminearum* are two of the most prevalent stalk rot fungi in Nebraska and Iowa. Corn root worms (CRW) are the most costly pests of maize plants, causing damage to both above- and below-ground parts of the plant. CRW feeding on roots creates good invading sites for different fungal species. The goal of this research was to determine whether use of transgenic CRW-resistant maize hybrids will have reduced levels of *Fusarium* colonization of roots and stalks compared to their near-isogenic susceptible hybrids. Experiments were conducted at two locations each in 2007 and 2008 in fields with high populations of Western corn root worms, (*Diabrotica virgifera* LeConte): Bruner farm near Ames, IA (2007), ISU Southeast Research Farm near Crawfordsville, IA (2007-2008), and Agricultural Research and Development Center, Univ. of NE near Mead, Nebraska (2007-2008). Commercially available hybrids with CRW-resistance transgenes along with their near-isogenic CRW-susceptible hybrids were planted. The CRW events include DAS-59122 (Herculex® Xtra), MON863 (Yieldgard Plus®), MON88107 (Yieldgard VT Triple®) and MIR604 (Agrisure® RW/CB/LL). In 2008, insecticidal seed treatment also was tested for its effect on CRW feeding and subsequent *Fusarium* colonization. Sampling was done on roots in mid-July and stalks in mid-September in 2007 and in 2008 mid-September roots were also sampled. Root colonization was assessed by dilution plating of dried ground tissue, and by real-time polymerase chain reaction (PCR).

Primers and fluorogenic probes, specific to mycotoxin biosynthesis genes in *F. verticillioides* and *F. graminearum* were used to quantify root and stalk colonization. Standard calibration curves of both species showed linear correlation ($r^2=0.99$) between the fungal genomic DNA and the threshold cycle values. There were highly significant differences in colonization between resistant and near isogenic susceptible hybrids for events MON 88017 and MIR 604 in Nebraska July roots in 2007. *F. verticillioides* was at high levels in Nebraska whereas *F. graminearum* was more common in Iowa. Stalks from both locations were highly colonized with *F. graminearum*. The PCR results were compared to a microbiological dilution plating method. In 2007 the results of the two methods correlated well but in 2008 they did not follow the same pattern. In dilution plating results we could consistently see significantly lower *Fusarium* CFU/g tissue in hybrids with CRW resistance than in near-isogenic CRW susceptible hybrids. Insecticidal seed treatment did not have a significant effect on level of colonization of fungal species measured by PCR or dilution plating results. CRW feeding on maize roots sometimes enhanced the infection of roots by *Fusarium* species and transgenic CRW resistant hybrids suffered less colonization than susceptible hybrids in CRW infested fields. However, the interaction between *Fusarium spp* and CRW varied in their degree among *Fusarium* species. Known maize pathogens *F. verticillioides* and *F. graminearum* were not consistently affected by CRW resistance as much as the total *Fusarium* population. The impact of this interaction on stalk rots is not clearly demonstrated.

Introduction

Maize is one of the major staple foods and among the three important cereal crops in the world. It is an important crop for both human and livestock consumption. It is used for the production of different food commodities such as cereals, corn meal, vegetable oils, high fructose corn syrup, etc. It has become a major source of carbohydrates in the daily menu in some parts of the world. Today it draws many researchers' attention as a feedstock for the production of biofuel. Its high demand in the food, feed, and fuel industries has heightened the need for plant pathologists to work on diseases of maize.

Stalk rots in Maize

Stalk rots, the most serious and widespread diseases of maize, are caused by a complex of fungi and bacteria. Stalk rots cause loss in yield due to premature plant death and lodging of plants. Stalk rot development is enhanced by stress in the plant during the grain filling stage (Munkvold et al 2000). The major stalk-rotting fungal species include *F. verticillioides*, *F. graminearum*, *F. proliferatum*, *F. subglutinans*, *Colletotrichum graminicola*, *Macrophomina phaseolina* and *Stenocarpella maydis*. All species overwinter in soil or plant debris, and upon favorable conditions, begin to grow or sporulate before infecting host plants. Most stalk rots share several similar symptoms, but some symptoms are specific to individual fungi. The common symptoms include pith tissue disintegration, discoloration of pith tissue and nodes, lodging of stalks, water soaked appearance and above ground symptoms that include wilting and scorching of leaves.

Fusarium species in Maize

Fusarium stalk rots are caused by several *Fusarium* species that overwinter as mycelium or chlamydospores in the debris or soil. Stalk rot causing fungi infect stalks through root colonization, insect wounds or natural openings. The *Fusarium* spp. isolated from debris and soil differed among host plants (maize, sorghum and soybean) in the central and eastern parts of USA, which demonstrates the diverse species distribution in the US (Leslie et al 1990). Insect injury to stalks enhances stalk infection by *Fusarium* spp. (Gatch et al 2002). *Fusarium* species causes both stalk and root rot in maize. The species that invade root systems also can reach the stalks and ears causing stalk rot and ear rot (Logrieco et al 2002). Many of the pathogenic *Fusarium* species enter through the roots, causing root rot prior to pollination and stalk rot later in the season (Munkvold 1996). The infection of maize with *Fusarium* spp. is widespread in all parts of world (Francis et al 1975 in Australia, Chambers 1987 in South Africa). Several species are seed borne and cause systemic infections that spread through the stalks and into the ears (Munkvold et al 1997).

Root infecting *Fusarium* species move to the base of the plant resulting in stalk rot. *F. graminearum* caused stalk rot with significant production of cellulase but did not reduce yield in *in vitro* studies (Chambers 1987). In recent studies, it was proved that *F. graminearum* was the predominant stalk rot pathogen in Iowa (Gatch et al 2002). Root decay is considered to be an important step in causing of stalk rots (Munkvold et al 1999). But there is no clear evidence or study how the root rot infection develops into stalk rot. Stalk rot is expected after harvest, and is important for the recycling of nutrients and organic matter; but if it occurs before physiological maturity then it leads to yield loss due to poorly developed

ears. *Fusarium* stalk rots and root rots are also induced by environmental stress; in some cases, reduced tillage practices can reduce disease severity by conserving soil moisture and preventing drought stress in the plants (Dodd 1980). Stalk rot also depends on the stress related to the soil conditions and environment which can be optimized by tillage regimes since tillage practices alter the chemical and physical properties of the soil.

The *Fusarium* species are most common species infecting maize kernels in North America leading to yield loss and the production of secondary metabolites that are hazardous to both human and domestic animals. The predominant species include *F. verticillioides*, *F. graminearum*, *F. proliferatum* and *F. subglutinans* depending on the region of cultivation. The *Fusarium* species can produce mycotoxins in cereal grains. *Fusarium* infection in maize is a concern for the food and livestock feed industries due to production of mycotoxins by several species in the grain. Damage to plants by insects increases the toxin accumulation due to more chances for fungal attack (Blaney et al 1986). This effect has been documented for ear damage; increased mycotoxin accumulation may also occur as a result of root or stalk damage, but this has not been demonstrated. *F. graminearum* causes Gibberella ear rot which leads to accumulation of deoxynivalenol and zearalenone in ears (Reid et al 1999). *F. verticillioides*, *F. proliferatum* and *F. subglutinans* cause Fusarium ear rot, and produce fumonisins in the ears (Munkvold, 2003).

Important stalk rots also can be caused by fungi other than *Fusarium* spp. Anthracnose stalk rot is caused by *C. graminicola*, which enters the corn through roots, through wounds caused by insects, or directly through the stalk epidermis. The disease affects all parts of the plant and at any time during the growing season. The review by

Nicholson et al, 2003 details the interaction of maize with the pathogen, focusing on the development of new management strategies in the improvement of plant protection. Recent studies show that root infection with *C. graminicola* leads to the systemic colonization of the plant (Sukno et al 2008). Authors used a green-fluorescent-protein tagged strain to monitor root colonization and the asymptomatic infection process *in vivo*. Diplodia stalk rot is caused by *S. maydis*; pycnidia are the survival structures which produce spores during wet conditions. Infection may occur through mesocotyl, crown, roots and lower nodes. Charcoal rot is caused by *M. phaseolina* but it is not common in Iowa. Pythium stalk rot caused by *P. aphanidermatum* and bacterial stalk rot caused by *Erwinia dissolvens* develop similar symptoms under warm, wet conditions and can occur at any time during the season, though they are not usually economically important (Munkvold et al 2000).

Root rots in Maize

Root rots are less studied diseases which occur on maize plants in all fields in each year. Yield losses due to root rot infections are unclear but researchers were able to identify the species which cause seedling blight infection and stalk rots. Many scientists believe root rots are complex of diseases caused by different fungi and bacteria even nematodes and root infecting insects. Fungi vary in their ability to cause disease and the species composition on roots depends upon the growth stage of the host plants, environmental conditions, genotype and previously grown crops (White, 1999).

Pythium root rots lead to low yield in poorly drained soils with continuous cultivation of maize and reduced tillage practices. Fungi overwinter in the soil and plant debris as oospores; upon favorable conditions, oospores germinate and produce mycelium and

zoospores, both of which infect the maize plants. Fourteen different *Pythium spp.* cause both seedling blight and root rot. *Rhizoctonia solani* causes crown and brace root rot in maize. It survives as sclerotia in the soil and colonizes on the roots, plant debris or weeds. Root rots caused by *Fusarium spp.* are mainly after seedling stage. *Fusarium* species overwinter on the seed, in the soil or crop debris. The infection starts when the roots come in contact with the fungal propagules in the soil. The contamination of seed with fungal species also can result in damping off. Root rot infection becomes severe as dry conditions favor the vegetative growth of hyphae and wet conditions induce reproductive phase.

Corn root worms

Western Corn Rootworm, *Diabrotica virgifera* LeConte (WCR) and northern corn root worm, *Diabrotica barbari* (NCR) are the most costly insect pests of maize in North America. Root worms attack plants both above and below ground, but the most serious losses are due to feeding on roots, which damages root function and causes lodging of plants (Levine et al 1991). These two beetles mostly feed on maize plants. Another species named southern corn root worm, *Diabrotica undecimpunctata howardi*, is prevalent in the southern states of the USA and attacks maize and sorghum (Weidberg, 1996). Female rootworms lay eggs in the fields of maize; eggs remain dormant in the winter and hatch when temperatures increase during spring. Rootworms hatch and are immediately attracted by the young corn roots which release CO₂ (Reidell et al 1999). Larvae start feeding on roots and mature to adult with three different stages, or instars. Each instar lasts from seven to ten days. The size of larvae increases from 1/8 to 1/2 inch in length. Since root feeding is the only source for survival of larvae, extensive damage occurs to the root system. Larvae pass through the

prepupal stage on the roots and pupate in the soil. Pupae are white and translucent (Weidberg, 1996). During the pupal stage they remain dormant and do not feed. Adult beetles emerge from the soil in mid July or early August and start feeding on the different aerial regions of plant. Adult feeding on leaves, pollen, silks, and kernels results in damage to ear formation. Male beetles appear before the females, which feed for about two weeks, then try to find egg-laying sites in the cracks of the soil. Each female beetle lays approximately 20-30 eggs and remains in the soil until it dies. These eggs overwinter and continue for another life cycle in the spring. WCR lays eggs into deep soil (up to 30 cm) whereas NCR lays eggs nearer the surface (approximately 20 cm) (Wright et al 1999).

