EXPLORING HORMONE CROSSTALK IN *FUSARIUM VERTICILLIOIDES* INFECTION OF MAIZE

An Undergraduate Research Scholars Thesis

by

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TABLE OF CONTENTS

Page

LIST OF FIGURES

Figure Page

ABSTRACT

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Fusarium verticillioides is a major pathogen of a broad range of field crops. Seeds infections lead to contamination by hazardous mycotoxins, such as fumonisin. Fumonisin is known to cause developmental defects in humans and animal when consumed. Previously, *acs2 acs6*, an ethylene biosynthetic mutant of maize has been found to be more resistant to *Fusarium* infection, colonization, and mycotoxin production. However, the molecular mechanism behind this phenomenon is poorly understood. Hormones, such as ethylene, regulate diverse processes during plant development and defenses against biotic and abiotic stresses. These potent signals have complex crosstalk among each other with positive and negative interactions occurring. A metabolomic analysis comparing *acs2 acs6* double mutant and wild type revealed several metabolites to be differentially produced between mutant and wild type. Metabolites of interest were further explored by pharmacological and genetic approaches. Addition of exogenous auxin showed a direct effect on in vitro fungal growth. Available auxin deficient mutants of maize

were exploited in a kernel bioassay and colonization was assessed through ergosterol quantification by high-performance liquid chromatography. Collectively, our results suggest that auxin plays a role ethylene-induced susceptibility.

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NOMENCLATURE

ACS	1-aminocyclopropane-1-carboxylate synthase
ET	Ethylene
IAA	Indole-3-Acetic Acid

CHAPTER 1

INTRODUCTION

Field crops, like *Zea mays* are under constant threat by a multitude of biological pathogens. Every year nearly 125 million tons of major crops are lost to fungal infections (MC Fisher et al. 2012). One of the major players behind this crop loss is the ear- and stalk-rotting fungus *Fusarium verticillioides.*

Fusarium verticillioides (teleomorph *Gibberella fujikuroi*) is an endophytic fungus that belongs to the phylum Ascomycota. The fungus parasites a diverse variety of host plants, including corn (Estrada 578). The effects of *Fusarium* infection are seedling blight, root rot, stalk rot and kernel rot. Kernel rot is especially important because this results in yield loss and lower kernel quality. Mycotoxins, mainly fumonisins, produced by Fusarium are the main contributors to these problems (Kende 1993). The effects of fumonisins are far reaching and have been linked to illnesses in a variety of mammals including humans (Ali et al. 1998).

However plants possess various responses to defend against biotic stress. An early response against microbial threat is known as the innate immune system. In this system host encoded pattern recognition receptors (PRRs) perceive microbial / pathogen molecular patterns (MAMPs or PAMPs) resulting in PAMP triggered immunity (PTI). Pathogens have evolved methods of circumventing their recognition. These pathogens secrete virulence factors which suppress the PTI and allow successful infection to occur. However, resistant plants have a second layer of defense that overcomes this pathogenic strategy. This defense is known as effector-triggered immunity (ETI). ETI induces appropriate counter-measures to the microbial infection (Chisholm

et al. 2006). Some of these defensive responses alter secondary metabolism which can enhance growth and reduce susceptibility. Based on similar functionality, secondary metabolites can be categorized.

Phytohormones constitute one group that plays an integral role in regulation of diverse processes and are highly interconnected in signaling networks (Nernhauser *et* al. 2006). These compounds are involved in responses to both biotic and abiotic stresses (Bari and Jones 2009). The extent of this study focused primarily on the biotic stresses experienced by corn. Previous work has focused on salicylic acid, jasmonic acid and ethylene and revealed complex cross-talk among the phytohormone signaling pathways during pathogenic threat (Bari and Jones 2009). Recently, it has also been found that other hormones such as abscisic acid, auxin, gibberellic acid and cytokinins also contribute to successful defense (Bari and Jones 2009). However, in these plant-pathogen interactions, hormones can act both in a positive manner as resistance factors, but also negatively as susceptibility factors (Bari and Jones 2009). These molecular mechanisms are not well understood and in this study, the role of an auxin derivative known as indole-3-acetic acid (IAA) was explored to better understand how this hormone affects the outcome of infection in the maize-*Fusarium* pathosystem.

