MONITORING THE INFECTION PROCESS OF GFP-EXPRESSING FUSARIUM VERTICILLIOIDES IN HARDLOCK COTTON

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Abstract

Hardlock is a severe disease of cotton in the Coastal Plains of the southeastern United States. It is assumed to be associated with high nitrogen, high plant density, temperature and humidity, insect damage and seed rot. Prior studies have found that application of fungicide to cotton flowers reduces disease. Flower inoculation studies have indicated that hardlock is caused by the fungal pathogen *Fusarium verticillioides*, also known as *F. moniliforme*. We have been investigating the actual pattern of colonization that takes place from the flower stigma to the flower ovary and to the seed. Artificial inoculations of flowers with a green fluorescent protein tagged *F. verticillioides* isolate were conducted in a greenhouse. Fusarium was detected in the stigma and style two days after bloom (DAB) and seeds at 4, 6, 8, 10, 16, 20, 40 and 60 (open bolls) DAB. Results indicate flowers are easily colonized by *F. verticillioides*. This study allowed us to track the progress of pathogen *F. verticilloides* through the boll maturation period.

Introduction

Hardlock of cotton (*Gossypium hirsutum*) has been identified as a problem for the last several years. It is found in most humid regions of the cotton belt and may seriously affect yield in many areas of the southeastern US. Hardlock is a failure of locules in the boll to fluff out and instead remain compact (Figure 1) sometimes with discoloration of fiber. Symptoms are not observed until the boll opens. The quality of fiber is not severely affected but yields have been known to be reduced by 50 percent or more. In Florida, yield losses due to hardlock vary from 20 to 70 percent depending on the temperature of the year.

Figure 1. (A) Healthy Cotton (B) Hardlock cotton

Hardlock is assumed to result from environmental or interaction of pathogens. Fungi seem to be associated with symptoms and the incidence appears to increase with rainfall, high nitrogen, high plant density, temperature and humidity. *Fusarium verticillioides* (synonym, *Fusarium moniliforme*) is a causal agent of hardlock in cotton.

Recently, the green fluorescent protein (GFP) has become a commonly used tool in the analysis of fungal-plant interactions. Spores and hyphae of GFP expressing fungal isolates can be identified by fluorescence microscopy in intact tissues or tissue sections without extensive manipulation and provide highly informative data on processes of plant colonization.

In the present study we used a GFP-expressing *F. verticillioides* isolate to directly monitor the development of the fungus through flower into the bolls using PCR.

Materials and Methods

Greenhouse Study

Cotton variety DPL 555 BG/RR was used for flower inoculations in a greenhouse. Each plant was maintained in a 12 l pot of miracle grow potting mix and irrigated regularly. Temperature varied from 21 to 40°C and relative humidity was maintained at 50 % or higher.

Fungal Isolation

F. verticillioides strain A-00149 was isolated in California from maize and the insertion of the GFP gene occurred at Tel Aviv University, and Tel Aviv 69978, Israel (Oren et al; 2003). The isolate was maintained at 30°C on potato dextrose agar (PDA) and inoculums produced on cotton seed agar (CSA) media.

Fusarium Amplification

A DNA fragment specific to the GFP sequence was amplified by PCR from *F. verticillioides* genomic DNA by using the primer 5'TTAGTTGTACAGCTCGTCCATG'3 5'ATGGTGAGCAAGGGCGAGGA'3. The PCR amplification were carried out in a thermocycler (Eppendorf) in a 25 ul of reaction mixture containing 1x PCR Buffer (10mM), 2mM MgCl2 (25mM), 0.04mM of each dNTP, 0.4 M of both primers, 1 U of Taq Polymerase (5U/ul) (promega): A programmer of 30 cycles (94^{o}C 50s, 58^{o}C 50 s, 72^{o}C 50 s) was performed. Each PCR reaction contained 100–200ng of genomic DNA template. PCR product mixtures were analyzed on 1.2% agarose gel in 1x TBE buffer.

Microscopy

Fluorescence microscopy was performed with a Nikon eclipse 80i microscope. Each of the tissues examined was placed on a microscope slide, submerged in a water droplet, and covered with a glass cover slip was used for GFP detection. Light microscopy was performed with the same microscope without filters.

Experimental Procedures

The greenhouse inoculation experiments were conducted in 18 plants. Flowers, were inoculated at 10 AM on the first day of bloom with a suspension of *Fusarium* tagged GFP (10^6 conidia/ml), by placing 0.1 ml on the surface of the stigma**.** Control flowers were labeled with ribbons indicating the treatment and date. Stigma, style, base of the style, ovary and seed at 4, 6, 8, 10, 16, 20, 40 days after flowering, and open bolls (60 days) were amplified using Polymerase chain reaction.

Results and Discussion

Marois et al; (2002) sprayed fungicide on all plant, bloom, boll and no spray and found that fungicide spray during bloom showed reduction in hardlock. This study was supported by Marois and Wright (2003) and Marois and Wright (2004), Wright et al; (2004). Inoculation of conidia into flowers has been shown to increase hardlock (Marois et al; 2005) On the basis of these studies we hypothesized that *Fusarium* conidia germinate through the stigma and move to the ovules. The stigma was inoculated in presumption that *Fusarium* follows the same pathway as germinating pollen.