Although there is some evidence for interactions between corn rootworms and fungi that infect roots and stalks, it is not clear whether meaningful reductions in root colonization or stalk rot occurrence can be accomplished through corn rootworm control. Wounds caused by CRW can be good invading sites for pathogenic fungi causing root and stalk rot. Interactions between WCR and several *Fusarium* species including *F. verticillioides* and *F. graminearum* were reported (Palmer and Kommedahl, 1969). In that study, no symptoms of stalk rots were seen, but several pathogenic *Fusarium* species and other fungi were recovered from western corn root worm larvae. Several species that they collected from roots are capable of causing stalk rots. Since *Fusarium* spp. are saprophytes, they can overwinter in crop residue, which serves as a major source of inoculum. Planting date and maize hybrid influence the impact of root worm which ultimately relates to yield losses due to root damage and plant lodging (Michael et al 2000). Reduced tillage and application of nitrogen fertilizer at regular timing can decrease western corn root worm injury (Roth et al 1995). Fungi that

can cause stalk rot (*F. moniliforme* and *F. subglutinans*) were isolated from western corn rootworm beetles in Colorado (Gilbertson et al 1986).

Fusarium diseases that are enhanced by insect injury can be partially managed through insect resistance. Damage caused by European corn borers increased stalk colonization by *Fusarium spp.*, and the composition of fungal species in stalks differed between corn-borer resistant Bt hybrids and susceptible hybrids (Gatch et al 2002). The use of transgenic (Bt) resistance to European corn borers can reduce the risk of stalk rot infection. There were increased populations of insects in maize plants highly infected with *F. verticillioides* in West Africa (Mouhoubé et al 2003). Herbicides did not show effect on the *Fusarium* populations (Skoglund et al 1988). Control of corn root worm damage traditionally depended on insecticides and crop rotation, but transgenic insecticidal maize hybrids are now available for CRW control. The transgenic hybrids were developed using *Cry* genes from *Bacillus thuringiensis* which code for the release of *Cry* proteins in the maize. The *CryBb1* genes released proteins which are eight times more lethal to corn root worm larvae than regular wild protein (Vaughn et al 2005). The main advantages of using transgenic hybrids for rootworm control are increased crop protection and reduced use of insecticides (Rice 2004).

Real time PCR

In order to determine the impact of CRW resistance on CRW-*Fusarium* interactions, a measure of fungal biomass in the infected plant tissue is needed. The standard method for quantification of fungi from plant tissue is a traditional microbiological assay, using serial dilution of ground plant tissue onto a suitable medium. This method is time and labor-

intensive but an important advantage that is several species can be enumerated simultaneously. However, fungal biomass can be measured only on a relative basis, the results are heavily influenced by particle size of ground plant tissue, and identification of fungi based on colony morphology is not precise. More precise identification is possible using nucleic-acid based methods like PCR with species-specific primers. Traditional conventional PCR is also time consuming, requires post-processing of the product, and is not quantitative. Whereas, using real-time PCR we can accurately quantify the amount of fungal biomass present in the sample for specific *Fusarium* species.

Real-time PCR detects and quantifies target DNA in the samples using fluorescent probes. Real-time PCR can quantitatively measure the target pathogenic DNA in environmental samples accurately, by calibrating against a known amount of pure fungal DNA. Real-time PCR methods have been developed for the detection and quantification of particular fungal species not only in the pure fungal cultures but also in plant tissue (Nicholson et al 2003). Authors have shown the detection of economically important fungal pathogens and quantification of oomycetes (Bart et al 2006), and ectomycorrhizal mycelium (Landeweert et al 2003) in plant and soil samples. Therefore, we are using a TaqMan real-time PCR approach for a more accurate assessment of fungal biomass in roots compared to microbiological methods. The application of real time PCR method gives both qualitative and quantitative estimation of fungal biomass in the root and stalk tissues.

Molecular detection using PCR techniques is well established for *Fusarium* species that are difficult to distinguish morphologically (De weerd et al 2006). TaqMan real-time PCR has been used to quantify different *Fusarium* species in wheat (Waalwijk et al 2004).

Compared to conventional PCR, real time quantitative PCR is more accurate, less labor-intensive and does not require post handling of PCR product (Heid et al 1996). To detect the presence of foreign DNA molecule in the plant material, in a fast and easy way quantitatively, real-time PCR is the approach preferred by most researchers.

Authors have shown that real time PCR gives accurate results for quantification and monitoring colonization by *F. graminearum* in wheat grains, barley, and malt in controlled environments (Burlakoti et al 2007; Sarlin et al 2006). Species specificity requires two steps, selective primer amplification and probe hybridization to target DNA in the PCR product (Roe et al 2001). The TaqMan probe binds to specific target DNA which allows accurate quantification. The most promising way for the identification of different species in environmental samples is detection of probe hybridized PCR products, amplified using species specific primers (Wu et al 2002). *Tri* genes have been identified which are involved in the synthesis of type B trichothecenes. *Tri 5* gene is an important gene which encodes for the first oxygenase step of the pathway. *Tri 5* primers have been designed from the sequences of *Tri 5* encoding trichodiene synthase gene (Desjardins et al 1992). So the species which are capable of producing trichothecenes can be identified using these set of primers. Authors were able to quantify *F. graminearum* in harvested wheat grain with real time PCR using primers that amplify the *Tri 5* gene for better assessment of efficacy of fungicides on head blight disease (Zhang et al 2009).

The fumonisin biosynthetic genes encodes polyketide synthase pathway which is required for the production of fumonisins. *Fum 1* gene encodes for the initial catalytic step of fumonisin biosynthesis (Proctor et al 1999). Fumonisin-producing species were

distinguished from non fumonisin-producing *Fusarium* species which are commonly present in the maize using quantitative PCR. These authors found a good correlation between fungal DNA and fumonisin content (Waalwijk et al 2008). Quantitative TaqMan PCR assay is also proven in the detection of species-specific *Fusarium* DNA in air samples which is useful for assessing the risk of infection by toxigenic *Fusarium* species (Halstensen et al, 2006). Quantitative real time PCR assays have been used to identify different 11 *Fusarium* species, including the major mycotoxin producing species in cereals, *F. graminearum*, *F. culmorum*, *F. poae*, *F. langsethiae*, *F. sporotrichioides*, *F. equiseti*, *F. tricinctum*, *F. avenaceum*, *F. verticillioides*, *F. subglutinans* and *F. proliferatum*, based on one gene, elongation factor (EF 1 α), (Nicholsen et al 2008).

Studies on interactions among root worms, root- and stalk-rot fungi are needed to support integrated approaches to corn root and stalk health management. Our research work mainly focuses on *Fusarium* species because they are among the most important root and stalk-rotting fungi in Iowa and Nebraska. In order to quantify the impact of CRW resistance on these interactions, we measured the fungal biomass in the infected plant tissue. We hope that real-time PCR offers a more accurate assessment of fungal biomass in roots, compared to microbiological methods. Successfully implemented real-time PCR can be useful for assessing the impacts of many environment and management practices in addition to CRW interactions.

Hypotheses: Maize roots injured by feeding of corn rootworm larvae will have greater *Fusarium* colonization than roots with little or no CRW injury. Colonization of roots with *Fusarium spp.* leads to stalk colonization and decay. Therefore, the main hypothesis of

my project is hybrids with CRW resistance will have reduced *Fusarium* colonization of roots and stalks compared to susceptible hybrids when they are exposed to CRW infestation.

Objectives: The overall aim of my project is to determine whether use of transgenic CRW-resistant maize hybrids will reduce the level of *Fusarium* colonization of roots and stalks:

- 1) Quantitative specific molecular detection of root and stalk colonization by *F. verticillioides* and *F. graminearum* in plants that are susceptible or resistant to CRW feeding, through real-time PCR.
- 2) Quantification of the relative biomass of *Fusarium* species in roots and stalks of plants those are susceptible or resistant to CRW feeding, through dilution plating.

Materials and Methods

Preparation of fungal pure cultures

Stock cultures of *F. verticillioides* and *F. graminearum* (Table 1), originally isolated from maize kernels in Iowa, and were maintained on silica gel crystals at 4 °C and working cultures were maintained in PDA media at 4 °C. For DNA extraction, strains were grown on a potato dextrose agar (PDA) medium at 25 °C for 3-9 days.

Field experiments

Experiments were conducted in two Iowa locations (ISU Bruner Farm, Boone Co., and ISU Southeast Research Farm (ISU SERF), Washington Co.) and one in Nebraska (University of Nebraska-Lincoln Agricultural Research and Development Center (UNL ARDC), Saunders Co.). In each field there were two pairs of corn root worm (CRW) resistant and corresponding CRW-susceptible hybrids (Table 2) planted in a trap-crop area with moderate to high CRW pressure. Plots were four rows, spaced 76 cm apart, 5.33 m long. In each row, 28 to 42 seeds were planted. We collected roots and stalk tissues for measurement of CRW injury and analysis of *Fusarium* colonization by dilution plating and quantitative PCR twice during the season (mid July and mid September). Five plants were sampled from each plot by cutting the stalks approximately 25 cm from the soil surface and manually digging the root mass from every fifth plant in one inside row of each plot.

Processing the roots and stalks

Root and stalk tissues were sampled from the field locations and immediately transported to the laboratory. They were stored at 4 °C overnight until they could be processed the following day. The tissues were thoroughly rinsed with tap water to clean the

soil attached to the roots and CRW injury was evaluated by a standard method (Oleson et al 2005). The root tissues were trimmed from the stalks and surface-disinfested in 10% bleach for 2 min, washed twice with autoclaved deionized water, and dried with paper towels. The root tissues of each plant were equally separated for further processing. One-half of the roots were placed in a drying oven at 40 °C for 3-5 days, to prepare for dilution plating. The other half was prepared for quantitative PCR analysis by freeze-drying and homogenizing in a stainless steel blender. These samples were stored at –20 °C until further processing could be completed. The stalk samples consisted of the basal 6 to 8 cm of the stalk, aseptically cut in longitudinal halves. Each half was treated as described for the roots. Mid-season samples consisted of roots only. Late-season samples consisted of stalks for the 2007 samples but for 2008 samples both root and stalk tissues.

Dilution plating

The oven dried plant material was ground using a cyclone sample mill, UDY corporation. One gram of sample was weighed and suspended in 9 ml of autoclaved water. Serial dilutions were made to determine the most useful dilution for colony enumeration. The samples were plated thrice with two different dilutions, 10^{-1} and 10^{-2} on Nash-Synder medium (Leslie and Summerell, 2006). The plates were incubated at 25 °C for 4 days and putative *Fusarium* colonies were counted using a colony counter. Representative colonies were transferred to carnation leaf agar medium to confirm identification as *Fusarium* species (Leslie and Summerell, 2006).

Extraction of DNA from pure cultures and plant material (roots and stalks)

Pure cultures was harvested using sterile forceps, approximately 2-4 cm of mycelial surface was scraped from PDA plates for the extraction of genomic DNA. Mycelia were transferred into in 2-ml tubes half-filled with glass beads (0.5 μm in diameter) and physically disrupted with a bead-beater apparatus (FastPrep-24 System, MP Biomedicals, Solon, OH) for 45 s for the release of genomic DNA. For plant tissue, 200 mg of homogenized sample was taken for the extraction of genomic DNA for both roots and stalks.