Collectively auxins are well studied for their roles in plant growth and cell elongation and are vital for early plant development (Staswick et al. 2005). IAA is the most biologically active isoform and kernels are known to accumulate 308 pmols of IAA (Staswick et al. 2005; Epstein et al. 1980). The high level correlates with the relatively quick growth of the kernel, however, previous research suggests auxin induces susceptibility to diverse pathogens. During rice blight

caused by *Xanthomonas oryzae pv. oryzae* IAA activates expression of expansins that loosen the cell wall, allowing the bacteria to penetrate host cells easier. When *GH3-8,* a protein involved in reducing these expansins, is overexpressed, the rice was much more resistant. Therefore lowering expansin expression by reduction of IAA caused higher resistance (Ding et al. 2008). IAA-mediated processes also promote *Fusarium* wilt. Exogenous IAA enhanced *Fusarium oxysporum* colonization of Arabidopsis (Brenden 2011).

Previous work in our laboratory has shown that ethylene biosynthetic double mutant *acs 2 acs* 6 is more resistant to *Fusarium verticllioides* compared with wild-type (Park et al. *in* preparation). ACS is involved in the biosynthetic pathway of ethylene (ET) where it catalyzes methionine into 1-aminocyclopropane-1-carboxylate (Kende 1993). Literature describes ET signaling as responsible for the expression of many defense-related genes for resistance to biotic stress. It is usually associated with defense against nectrotrophic pathogens and herbivorous insects (Bari and Jones, 2009). In a previous experiment, liquid chromatography-mass spectrometry revealed lowered IAA levels in *acs 2 acs6* mutant kernels prompting the hypothesis that disruption of ET production reduces IAA accumulation which contributes to a reduction of susceptibility to *Fusarium verticillioides* infection.

CHAPTER II

METHODS

Plant material

The B73 genetic background was used for the exogenous IAA experiment. Seeds were harvested in College Station, TX, USA in 2012 and kept at 7 ºC for preservation. Auxin biosynthetic mutant, *de18,* and near-isogenic wild-type seeds were obtained from Dr. Prem Chourey at University of Florida (Bernardi et al. 2012).

In vitro fungal growth

Potato dextrose agar (PDA) culture tubes were prepared to concentrations of 0.6 nM and 28 nM of IAA (Research Products International Co., Prospect, IL, USA cat # 87-51-4). Concentrations were chosen based on previously described levels of endogenous kernel IAA and total auxin derivatives, respectively (Epstein et al. 1980). Tubes were inoculated with 10 µl of *Fusarium verticllioidies* conidia (10^6 spores/ml) suspended in 0.01% Tween-20. The tubes were kept in a humidity chamber at 27ºC with 12 hour light and dark cycles. Pictures were taken after 24 hours.

Kernel assays

For kernel bioassays with *Fusarium verticllioidies,* methods were followed as described by Christensen and Borrego et al.(2012**).** For this study two kernel assays were performed and briefly described hereafter. In the first assay, wild-type kernels were surface sterilized and wounded with a scalpel to produce a 0.5mm slit to facilitate infection. Kernels were placed in 20-ml glass vials and inoculated with 200 µl of 10^6 conidia/ml suspended in 0.01% Tween-20. The

vials were kept in a humidity chamber at 27ºC with 12 hour light and dark cycles. Each day the treated group received the equivalent of 308 pmols of indole-3-acetic acid per kernel, and the control group received 0.16% ethanol solution per kernel. Kernels were harvested at 2 and 4 days for later analysis.

In the second experiment, auxin biosynthetic mutant, *de18* (Bernardi et al. 2012) and wild-type kernels were prepared and inoculated as described above with the exclusion of exogenous IAA application. The vials were kept in a humidity chamber at 27ºC with 12 hour light and dark cycles. Kernels were harvested at 2 and 4 days.

Fungal quantification

Two different analyses were used to assess fungal colonization and sporulation. After each harvested day, 2.5 ml methanol was added to each kernel vial, vortexed and 150 ul from each sample was withdrawn and used for spore enumeration. 5 ml chloroform was then added to the kernel vials were incubated in the 2:1 chloroform: methanol solution for roughly twelve hours under dark. Spore enumeration was accomplished with the use of a hemacytometer with each sample measured twice. The remaining solution was then prepared for ergosterol quantification by filtering supernatant from each sample through a 0.45 um nylon membrane. Ergosterol quantification was done via High Performance Liquid Chromatography. Samples were directly injected into a Shimadzu HPLC LC-20AT system equipped with a 4.6 U ODS-C18 column (200 Å, 250 ± 4.6 mm) and a Shimadzu SPD-20A UV/VIS detector set to monitor at 282 nm. Methanol (100%) was used as the mobile phase at a flow rate of 1.5 mL/min. Ergosterol was

quantified by comparing peak areas of samples to a standard curve generated from HPLC-grade ergosterol.