Fusarium like structure was found in hardlocked cotton when stained with lactophenol**.** Locules were categorized into five different groups depending on severity of symptoms – Healthy, slightly hardlocked, moderately hardlock, highly hardlocked and severely hardlocked (Figure 2). Hardlocked cotton is associated with undeveloped hollow seeds (Figure 3). The number of healthy seeds differed between healthy and hardlock bolls. Healthy bolls have more than 30 solid seeds per boll while severely hardlocked bolls have 0-9 solid seeds (Table 1). Using PCR *Fusarium* was detected in the stigma and style 48 hours after inoculation but was not found in the base of the style or ovary at that time (Figure 4). It was detected in seeds in some samples (10%) at 4 DAB. By 8 DAB, it was present in > 80 % of samples and remained high at subsequent sampling times (Figure 5).

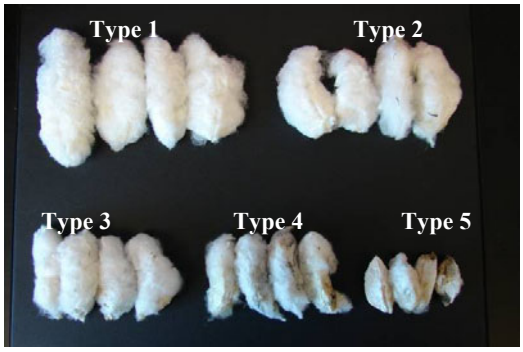

Figure 2 Visual comparison of locules collected from healthy and hardlocked cotton. 1 = healthy (No infection), 2 = slightly hardlocked, 3= moderately hardlocked, 4= highly hardlocked and 5 = severely hardlocked

Figure 3 (A) Seeds from non hardlock bolls and (B) Seeds from hardlock bolls

Table 1 Number of solid seeds present per boll on the basis of symptom

Symptoms of bolls	No. of seeds
Healthy	More than 30
Slightly hardlocked	25-29
Moderately hardlocked	20-24
Highly hardlocked	10-19
Severely hardlocked	0-9

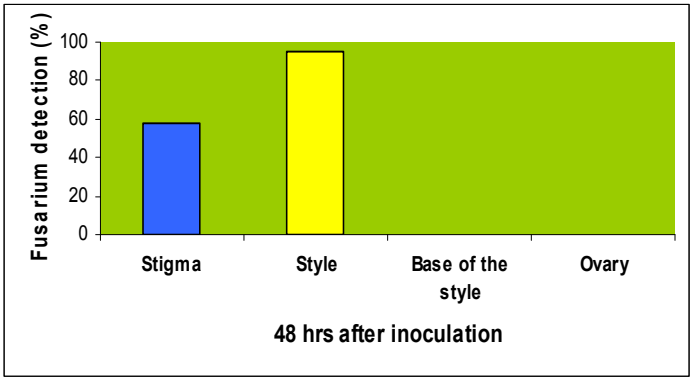

Figure 4 Progress of flower infection 48 hours after inoculation with *Fusarium* conidia

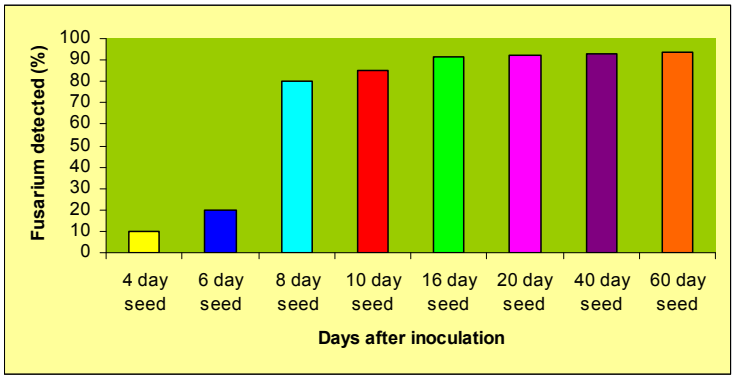

Figure 5 Progress of infection in seeds after inoculation with *Fusarium* conidia

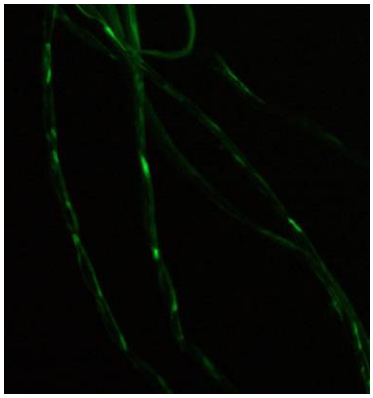

Figure 6 Cotton fibers with *Fusarium* expressing GFP under fluorescent microscope

Although the *Fusarium* was readily recovered from seeds by PCR, samples were plated to re-isolate the *Fusarium.* We detected fluorescent macroconidia and microconidia during microscopic examination of fusarium that developed in fibers (Figure 6) and from seeds inoculated into the media. Using fluorescence and confocal microscopy, the GFP expressing fungi were easily differentiated from the wild-type strains. The results show that the use of the GFP system is feasible to monitor *Fusarium* in flower and seeds.

This study confirms that *Fusarium verticillioides* is a causal agent of hardlock, and that infection can occur through flowers. Infection of seeds occurred at an early stage of development. The number of healthy seeds differed between *Fusarium* infected and healthy bolls and seed number is associated with severity of the hardlock symptom.

Acknowledgement

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