Selection of extraction method

The protocols used for the extraction of DNA were optimized using yield quality and quantity and fluorescence intensity or threshold value (Ct) value. We tested three protocols to optimize the conditions: cetyl trimethyl ammonium bromide (CTAB) (Allen *et al.*, 2006), sodium dodecyl sulphide (SDS) (Chow and Kafer, 1993) and fast prep-24 system machine ((FastPrep-24 System, MP Biomedicals, Solon, OH) extraction kit protocol. Genomic DNA was extracted from mycelium harvested from PDA plates and physically disrupted for all three protocols. Quantity and quality of DNA extracts were determined with an ND-1000 NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE) and purified DNA samples were stored in sterile de-ionized water at $-20\text{ }^{\circ}\text{C}$ until used. The optimized protocol was selected based on the quality, quantity and threshold value for the extraction of DNA from fungal Chow and Kafer method and for plant material Allen et al method.

Specificity of primers and probes

The specificity of primers and probes were optimized using DNA from pure cultures. The range of sensitivity of the PCR assay was determined using 50 ng of purified genomic

DNA from both *Fusarium* species with 10 fold serial dilutions. A real-time PCR TaqMan assay published previously (Bluhm *et al* 2004) was utilized in this study. Reactions were conducted in optical-grade 96-well PCR plates in an ABI Prism 7900 Sequence Detection System (ABI, Foster City, CA). The thermal profile was 2 min at 50 °C, 10 min at 95 °C followed by 40 cycles of 15 s at 95 °C and 60 s at 60 °C. Amplification data were analyzed by the SDS software v. 2.3 (Applied Biosystems, Foster City, CA). For *Fusarium* quantification, root and stalk tissues DNA was at the concentration of 250 ng/20µl PCR reaction.

Construction of standard calibration curve

Standard calibration curve for pure fungal genomic DNA of both species was constructed using tenfold serial dilution of quantified DNA. The concentrations of DNA varied from 50 ng-500 fg were used for generating the standard calibration curves. The concentration of DNA used in the reaction was plotted against threshold value to get standard quantification curve. The TaqMan PCR assay was repeated twice with each serial dilution reaction thrice.

Specificity of primer detection of target DNA in plant material

The specificity of primers to detect the presence of target fungal DNA in plant tissue was determined using the spiking of uncontaminated host tissue with known quantities of fungal amounts in each reaction. The plant tissue which did not amplify with the primers that specifically detect the presence of fungal species and positive PCR reaction for maize single copy gene alcohol dehydrogenase was used to study the primer specificity in the detection of target fungal DNA in the host plant. A range of fungal DNA concentrations (250 ng to 2.5

fg) was spiked into fixed amounts of host plant tissue. The assay was repeated thrice with three replications each time.

The amount of fungal concentrations present in the plant samples were statistically correlated between CRW-resistant and CRW-susceptible hybrids. The data were statistically analysed using SAS software version 9.1 with general linear model (GLM). The figures were drawn using Sigma plot software version 9.0.

Results

Real time PCR TaqMan assay optimization

The real-time PCR TaqMan assay specificity was established with published (Bluhm et al 2004) primers for *Fum 1* and *Tri 6* genes in *F. verticillioides* and *F. graminearum*, respectively, using the primer pairs (*Fum 1 F/ Fum 1 R* and *Tri 6 F/Tri 6 R*) listed in the methods section. The specificity of primers was confirmed using genomic DNA from different *Fusarium* spp. and the concentration of primers was established by performing PCR assays with different concentrations (Table 3). Trichothecene-producing species had positive PCR reactions for *Tri 6* set of primers and fumonisin-producing species had positive PCR reactions for *Fum 1* set of primers; both showed positive response for the internal control ITS product which has been designed from *Fusarium* rDNA sequences (Table 1).

Standard Calibration curve for the pure genomic DNA from fungal cultures

The sensitivity of the PCR assay was established using standard curves generated by ten-fold serial dilutions of purified genomic DNA of *F. verticillioides* and *F. graminearum*. In the real-time PCR TaqMan assay, a higher concentration of DNA gives a low Ct value and a lower concentration of DNA gives a high Ct value. There was a negative correlation when the Ct value was plotted against the different fungal DNA concentrations ($R^2= 0.993$ and $R^2=0.9985$). The limit of detection of the assay was 500 fg of purified fungal genomic DNA per reaction (Fig 1 & 2).

Spiking studies of fungal DNA

The aim of the assay was to determine whether the presence of overwhelming amounts of host tissue would interfere in the detection of target DNA in the reaction. To

confirm the capability of the assays for detection and quantification of target DNA in environmental field samples, a negative field sample was spiked with varying amounts of fungal DNA, to mimic different colonization levels of actual field sample root and stalk tissues. Negative (non-infected) host tissue samples that had no reaction with the Fum1 and Tri6 probes, but had positive reactions with a probe for the *Adh1* host gene was selected for the assay. The signal generated using the primers and probe in response to the different varying amounts of fungal tissue was adequately sensitive and was able to detect and quantify the target DNA in the host tissues. There was a negative correlation when the Ct value was plotted against the different fungal DNA concentrations ($R^2=0.9987$ and $R^2=0.9946$) (Table 5). The limit of quantification of fungal DNA in the host tissue is 2.5 pg. We can conclude that the presence of host tissue did not interfere the detection and quantification of target DNA using specific primers and probes (Fig 3 & 4).

Roots and Stalk Sample Analysis:

PCR Results

The fungal biomass in the root and stalk tissues was quantified using the standard curves constructed with known concentrations of DNA from pure cultures of *F. verticillioides* and *F. graminearum* (Fig 1 & 2). The standard curve for the both fungi had a linear correlation coefficient of $r=0.99$, which indicates a high level of reproducibility.

At ISU SERF in 2007 the colonization level of fungal species was evaluated in the mid-July root tissues. There was a high level of variability in the colonization of roots with *F. verticillioides* and *F. graminearum*. Overall there was no significant difference in levels of

colonization in resistant CRW hybrids when compared to susceptible CRW hybrids within the treatments (Table 5).

At ISU Bruner farm, the level of colonization of roots with both fungal species was highly variable within the treatments, and there were no significant differences among treatments. In stalks there was a high colonization of *F. graminearum* in both Bruner and Crawfordville farms (Table 5 & 6), which is consistent with the previous studies indicating that stalk rot caused by *F. graminearum* is common in Iowa.

In the UNL ARDC location there was a high level of colonization by *F. verticillioides* in roots in susceptible CRW hybrids while CRW-resistant hybrids had low levels of colonization. Overall there was a significant difference ($P=0.049$) between CRW-resistant and susceptible hybrids in the colonization of roots with *F. verticillioides*; all the resistant hybrids showed less colonization than susceptible hybrids in all events; however, high variability among treatments masked significant treatment effects in several cases. In event MIR 604 there was significantly less colonization by *F. verticillioides* both in the roots and stalks ($P=0.03$ and $P=0.04$ respectively). There was a higher level of colonization of roots than stalks. Colonization of stalks by *F. graminearum* was at low levels and did not differ significantly among hybrids (Table 7).

In 2008, at the ISU SERF location, we used only two events, DAS 59122 and MON 88017, and their susceptible counterparts, treated with different levels of clothianidin (Poncho seed treatment, Bayer CropScience) and we observed colonization level in mid-July roots, mid-September roots and stalks. Due to high level of variability within treatments, the treatments did not show significant difference between resistant and susceptible hybrids in

the level of colonization. Only in the event DAS 59122 showed significant difference (P value is 0.04) between resistant and susceptible CRW hybrids in the colonization of *F. graminearum* in July root tissues (Table 8).

At UNL ARDC 2008, mid-July roots, the event MON 863 resistant hybrid had low level of colonization of *F. verticillioides* and of *F. graminearum* compared with its near-isogenic susceptible hybrid and showed significant difference ($P=0.05$ and $P=0.001$). The MON 88017 event (DKC 63-42) had significantly less colonization with *F. graminearum* in July roots ($P=0.001$). The stalks were highly colonized with *F. graminearum* irrespective of hybrid or transgenes (Table 9). In both Nebraska and Iowa locations, stalks were highly colonized with *F. graminearum* and neither seed treatment nor resistant transgenes had an effect on colonization by *F. graminearum* in stalks (Table 8 & 9).

Serial dilution plating results

The colonization of roots and stalks with *Fusarium* species were significantly lower in CRW resistant hybrids than in the near-isogenic CRW susceptible hybrids. In Iowa at Bruner farm 2007, in all resistant CRW hybrids suffered significantly less colonization than the susceptible hybrids (P value for Bt is 0.01 (roots-fig 5) & 0.04 (stalks-fig 6)). In stalks event DAS 59122 had significantly less colonization in resistant vs. their near susceptible hybrid ($P=0.04$) (Fig. 6).

In UNL, ARDC the colonization level in roots with DAS 59122 and MON 88017 events showed significant difference between resistant and susceptible hybrids ($P \leq 0.05$). Within all treatments there was significant difference ($P=0.004$ for Bt) between resistant and susceptible hybrids for the colonization of roots (Fig 7). Events DAS 59122 and MON 88017

showed significant differences between the resistant and susceptible hybrids ($P=0.01$ and $P=0.03$) in the colonization of roots. In the stalks there was no significant difference between the resistant and susceptible hybrids in the level of colonization (Fig. 8).

In 2008 the colonization level on roots was measured in summer (mid-July) and fall (mid-September) and stalks. In ISU SERF, DAS 59122 with both levels of clothianidin treatment and MON 88017 with both levels of clothianidin treatment showed significant difference in the colonization between CRW-resistant and CRW-susceptible hybrids in summer root ($P=0.01$ for Bt) (Fig. 9) and fall root ($P=0.001$ for Bt) (Fig. 10) tissues and also in stalks ($P=0.003$ for Bt) (Fig. 11). In fall, root and stalk tissues showed significantly lower colonization in lower level of clothianidin treatment for the DAS 59122 event ($P=0.001$) (Fig. 10 & 11).

In UNL ARDC 2008, there was significant difference between resistant and susceptible CRW hybrids among all events for level of colonization of fall roots ($P=0.008$ for Bt) and stalks ($P=0.01$ for Bt). In event DAS 59122 there was a significant difference between resistant and susceptible hybrids in summer roots ($P=0.001$) (Fig. 12), fall roots ($P=0.001$) (Fig. 13) and stalks ($P=0.007$) (Fig. 14). In event MON 88017 there was a significant difference in the colonization level between resistant and susceptible hybrids in all summer roots, fall roots and stalks ($P=0.016$) (Fig. 13). In event MON 863 there was a significant difference in fall roots ($P=0.014$).

Discussion

Root feeding by corn rootworm increases the chances of invasion by pathogenic organisms, but this effect has not been described in detail. Previous reports (Palmer and Kommedahl, 1967, 1969) related corn rootworm infestation to an increased incidence of *Fusarium* species in roots, but root infection was not quantified and the influence of rootworm feeding on individual *Fusarium* species was not reported. In our research, we quantified root and stalk infection by *Fusarium* spp. (through dilution plating) and infection by fumonisin- and trichothecene-producing species through the use of a real-time PCR TaqMan assay. These approaches facilitated the quantification of fungal biomass in maize roots and stalks with relation to transgenic protection against corn root worms.

We are specifically interested in the quantification of two species, *F. verticillioides* and *F. graminearum*, because these two species were previously reported as predominant stalk rot pathogens in Iowa and other parts of the Midwestern US (Kommendahl and Windels, 1981; Smith and White, 1988; White, 1999). To detect and quantify these two species many researchers recently have used specific primers for genes which are involved in mycotoxin biosynthesis. The best estimate for quantification of *F. verticillioides* and *F. graminearum* is obtained by conducting real-time Taqman PCR using these primers. The use of fluorogenic probe in the assay increases the sensitivity and enables the specific species identification and quantification of target fungal organisms. This TaqMan assay provided accurate quantitative detection of these *Fusarium* spp. in maize tissues, which allowed for better knowledge of the effects of corn root worm resistant hybrids on the level of *Fusarium* colonization.