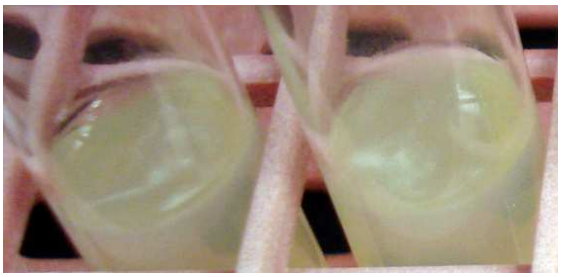
CHAPTER III

RESULTS

Effect of IAA incorporated potato dextrose agar(PDA) on in vitro growth

Figure 1 shows *F. verticillioides* growth with two levels of IAA. In the presence of IAA, there was enhanced fungal growth (b and c). IAA concentrations explored were 0.6 nM and 28 nM (b and c).

A)



B)

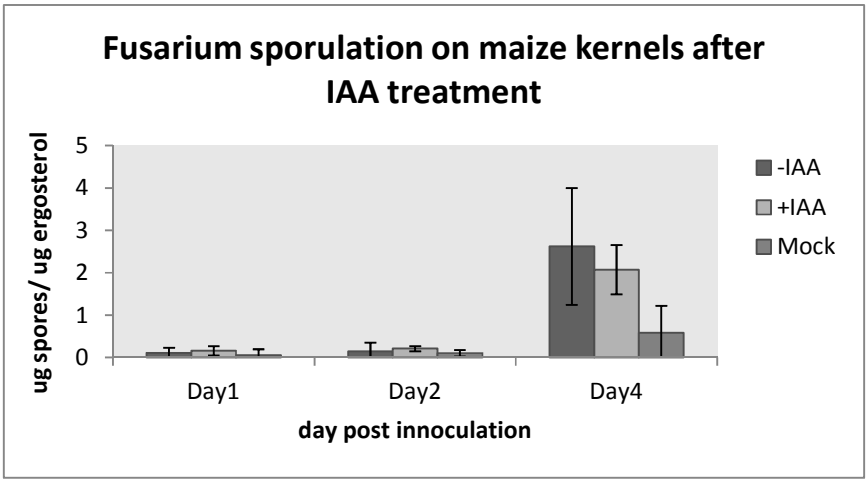
C)

Figure 1: *Fusarium* growth on IAA incorporated PDA, at concentrations of 0.0 nM (a), 0.6nM (b), and 28 nM (c).

Effect of exogenous IAA application on colonization and sporulation on maize kernels

Treatment of wild-type kernels with 308 pmols of IAA had an effect on *F. verticillioides* (fig. 2). Sporulation of the fungus was relatively unaffected by the addition of IAA (a). However, application of exogenous IAA did cause enhanced fungal colonization (b). At day 1 and 2 post inoculation there was no difference in fungal biomass between treated and control. After day 4 post inoculation, there was a statistically significant ($p<0.01$) increase in fungal biomass on treated kernels compared with untreated kernels.

A)



B)