The concentration of primers in each reaction was optimized using different concentrations in the PCR assay. Standard curves of target DNA are required for the quantification of target species in unknown sample tissues (Kuhne et al 2002). Both TaqMan assays had a wide dynamic range in detection and were highly sensitive and reproducible. The assay was capable of detecting the target DNA in femtograms even in the environmental samples. The assay was established by spiking known amounts of fungal organisms in the host tissue, which gives the specificity and sensitivity of the primers in the detection of target DNA in the presence of large amounts of host tissue.

The quantification of target DNA in the root and stalk samples using standard calibration curve gave efficient and accurate values, based on the high coefficient of determination for the regression curve ($r^2=0.998$). We also tested the accuracy by spiking known amounts of target DNA in root tissue, and there was a linear correlation between the concentration and cycle threshold values ($r^2=0.9984$). The soil DNA or plant DNA will not significantly interfere in the PCR Taqman assay while we quantify the target fungal DNA present in the plant tissue, even specific amount of target DNA from 0.12 to 1.31 ng in 1 gram of soil was detected (Bart et al 2006). The real time PCR assay was able to detect and quantify the target *Fusarium* species in wheat samples. The assay was able to quantify, *F. graminearum* from 0-4.0 pg DNA/ng of plant DNA, *F. culmorum* from 0-16.4 pg DNA/ng of plant DNA, *F. poae* from 0-26.2 pg DNA/ng of plant DNA, *F. langsethiae* from 0-0.8 pg DNA/ng of plant DNA, *F. avenaceum* from 0.1-8.6 pg DNA/ng of plant DNA and *F. tricinctum* from 0-2.6 pg DNA/ng plant DNA (Nicolaisen et al 2009). There was no evidence for PCR inhibitors in the assay which might have hindered the PCR reaction and affected the

quantification of target DNA in the root tissues. The real time PCR assay using a Taqman hybridization probe is a fast and reliable method for species specific identification and quantification of *F. graminearum* in planta (Reischer et al 2004) and FUM1 gene in maize grains (Rangel et al 2005).

We estimated fungal biomass by a traditional method, using dilution plating, and a more specific method, using real-time PCR TaqMan assay. The major advantage of real time PCR is quantification of target DNA quickly, in a precise way with high sensitivity and without post processing of the amplification product. The disadvantage is it does not simultaneously quantify the DNA of all species of interest. The advantage of dilution plating is the ability to simultaneously quantify all *Fusarium* species present in the sample but it is time consuming and labor intensive, it is less sensitive and less accurate than PCR, and does not yield precise information at the species level.

PCR Results

The PCR assay results did not consistently indicate statistically significant differences in colonization between resistant and susceptible hybrids, although in most cases resistant hybrids had lower means. Due to high variability among the replications, these differences often were not statistically significant. In 2007, at the Bruner farm, biomass of *F. verticillioides* in stalks was significantly lower in the hybrid with Bt event MIR 604 compared to its susceptible counterpart. In the UNL, ARDC location there was a significant effect of Bt on the colonization of roots by *F. verticillioides*; the resistant hybrids with all the events showed less colonization than susceptible hybrids. In the event MIR 604, it had a significant effect on colonization by *F. verticillioides* both in the roots and stalks and also in

the colonization of roots with *F. graminearum*. There was a significant effect of CRW resistance on the level of colonization by *F. verticillioides* in both roots and stalks at UNL ARDC and by *F. graminearum* at the ISU Bruner farm.

In 2008, at the ISU SERF location, hybrids with two events, DAS 59122 and MON 88017, were planted and were treated with two different rates of clothianidin (Poncho® seed treatment): 0.25 mg a.i./seed, or 1.25 mg a.i./seed. Event DAS 59122 with higher rate of treatment showed significant difference between the CRW-resistant and -susceptible hybrids in the colonization of *F. graminearum* in July root tissues. In Nebraska the MON 863 resistant hybrid had a significantly lower level of colonization of *F. verticillioides* and of *F. graminearum* in the mid-July roots and also event MON 88017(DKC 63-42) also had significantly less colonization with *F. graminearum* compared with their susceptible counterpart. Overall from two years, PCR results illustrated that stalks were highly colonized with *F. graminearum*. At the UNL ARDC location, *F. verticillioides* was the predominant species and *F. graminearum* was predominant at ISU SERF.

Dilution plating results

In 2007 in the dilution plating results there was consistently lower colonization in resistant hybrids compared to their susceptible counterparts. At UNL ARDC, there was significant effect of Bt on the colonization of roots. The colonization level in roots was significantly lower for DAS 59122 and MON 88017 events.

In 2008, At ISU SERF, there was a significant effect of Bt on the colonization of both summer and fall roots and stalks. At UNL ARDC, there were significant effects of Bt

between resistant and susceptible CRW hybrids across all events in level of colonization of fall roots and stalks.

The results confirm that feeding of roots by corn root worms increases colonization by *Fusarium* spp. In most events there was difference between the levels of colonization of roots and stalks with *Fusarium* spp. between resistant and susceptible CRW hybrids, according to dilution plating evaluation. But whereas we are only quantifying the species involved in the synthesis of trichothecenes and fumonisins using real-time PCR TaqMan assay, the difference between resistant and susceptible hybrids were not significant in all events. The difference in results between PCR and dilution plating suggest that CRW injury may increase colonization of roots by *Fusarium* species that are not trichothecene or fumonisin-producing species. There was a higher level of colonization in roots than stalks for all locations. Dilution plating is not a strict measurement of fungal biomass since it is the visual calculation of colony-forming units, which may represent a range of fungal propagules, hyphal fragments or mycelial clusters. In addition, colonies consisted of numerous *Fusarium* species, not only trichothecene- or fumonisin-producing species. Therefore we could not correlate the PCR results with dilution plating results. Also in a recent study of quantification of *F. solani* f. sp. *phaseoli* in mycorrhizal bean plants and the surrounding soil, the enumeration of fungal colonies using dilution plating method did not correlate with PCR results (Filion et al 2003). Possibly statistically significant differences could be detected more consistently with more intensive sampling, in order to offset the high level of variation among plants. However, the cost of processing a high number of samples can be prohibitive.

Further progress in strengthening data to support the hypothesis might be achieved by using the same resistant and susceptible hybrids within all selected fields and also doing controlled experiments in a green house with root worm resistance and non resistant plants, with and without corn rootworm infestation, so that we can compare the colonization in the presence and absence of the root worm resistance with respective of Bt events. Primers designed on sequences of genes other than the mycotoxin biosynthesis genes might better serve to quantify only target species in the samples, instead of all trichothecene-producing or fumonisin-producing species.

Conclusions

The corn root worm feeding enables different soil microflora to enter in to the plants through roots by creating wounds or invasive courts. To better understand the mechanism of colonization of *Fusarium spp.* and study of interaction of roots with *Fusarium* species for the development of good management practices we proposed to quantify *Fusarium spp.* in roots. The quantification was measured by both traditional microbiological dilution plating method and real-time PCR TaqMan assay. In 2007, only roots were sampled in the summer and only stalks in the fall but for the 2008 we sampled roots also in fall. The colonization in CRW susceptible hybrids was high when compared to resistant hybrids but it did not show the same trend in all transgenic events.

In 2007, Iowa location PCR results showed much variation in colonization in-between resistant and susceptible CRW hybrids, whereas dilution plating results showed significant reduction in the resistant CRW hybrids mainly in DAS59122, MON 863 and MON 88107 events. But the Iowa locations had high levels of *F. graminearum* (or other trichothecene-producing species) and the Nebraska location had higher levels of *F. verticillioides*. We can conclude upon these results that feeding of maize roots with corn root worms enhances the infection of roots with *Fusarium spp.* and resistant hybrids are less colonized when compared to susceptible hybrids.

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Tables

Table 1: List of *Fusarium* isolates used to determine primer specificity

Species	Isolate	Origin	Host	Primer Specificity		
				FUM 1	ITS	TRI 6
<i>F. graminearum</i>	A66A	Iowa	Maize	----	+++	+++
<i>F. graminearum</i>	A66B	Iowa	Maize	----	+++	+++
<i>F. graminearum</i>	A72A	Iowa	Maize	----	+++	+++
<i>F. oxysporum</i>	120 T 7	Iowa	Soybean	----	+++	----
<i>F. oxysporum</i>	120 L 6	Iowa	Soybean	----	+++	----
<i>F. proliferatum</i>	114 L 5	Iowa	Soybean	+++	+++	----
<i>F. proliferatum</i>	15 T 1	Iowa	Soybean	+++	+++	----
<i>F. semitectum</i>	5 L 5	Iowa	Soybean	----	+++	----
<i>F. sporotrichoides</i>	194 L 5	Iowa	Soybean	----	+++	+++
<i>F. sporotrichoides</i>	29 L 6	Iowa	Soybean	----	+++	+++
<i>F. subglutinans</i>	19 L 10	Iowa	Soybean	+++	+++	----
<i>F. subglutinans</i>	34 L 5	Iowa	Soybean	+++	+++	----
<i>F. subglutinans</i>	42 T 7	Iowa	Soybean	+++	+++	----
<i>F. verticillioides</i>	94-157	Iowa	Maize	+++	+++	----
<i>F. verticillioides</i>	m3120	Iowa	Maize	+++	+++	----

Table 2: Hybrids used in the field experiments

Company	Hybrid Name	Maturity	Traits	Bt event (Coleoptera)	Cry endotoxin (Coleoptera)
Pioneer	34A20	109	RR2/HXX/LL	DAS-59122-7	Cry 34/Cry 35
Pioneer	34A14	108	RR2		
Pioneer	35T08	107	RR2/HXX/LL	DAS-59122-7	Cry 34/Cry 35
Pioneer	35T05	105	RR2		
Syngenta	N51-T6	104	AgriSure RW/CB/LL	MIR 604	Cry 3A
Syngenta	N51-T8	104	AgriSure CB/LL		
Dekalb	DKC 61-69	111	VT3	MON 88017	Cry 3Bb
Dekalb	DKC 61-72	111	RR2		
Dekalb	DKC 63-46	113	RR2/YGCB		
Dekalb	DKC 63-39	113	RR2/YGPL	MON 863	Cry 3Bb
Dekalb	DKC 63-42	113	VT3	MON 88017	Cry 3Bb
Dekalb	DKC 58-13	108	RR2/YGPL	MON 863	Cry 3Bb
Dekalb	DKC 58-19	108	RR2		

Table 3: Optimization of primers concentration in the PCR assay

Primers Concentration/in the 20 µl PCR reaction	<i>F. verticillioides</i> Mean Ct/Std Dev	<i>F. graminearum</i> Mean Ct/Std Dev
0.3 uM	21.59/0.07	20.88/0.30
0.45 uM	21.87/0.21	21.57/0.08
0.9 uM	21.66/0.26	21.36/0.55

Table 4: Regression equation of standard curves and spiked DNA for *F. verticillioides* and *F. graminearum*

Sample	<i>F. verticillioides</i>	<i>F. graminearum</i>
Pure culture DNA	y = 3.698x + 14.783 R ² = 0.993	y = 3.4516x + 15.258 R ² = 0.9985
Spiked DNA in the 250 ng of Host tissue	y = 3.4126x + 17.026 R ² = 0.9987	y = 3.4329x + 14.843 R ² = 0.9946

Table 5. *Fusarium* DNA concentrations (pg DNA/200mg host tissue) for maize hybrids with or without transgenes for resistance to corn rootworm feeding. Data are from ISU Southeast Research Farm near Crawfordsville, IA, in 2007.

Hybrid	Event	2007- July Roots	
		<i>F. vert</i> conc (pg)	<i>F. gram</i> conc (pg)
34A20	DAS 59122	< 0.5	1.8
34A14	None	< 0.5	1.2
DKC 61-69	MON 88017	< 0.5	1.6
DKC 61-72	None	< 0.5	< 0.5

The resistant and near-isogenic susceptible hybrids are grouped between horizontal lines. Hybrids with Bt events are resistant and without Bt events are susceptible. The limit of quantification was 0.5 pg. Means are according to the LSMEANS calculation (SAS PROC GLM) and means were compared by orthogonal contrasts between the resistant and the near isogenic susceptible hybrid within the event. ND=Not detected.

Table 6. *Fusarium* DNA concentrations (pg DNA/200mg host tissue) for maize hybrids with or without transgenes for resistance to corn rootworm feeding. Data are from Iowa State University Bruner farm in Boone Co., IA, in 2007.

Hybrid	Event	2007- July Roots		2007- Sept Stalks	
		<i>F. vert</i> conc (pg)	<i>F. gram</i> conc (pg)	<i>F. vert</i> conc (pg)	<i>F. gram</i> conc (pg)
35T08	DAS 59122	19.9	5.5	ND	1.48
35T05	None	38.6	15.8	< 0.5	13.7
DKC61-69	MON 88017	32.6	51.1	< 0.5	< 0.5
DKC61-72	None	57.8	17.6	< 0.5	ND
N51-T6	MIR 604	20.6	20.9	< 0.5	1.0
N51-T8	None	6.6	22.2	< 0.5	6.6
DKC58-13	MON 863	30.9	61.0	< 0.5	4.0
DKC58-19	None	12.6	20.8	< 0.5	0.8

The resistant and near-isogenic susceptible hybrids are grouped between horizontal lines. Hybrids with Bt events are resistant and without Bt events are susceptible. The limit of quantification was 0.5 pg. Means are according to the LSMEANS calculation (SAS PROC GLM) and means were compared by orthogonal contrasts between the resistant and the near isogenic susceptible hybrid within the event. ND=Not detected.

Table 7. *Fusarium* DNA concentrations (pg DNA/200mg host tissue) for maize hybrids with or without transgenes for resistance to corn rootworm feeding. Data are from University of Nebraska Research and Agricultural Development Center Farm near Mead, NE, in 2007.

Hybrid	Event	2007- July Roots		2007- Sept Stalks	
		<i>F. vert</i> conc (pg)	<i>F. gram</i> conc (pg)	<i>F. vert</i> conc (pg)	<i>F. gram</i> conc (pg)
34A20	DAS 59122	< 0.5	< 0.5	ND	0.7
34A14	None	37.4	< 0.5	4.8	< 0.5
DKC 58-13	MON 863	28.5	< 0.5	< 0.5	ND
DKC 58-19	None	131.9	< 0.5	< 0.5	ND
DKC 61-69	MON 88017	3.3	< 0.5	< 0.5	< 0.5
DKC 61-72	None	307.0	< 0.5	< 0.5	ND
N51-T6	MIR 604	31.9*	< 0.5	2.2*	7.4
N51-T8	None	567.4	< 0.5	15.3	3.4

The resistant and near-isogenic susceptible hybrids are grouped between horizontal lines. Hybrids with Bt events are resistant and without Bt events are susceptible. The limit of quantification was 0.5 pg. Means are according to the LSMEANS calculation (SAS PROC GLM) and means were compared by orthogonal contrasts between the resistant and the near isogenic susceptible hybrid within the event. * Asterisks indicate means that are significantly different ($P \leq 0.05$) from near-isogenic susceptible hybrid for that particular event. ND=Not detected.

Table 8. *Fusarium* DNA concentrations (pg DNA/200mg host tissue) for maize hybrids with or without transgenes for resistance to corn rootworm feeding. Data are from ISU Southeast Research Farm near Crawfordsville, IA, in 2008.

Hybrid	Event	Rate of treatment (mg/seed)	2008 - July Roots		2008 - Sept Roots		2008 - Sept Stalks	
			<i>F. verti.</i> conc (pg)	<i>F. gram.</i> conc (pg)	<i>F. verti.</i> conc (pg)	<i>F. gram.</i> conc (pg)	<i>F. verti.</i> conc (pg)	<i>F. gram.</i> conc (pg)
34A20	DAS							
	59122	0.25	< 0.5	< 0.5	15.7	24.7	ND	335.4
34A14	None	0.25	< 0.5	< 0.5	4.5	1.6	80.3	513.3
34A20	DAS							
	59122	1.25	< 0.5	< 0.5	14.1	3.5	ND	139.8
34A14	None	1.25	< 0.5	< 0.5	12.3	17.1	122.8	760
DKC61-69	MON							
	88017	0.25	< 0.5	< 0.5	6.2	15.8	47	162.2
DKC61-72	None	0.25	< 0.5	< 0.5	ND	1.4	217	108.5
DKC61-69	MON							
	88017	1.25	< 0.5	< 0.5*	2.0	4.9	ND	719.6
DKC61-72	None	1.25	< 0.5	1.2	1.0	4.4	ND	1307.7

The resistant and near-isogenic susceptible hybrids are grouped between horizontal lines. Hybrids with Bt events are resistant and without Bt events are susceptible. The limit of quantification was 0.5 pg. Means are according to the LSMEANS calculation (SAS PROC GLM) and means were compared by orthogonal contrasts between the resistant and the near isogenic susceptible hybrid within the event. * Asterisks indicate means that are significantly different ($P \leq 0.05$) from near-isogenic susceptible hybrid for that particular event. The rate of concentration of clothianidin treatment was 0.25 mg/seed or 1.25mg/seed. ND=Not detected.

Table 9. *Fusarium* DNA concentrations (pg DNA/200mg host tissue) for maize hybrids with or without transgenes for resistance to corn rootworm feeding. Data are from University of Nebraska Research and Agricultural Development Center Farm near Mead, NE, in 2008.

Hybrid	Event	July Roots-2008		Sept Roots-2008		Sept Stalks-2008	
		<i>F. vert</i> conc (pg)	<i>F. gram</i> conc (pg)	<i>F. vert</i> conc (pg)	<i>F. gram</i> conc (pg)	<i>F. vert</i> conc (pg)	<i>F. gram</i> conc (pg)
34A20	DAS 59122	ND	< 0.5	< 0.5	< 0.5	501.6*	12036.8
34A14	None	< 0.5	< 0.5	< 0.5	< 0.5	5787.1	19097.5
DKC 61-69	MON 88017	ND	ND	< 0.5	< 0.5	35.9	2454.6
DKC 61-72	None	< 0.5	< 0.5	< 0.5	0.734	1183.4	6375.5
DKC 63-46	None	1.7	< 0.5	ND	< 0.5	153.2	3326.9
DKC 63-39	MON 863	< 0.5	ND	ND	< 0.5	ND	4085.3
DKC 63-42	MON 88017	ND	ND	ND	< 0.5	6.8	2159.9

The resistant and near-isogenic susceptible hybrids are grouped between horizontal lines. Hybrids with Bt events are resistant and without Bt events are susceptible. The limit of quantification was 0.5 pg. Means are according to the LSMEANS calculation (SAS PROC GLM) and means were compared by orthogonal contrasts between the resistant and the near isogenic susceptible hybrid within the event. * Asterisks indicate means that are significantly different ($P \leq 0.05$) from near-isogenic susceptible hybrid for that particular event. ND=Not detected.

Figures

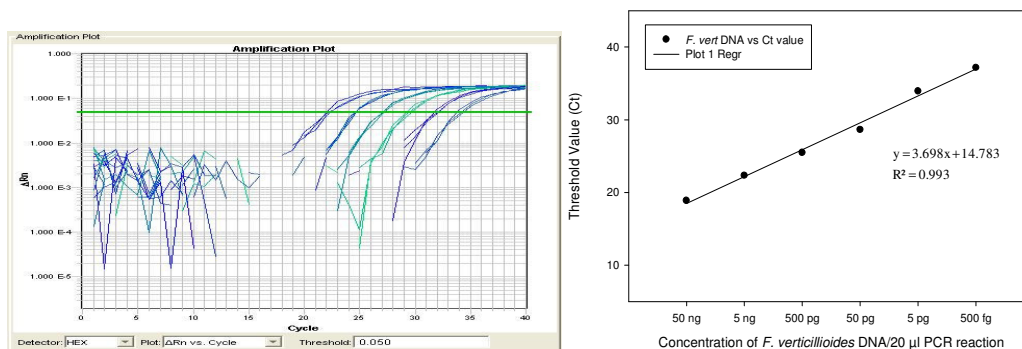


Figure 1. Amplification of tenfold serial dilution of *F. verticillioides* DNA by real-time PCR. The corresponding standard curve for *F. verticillioides* DNA, where threshold cycle value was plotted against DNA concentration used as template in 20 μ l reaction mixture.

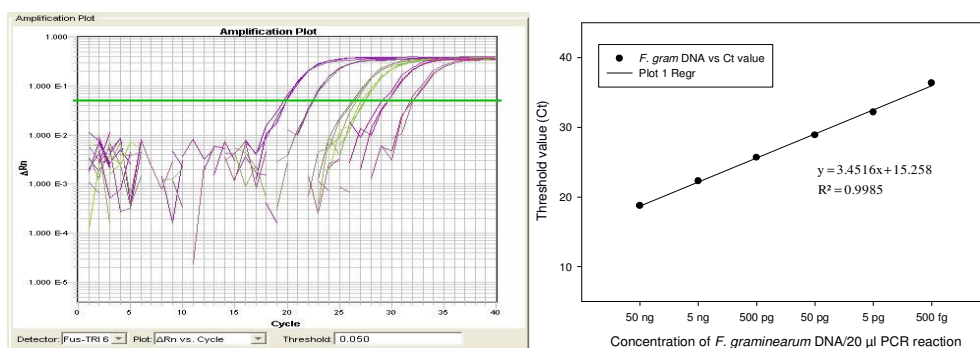


Figure 2. Amplification of tenfold serial dilution of *F. graminearum* DNA by real-time PCR. The corresponding standard curve for *F. graminearum* DNA, where threshold cycle value was plotted against DNA concentration used as template in 20 μ l reaction mixture.

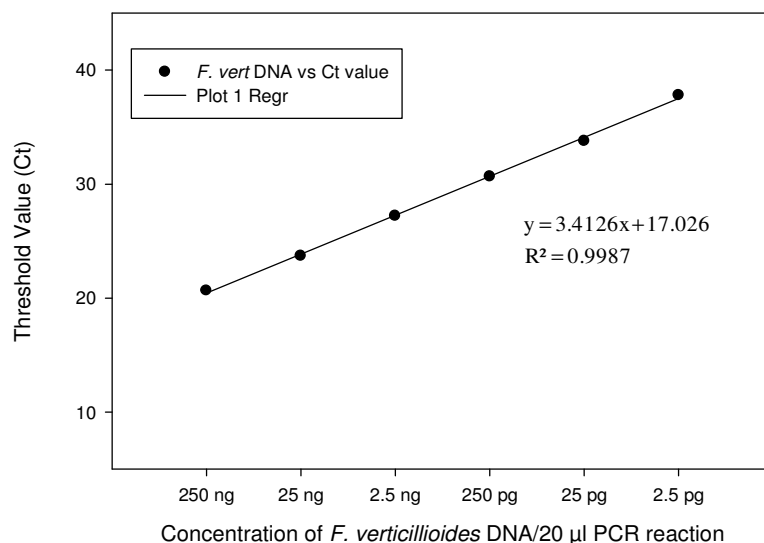


Figure 3. The curve is for the detection of known spiked amount of *F. verticillioides* DNA in 250ng of Host tissue, where threshold cycle values are plotted against fungal DNA concentration in 20 µl PCR reaction mixture.