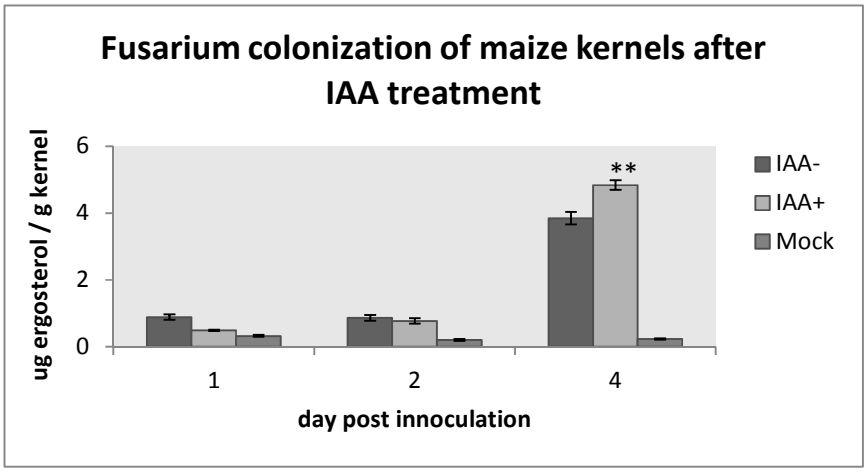
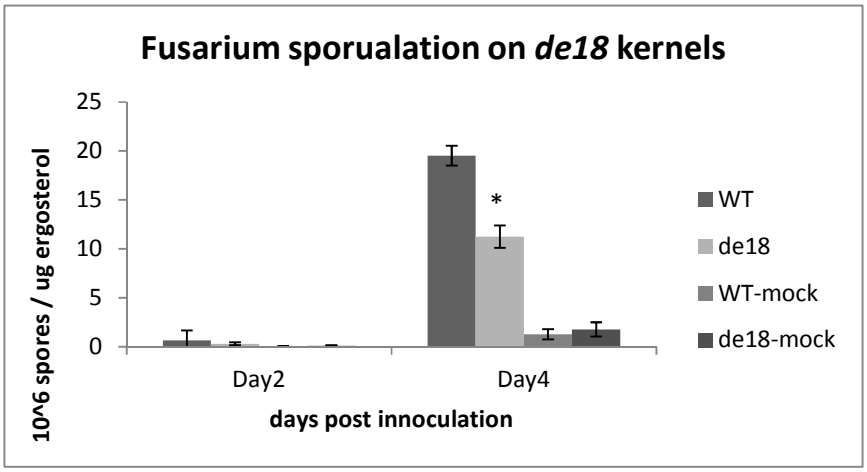


Figure 2: (a) Spore enumeration following *Fusarium verticillioides* infection and IAA application. **(b)** HPLC analysis of ergosterol on day 1, 2 and 4 after inoculation

Response of auxin deficient mutant *de18* on colonization and sporulation following *F. verticillioides* infection

Auxin biosynthetic mutant did have some effect on *F. verticilliodies* (fig. 3). Infection of auxin biosynthetic mutant with *F. verticillioides* did showed decreased fungal sporulation (a). On day 2 after inoculation sporulation of wild-type and mutant kernels was relatively equal. At day 4 after inoculation sporulation of the mutant kernels was nearly half that of the wild-type kernels. Fungal colonization was not significantly affected in this kernel assay (b).

A)



B)

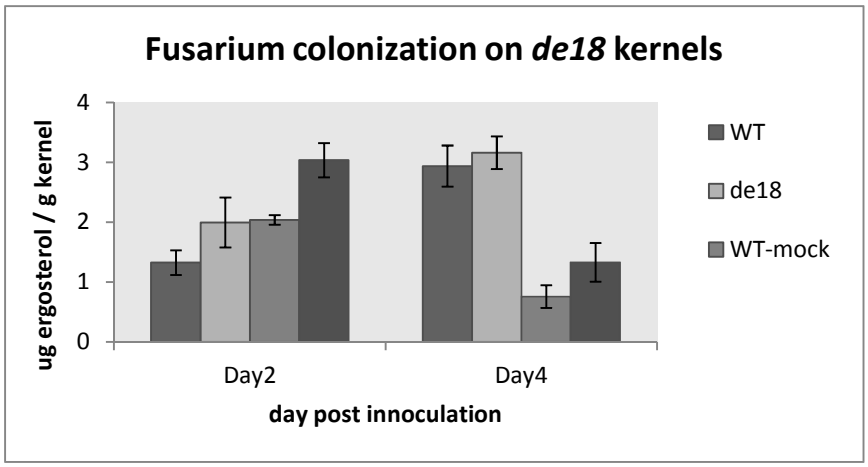


Figure 3(a) Spore enumeration following *F. verticillioides* infection on auxin biosynthetic mutants kernels. **(b)** HPLC analysis of ergosterol on day 2 and 4 of infection.

CHAPTER IV

CONCLUSION

Results from the IAA incorporated PDA confirmed that in the presence of IAA, *F. verticillioides* does show enhanced colonization. IAA promoted *in vitro* fungal growth in a dose-dependent manner. This suggests that IAA directly acts on *Fusarium* perhaps in analogous fashion to perception and response activity in plants (Woodward et al. 2005). Furthermore, it suggest that during ear-rotting of maize kernels by *Fusarium*, auxin may indeed be a susceptibility factor prompting fungal invasion and colonization.