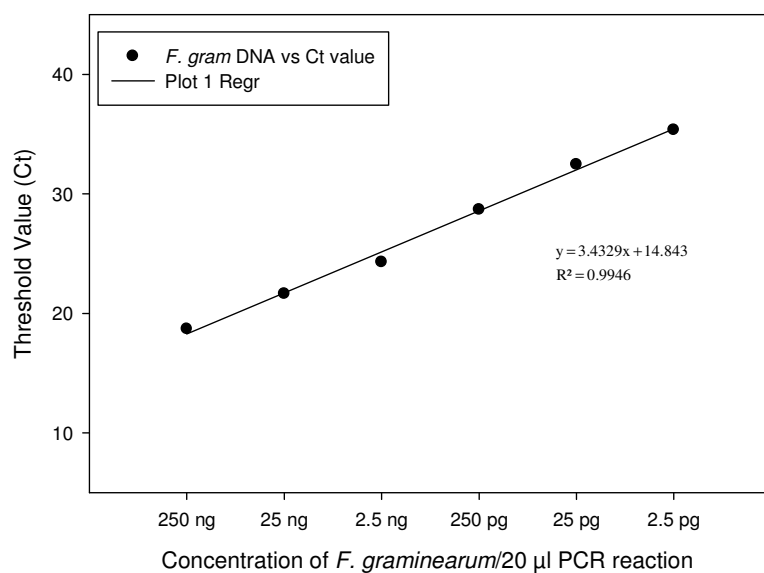


Figure 4. The curve is for the detection of known spiked amount of *F. graminearum* DNA in 250ng of Host tissue, where threshold cycle values are plotted against fungal DNA concentration in 20 µl reaction mixture.

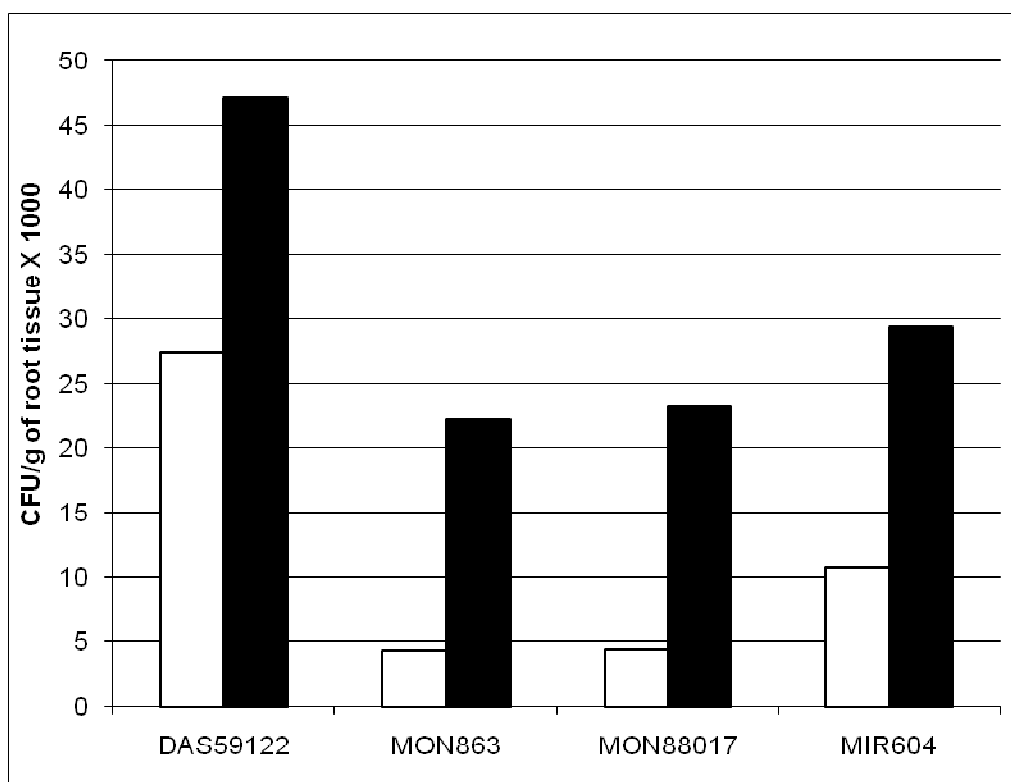


Figure 5. Results for dilution plating, *Fusarium* cfu/g of root tissue for maize hybrids with or without transgenes for resistance to corn rootworm feeding. Data are from Iowa State University Bruner farm in Boone Co., IA, in 2007. White bars are for transgenic CRW-resistant hybrids and black bars are for near isogenic CRW-susceptible hybrids. Means are according to the LSMEANS calculation (SAS PROC GLM) and means were compared by orthogonal contrasts between the resistant and the near isogenic susceptible hybrid within the event.

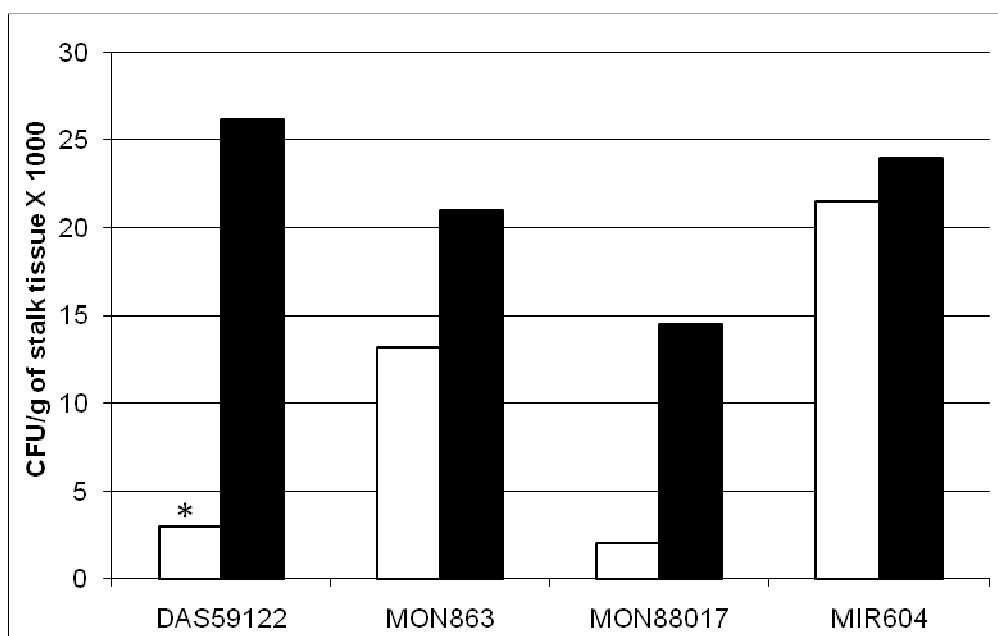


Figure 6. Results for dilution plating, *Fusarium* cfu/g of stalk tissue for maize hybrids with or without transgenes for resistance to corn rootworm feeding. Data are from Iowa State University Bruner farm in Boone Co., IA, in 2007. White bars are for transgenic CRW-resistant hybrids and black bars are for near isogenic CRW-susceptible hybrids. Means are according to the LSMEANS calculation (SAS PROC GLM) and means were compared by orthogonal contrasts between the resistant and the near isogenic susceptible hybrid within the event. * Asterisks indicate means that are significantly different ($P \leq 0.05$) from near-isogenic susceptible hybrid for that particular event.

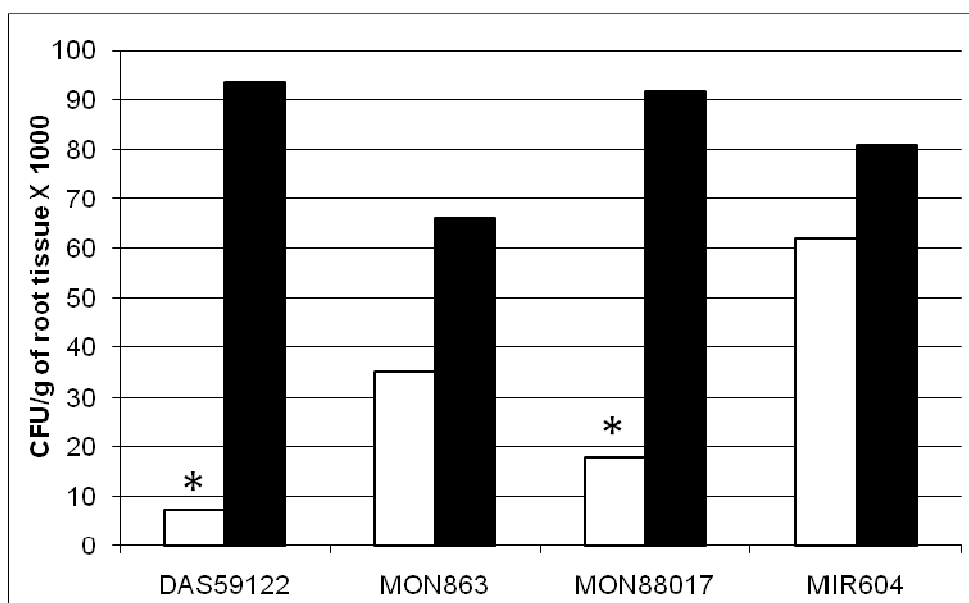


Figure 7. Results for dilution plating, *Fusarium* cfu/g of root tissue for maize hybrids with or without transgenes for resistance to corn rootworm feeding. Data are from University of Nebraska Research and Agricultural Development Center Farm near Mead, NE, in 2007. White bars are for transgenic CRW-resistant hybrids and black bars are for near isogenic CRW-susceptible hybrids. Means are according to the LSMEANS calculation (SAS PROC GLM) and means were compared by orthogonal contrasts between the resistant and the near isogenic susceptible hybrid within the event. * Asterisks indicate means that are significantly different ($P \leq 0.05$) from near-isogenic susceptible hybrid for that particular event.

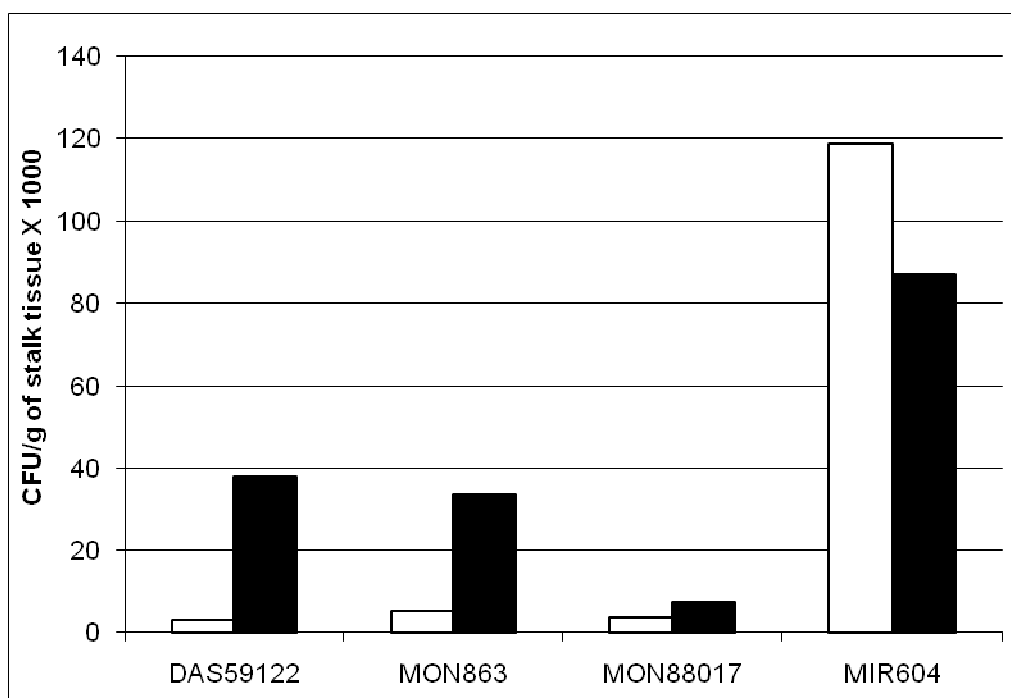


Figure 8. Results for dilution plating, *Fusarium* cfu/g of stalk tissue for maize hybrids with or without transgenes for resistance to corn rootworm feeding. Data are from University of Nebraska Research and Agricultural Development Center Farm near Mead, NE, in 2007. White bars are for transgenic CRW-resistant hybrids and black bars are for near isogenic CRW-susceptible hybrids. Means are according to the LSMEANS calculation (SAS PROC GLM) and means were compared by orthogonal contrasts between the resistant and the near isogenic susceptible hybrid within the event.

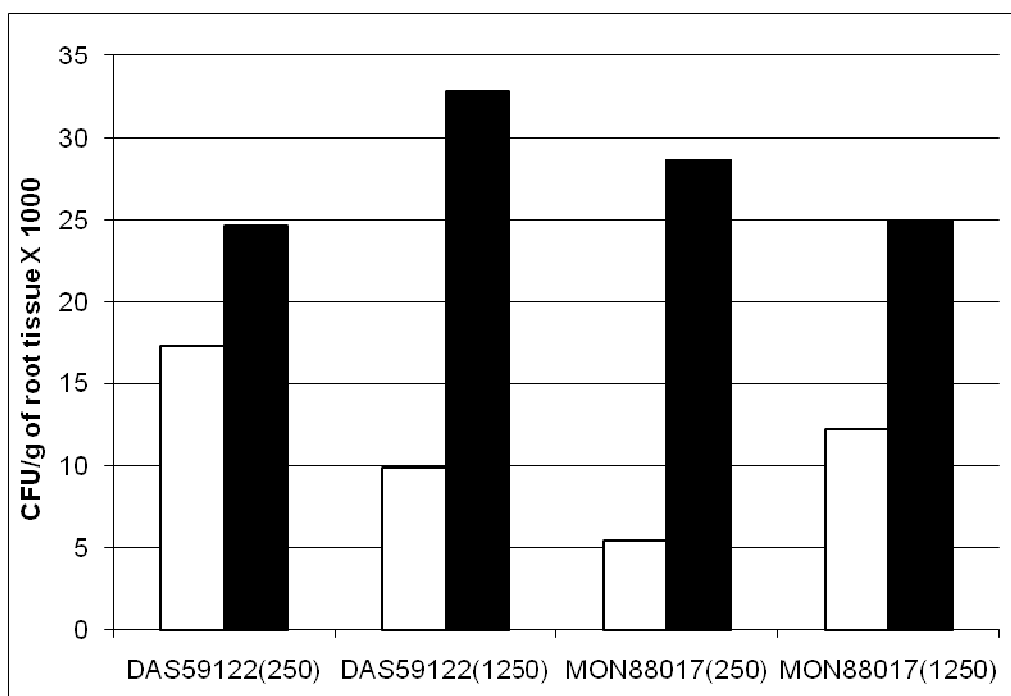


Figure 9. Results for dilution plating, *Fusarium* cfu/ g of root tissue (July) for maize hybrids with or without transgenes for resistance to corn rootworm feeding. Data are from ISU Southeast Research Farm near Crawfordsville, IA, in 2008. White bars are for transgenic CRW-resistant hybrids and black bars are for near isogenic CRW-susceptible hybrids. The rate of concentration of clothianidin treatment was 0.25 mg/seed or 1.25mg/seed. Means are according to the LSMEANS calculation (SAS PROC GLM) and means were compared by orthogonal contrasts between the resistant and the near isogenic susceptible hybrid within the event.

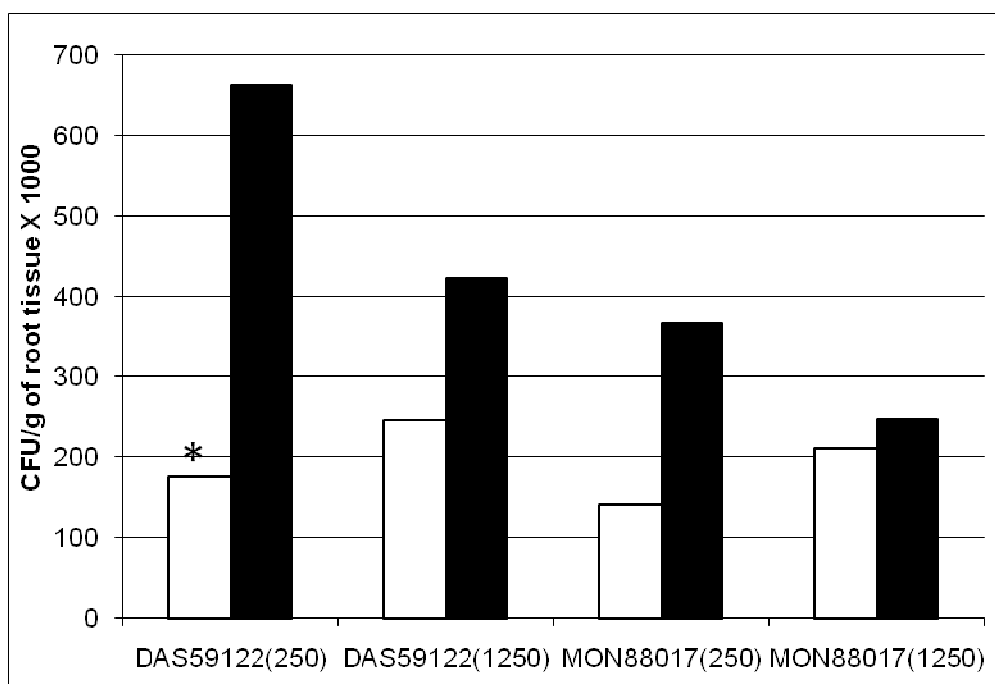


Figure 10. Results for dilution plating, *Fusarium* cfu/ g of root tissue (September) for maize hybrids with or without transgenes for resistance to corn rootworm feeding. Data are from ISU Southeast Research Farm near Crawfordsville, IA, in 2008. White bars are for transgenic CRW-resistant hybrids and black bars are for near isogenic CRW-susceptible hybrids. The rate of concentration of clothianidin treatment was 0.25 mg/seed or 1.25mg/seed. Means are according to the LSMEANS calculation (SAS PROC GLM) and means were compared by orthogonal contrasts between the resistant and the near isogenic susceptible hybrid within the event. * Asterisks indicate means that are significantly different ($P \leq 0.05$) from near-isogenic susceptible hybrid for that particular event.

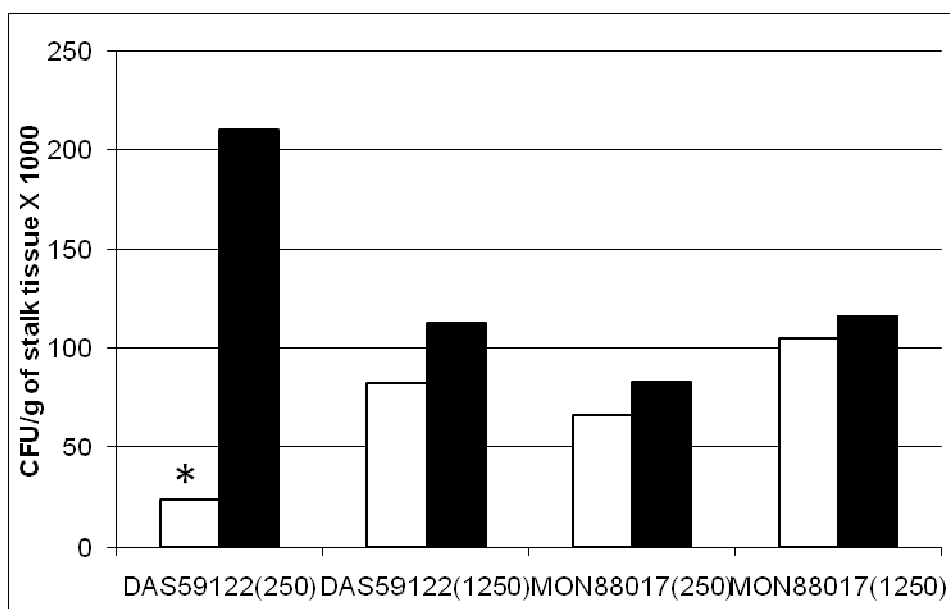


Figure 11. Results for dilution plating, *Fusarium* cfu/ g of stalk tissue for maize hybrids with or without transgenes for resistance to corn rootworm feeding. Data are from ISU Southeast Research Farm near Crawfordsville, IA, in 2008. White bars are for transgenic CRW-resistant hybrids and black bars are for near isogenic CRW-susceptible hybrids. The rate of concentration of clothianidin treatment was 0.25 mg/seed or 1.25mg/seed. Means are according to the LSMEANS calculation (SAS PROC GLM) and means were compared by orthogonal contrasts between the resistant and the near isogenic susceptible hybrid within the event. * Asterisks indicate means that are significantly different ($P \leq 0.05$) from near-isogenic susceptible hybrid for that particular event.