Application of exogenous IAA reinforced the role of this IAA in disease development. The exogenous IAA stimulated *F. verticllioides* growth on wild-type maize kernels. No effect was observed on sporulation suggesting IAA has a specific role on fungal colonization during disease development and not fungal reproduction. It can be concluded from this experiment that IAA does play a significant role in increased fungal colonization.

Colonization of *de18* mutant and wild-type kernels were similar. It is currently unknown if this mutant is defective in auxin accumulation following infection by *Fusarium*. Alternative auxin biosynthetic pathways may be up-regulated following infection (Llorente et al. 2008). However, sporulation was significantly different between wild-type and mutant kernels suggesting additional metabolites may be perturbed in this background contributing towards fungal reproduction. Further analysis is warranted on this mutant.

REFERNCES

Ali, N., Sardjono, Yamashita, A., & Yoshizawa, T. (1998). Natural co-occurrence of aflatoxins and Fusarium mycotoxins (fumonisins, deoxynivalenol, nivalenol and zearalenone) in corn from Indonesia. Food Additives and Contaminants, 15(4), 377e384.

Bari, Rajendra, and Jonathan D. Jones.(2009) "Role of Plant Hormones in Plant Defense Responses." Plant Molecular Biology 69:473-488

Bernardi, J., Lanubile, A., Li, Q. B., Kumar, D., Kladnik, A., Cook, S. D., Ross, J. J., Marocco, A. and Chourey, P. S. (2012). Impaired auxin biosynthesis in the defective endosperm18 mutant is due to mutational loss of expression in the ZmYuc1 gene encoding endosperm-specific YUCCA1 protein in maize. Plant Physiol. 160, 1318-1328.

Brendan, Kidd N., Kadoo Y. Narendra, Bruno Dombrecht, Mucella Tekeoglu, and Donald M. Gardiner. (2011) "Auxin Signaling and Transport Promote Susceptibility to the Root-Infecting Fungal Pathogen Fusarium Oxysproum in Arabidopsis." Molecular Plant-Microbe Interaction 24.6:733-748

Chisholm ST, Coaker G, Day B, Staskawicz BJ (2006) Host-microbe interactions: shaping the evolution of the plant immune response. Cell 124:803–814

Christensen S, Borrego E, Shim WB, Isakeit T, and Kolomiets M (2012) Quantification of fungal colonization, sporogenesis, and production of mycotoxins using kernel bioassays. J Vis Expe3727.

Ding X, Cao Y, Huang L, Zhao J, Xu C, Li X, Wang S (2008) Activation of the indole-3-acetic acid-amido synthetase GH3-8 suppresses expansin expression and promotes salicylate- and jasmonate-independent basal immunity in rice. Plant Cell 20:228–240. doi:10.1105/tpc.107.055657

Epstein E, Cohen JD, Bandurski RS (1980) Concentration and metabolic turnover of indoles in germinating kernels of Zea mays L. Plant Physiol 65: 415–421

Estrada, A.E.R., Hegeman, A., Kistler, H.C., May, G., 2011. In vitro interactions between *Fusarium verticillioides* and Ustilago maydis through real-time PCR and metabolic profiling. Fungal Genetics and Biology 48, 874–885

Kende, Hans. (1993) "Ethylene Biosynthesis." Annual Review of Plant Physiology and Plant Molecular Biology 44:283-307.

Llorente, F., Muskett, P., Sanchez-Vallet, A., Lopez, G., Ramos, B., SanchezRodriguez, C., Jorda, L., Parker, J. and Molina, A. (2008) Repression of the auxin response pathway increases Arabidopsis susceptibility to necrotrophic fungi. Mol. Plant, 1, 496–509.

MC Fisher et al. "Emerging fungal threats to animal, plant and ecosystem health." Nature, 12 April 2012. DOI 10.1038/nature10947

Nemhauser JL, Hong F, and Chory J (2006) Different plant hormones regulate similar processes through largely nonoverlapping transcriptional responses. Cell 126:467-475.

Staswick PE, Serban B et al (2005) Characterization of an Arabidopsis enzyme family that conjugates amino acids to indole-3-acetic acid. Plant Cell 17:616–627. doi:10.1105/tpc.104.026690

Woodward, A. W. and Bartel, B. (2005). Auxin: regulation, action, and interaction. Ann. Bot. 95, 707-735.

Yong-Soon Park*, Xiquan Gao*, Eli Borrego, et al. (in preparation) Mycotoxigenic fungi induce ethylene production in host via G-protein signaling to promote seed colonization

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