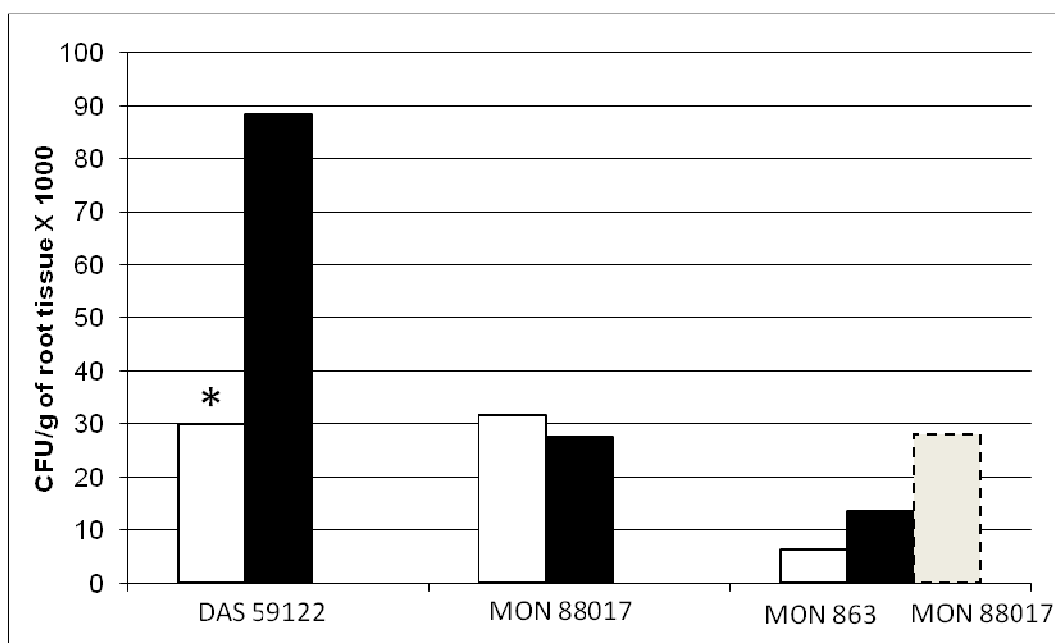


Figure 12. Results for dilution plating, *Fusarium* cfu/g of root tissue (July) for maize hybrids with or without transgenes for resistance to corn rootworm feeding. Data are from University of Nebraska Research and Agricultural Development Center Farm near Mead, NE, in 2008. White and dotted line bars are for transgenic CRW-resistant hybrids and black bars is for near isogenic CRW-susceptible hybrids. Means are according to the LSMEANS calculation (SAS PROC GLM) and means were compared by orthogonal contrasts between the resistant and the near isogenic susceptible hybrid within the event. * Asterisks indicate means that are significantly different ($P \leq 0.05$) from near-isogenic susceptible hybrid for that particular event.

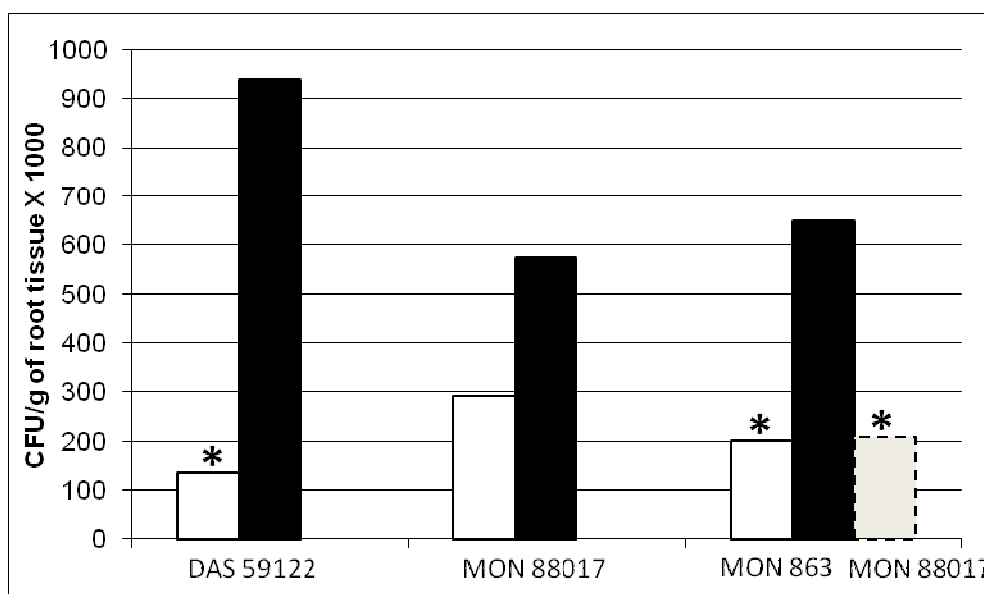


Figure 13. Results for dilution plating, *Fusarium* cfu/g of root tissue (September) for maize hybrids with or without transgenes for resistance to corn rootworm feeding. Data are from University of Nebraska Research and Agricultural Development Center Farm near Mead, NE, in 2008. White and dotted line bars are for transgenic CRW-resistant hybrids and black bar is for near isogenic CRW-susceptible hybrids. Means are according to the LSMEANS calculation (SAS PROC GLM) and means were compared by orthogonal contrasts between the resistant and the near isogenic susceptible hybrid within the event. * Asterisks indicate means that are significantly different ($P \leq 0.05$) from near-isogenic susceptible hybrid for that particular event.

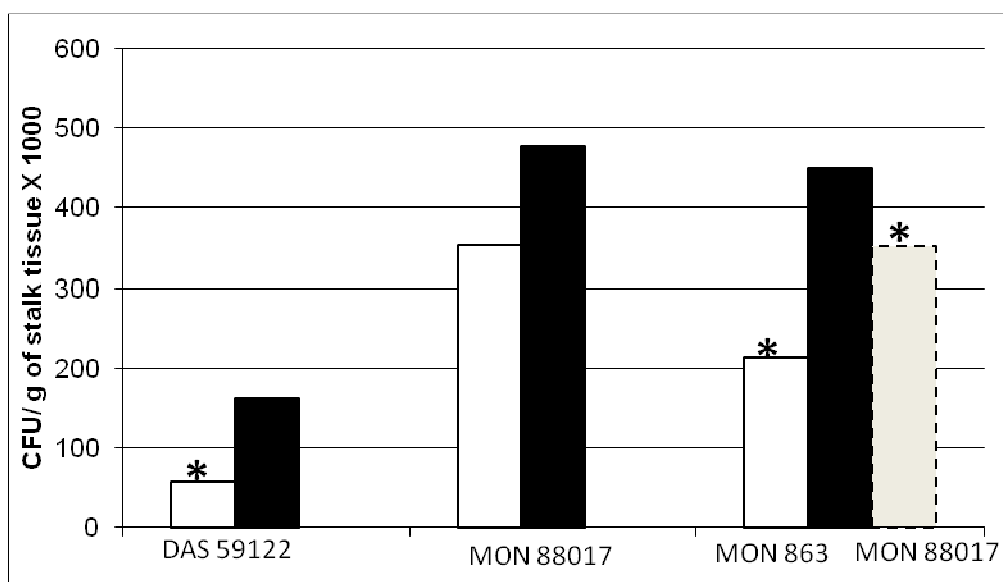


Figure 14. Results for dilution plating, *Fusarium* cfu/g of stalk tissue (September) for maize hybrids with or without transgenes for resistance to corn rootworm feeding. Data are from University of Nebraska Research and Agricultural Development Center Farm near Mead, NE, in 2008. White and dotted line bars are for transgenic CRW-resistant hybrids and black bar is for near isogenic CRW-susceptible hybrids. Means are according to the LSMEANS calculation (SAS PROC GLM) and means were compared by orthogonal contrasts between the resistant and the near isogenic susceptible hybrid within the event.* Asterisks indicate means that are significantly different ($P \leq 0.05$) from near-isogenic susceptible hybrid for that particular event.

Appendix:**Real time PCR primers and probes for the detection of *Fusarium* species****Internal Standard**

ITS forward: 5' – AAC TCC CAA ACC CCT GTG AAC ATA – 3'

ITS reverse: 5' – TTT AAC GGC GTG GCC GC – 3'

ITS probe: 5' – CGC TCG AAC AGG CAT GCC CGC CAG AAT AC – 3'

***Fusarium verticillioides* detection**

FUM 1 forward: 5' – CCA TCA CAG TGG GAC ACA GT – 3'

FUM 1 reverse: 5' – CGT ATC GTC AGC ATG ATG TAG C – 3'

FUM 1 probe: 5' – TCT CAA GGC CAG CCA AGG AGT CGG CGC – 3'

***Fusarium graminearum* detection**

TRI 6 forward: 5' – TGA TTT ACA TGG AGG CCG AAT CTC A – 3'

TRI 6 reverse: 5' – TTC GAA TGT TGG TGA TTC ATA GTC GTT – 3'

TRI 6 probe: 5' – TTG CCC CTC TTT GAT CGA GTT GCG TCT CCC – 3'

Protocols:***Extraction of DNA from corn roots and stalk tissues using CTAB method***

200 mg of sample was weighed and transferred to a 1.5 mL centrifuge tube, add 600 μ L of CTAB buffer of pH:8.0 (100mM Tris HCl, 20mM EDTA, 2% CTAB and 1% mercapto ethanol freshly added to stock solution) to the tube and vortexed until the solution was homogenized. Samples were incubating at 62 °C for 60 minutes. Add 600 μ L of phenol/chloroform (1:1) to each tube and mix by inverting several times till the solution looks homogeneous. The samples were centrifuged at 14,000 \times g for 12 minutes. The aqueous phase was transferred to a new tube. To this tube half the volume of 5M sodium chloride and an equal volume of ice cold *iso*-propanol was added and mixed gently by inverting. Samples were incubated at -20 °C for 30 minutes and samples can be left at 4 °C for overnight. Centrifuge the samples at 5,000 \times g for 5 minutes and discard the supernatant carefully not to drop the DNA pellets. The pellets were rinsed with 70% ethanol. Pellets were air dried and dissolved in autoclaved deionised water. Ribonuclease enzyme was added at concentration of 20 μ g/ml to each sample and incubate at 37 °C for 15 minutes. Quality and quantity of the DNA was measured using nanodrop. DNA is stored at -20 °C.

The protocol for extraction of fungal mycelium from PDA plates using SDS method was followed as described by Chow and Kafer detailed below.

A sample of 100 mg of fungal mycelia was scraped directly from the surface of PDA media and homogenize in the FastPrep® instrument for 40 seconds at a speed setting of 6.0. 400 µL of extraction buffer (100mM Tris-HCl (pH 8.0), 20 mM Na₂EDTA, 0.5 M NaCl and 1% sodium dodecylsulfate) and 200 µL of buffered phenol/CHCl₃/isoamyl alcohol were added and the solution was homogenized thoroughly. Samples were then centrifuged at 16,000 × g for 10 minutes at room temperature. Supernatant was transferred to a fresh tube and ½ volume of 7.5 M ammonium acetate and 2.5 volumes of 95% EtOH were added and incubate for 30 minutes at −20 °C. The samples were then centrifuged at 16,000 × g for 15 minutes. The supernatant solution was decanted and DNA pellet was rinsed with 95% EtOH, air dried and re-suspended in 100 µl of TE buffer (10 mM Tris-HCl, 1 mM Na₂EDTA, pH 8.0). Quality and quantity of the DNA was measured using nanodrop. DNA is stored at −20 °C.

Protocol for Fast Prep as described by instrument kit:

100 mg of fungal sample was measured and 1mL CLS-Y solution was added, homogenize in the FastPrep[®] Instrument for 40 seconds at a speed setting of 6.0. Samples were then centrifuged at $16,000 \times g$ for 10 minutes at room temperature to pellet debris. Supernatant was transferred to a new tube and an equal volume of binding matrix was added, invert to mix and incubate for 5 min with gentle agitation at room temperature on a rotator. The samples were then centrifuged at $14,000 \times g$ for 10 seconds to pellet binding matrix. Discard the supernatant and add 500 μ l sewis-M solution and gently resuspended the pellet using the force of the liquid from the pipet tip. Transfer the resuspended binding matrix to a spin module. The samples were then centrifuged twice at $14,000 \times g$ for 1 minute and replace catch tube with a recovery tube. Elute DNA by gently resuspending binding matrix above the spin filter in 100 μ L of DES. Incubate for 5 minutes at 55 °C in a water bath. Centrifuge for 1 min at $14,000 \times g$ to bring eluted DNA into the recovery tube and discard the spin filter. Quality and quantity of the DNA was measured using nanodrop. DNA is stored at -20°C .