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# FREQUENCY AND PATHOGENICITY OF MICROORGANISMS ASSOCIATED WITH COTTON SEED ROT IN SOUTH CAROLINA

**Alois A. Bell and Enrique Gino Medrano**
**USDA-ARS**
**College Station, TX**
**Michael A. Jones**
**Pee Dee Research & Education Center**
**Florence, SC**

## Abstract

More than 50% of bolls from 'Acala Maxxa' cotton grown at the Pee Dee Research Station in South Carolina showed internal discoloration and rot of seed without external symptoms on the boll. Various isolation and inoculation techniques were used to identify pathogenic fungi and bacteria associated with seed discoloration. The number of pathogenic fungal isolates obtained asceptically from ca. 60 bolls were: *Phoma exigua*, 20; *Verticillium nigrescens*, 13; *Alternaria alternata*, 7; *Fusarium semitectum*, 1; and *Curvularia lunata*, 1. The number of pathogenic bacterial isolates obtained from 20 seeds, each from different bolls, were: *Pantoea agglomerans*, 10; a bacterium putatively identified as *Pantoea stewartii*, 4; and *Agrobacterium tumefaciens*, 2. Each of these pathogens, when introduced through puncture wounds (28 gauge needle), caused seed rot similar to that found in young and middle-aged bolls in the field. None of these pathogens infected bolls through intact or scratched sutures, nectaries, bracts, or calyxes or through punctured peduncles. The same pathogens caused spotting of fiber, tight locks, or completely macerated and discolored locks depending on the age of bolls at infection. The seed rot in Acala apparently was due to a large variety of pathogens taking advantage of boll wounds that disrupted the endocarp, such as wounds caused by *Lygus* bugs or boll weevils.

## Introduction

In 2001, Jones and Edmister described a cotton seed rot that occurred extensively (5-40% of bolls) in various counties in North and South Carolina. While seed were discolored and embryos either killed or stunted, there were no external symptoms of infection. Studies of various bacterial species isolated from discolored seed did not conclusively show a cause for the disorder (Kluepfel, et al., 2001; Yan et al., 2002). Seed rot again appeared extensively in 'Acala Maxxa', a highly susceptible cultivar (Jones et al., 2002), in South Carolina in 2003. We report here our studies of the probable causes of this seed rot symptom.

## Methods and Materials

### Isolation Protocols

Bolls were washed in a 5.25% sodium hypochlorite solution for 2 minutes and rinsed 3x with sterile water. Carpel walls were aseptically removed and a glass rod was rubbed on individual discolored seed and streaked on trypticase soy agar (TSA) or D-1 medium that is selective for *Agrobacterium.* Individual seeds, stigma scars, suture tissue and placental tissue were placed on Komada Fusarium medium and potato dextrose agar (PDA) amended with chloramphenicol (100mg/L) and tetracycline (50mg/L). Other seed were submerged overnight in sterile water and then placed on Komada or dilute Schyzophyllum medium; and supernatants were spread on TSA. Tissues were incubated until microbes appeared, but not more than 3 weeks. Microbes were purified by diluting in water, and spreading the dilutions on PDA and TSA to obtain pure isolates.

### Inoculation Protocols

One isolate of each fungal species and 1-3 isolates of each bacterial species from each boll yielding the pathogens were used to test for pathogenicity. Fungi were grown for 14 days on dilute Schizophyllium medium at 22°C, and a 25x30 mm section of culture was agitated in 2mL sterile water to produce a spore suspension. Bacteria were streaked and grown on PDA containing 0.8g/L of fine $CaCO_3$ for 24 hours at 30°C, and a 10 µl loop full of bacteria was suspended in 2 mL sterile water to give a bacterial suspension. Microbial suspensions were placed on stigma scars, wall sutures, and scratch wounds on bracts, calyx and boll, or a drop was placed on boll wall, suture or pedicel and a 28 gauge needle was placed through the drop and into the bract or pedicel. Bolls were sectioned 7-14 days after inoculation to observe disease progress or bolls were allowed to mature before final symptoms were observed.

## Results and Discussion

### Identification and Frequency of Seed Rot Pathogens

The identification and frequency of fungal and bacterial species associated with seed rot are shown in Tables 1 and 2, respectively. The pathogenicity of each species and isolate was confirmed by Koch's postulates. Other bacteria and fungi that were

not pathogenic are not listed. The fungal pathogens and *P. agglomerans* have previously been reported as boll rot pathogens that require wounds for infection of the boll. (Roncadori, 1969; Pinckard et al., 1981; Hillocks, 1992; Batson, 2001).

**Effects of Inoculation Techniques**

The frequency of seed rot caused by *Pantoea agglomerans* using different inoculation techniques is shown in Table 3. The same test was repeated with all other pathogens (Table 1 and 2) with the same result. Infections only occurred when wounds breached the endocarp of the boll either through the carpel wall or the suture between carpel sections. Wounds of the boll epicarp, bract, calyx or peduncle did not allow infection by any of these pathogens.

**Factors Affecting Seed Rot Severity**

Variation in seed rot severity caused by different isolates of fungal species, *Pantoea* species, and *Agrobacterium tumefaciens* isolates are shown in Tables 4, 5 and 6, respectively. The effects of boll age at inoculation on the severity of seed rot symptoms caused by bacteria is shown in Table 7.

All fungi, except *Verticillium nigrescens* and some *Phoma exigua* isolates caused complete rot of seed within the inoculated locule. This was true regardless of boll age at infection. *V. nigrescens* caused more rot of bolls under 15-days-old at infection than of older bolls. The most virulent strains of the three bacterial species caused similar severity of seed rot which decreased as bolls aged (Table 7).

**Symptoms at Boll Opening**

Symptoms of seed and boll rot caused by inoculation of middle-aged bolls are summarized in Table 8. The degree to which pathogens spread to other locks is shown in Table 9.

All fungal species, except *V. nigrescens* caused rot of the entire lock yielding tight (or hard) locks at boll opening. Tight locks caused by *Alternaria* and *Phoma* were picturesque and readily lost from the open boll, similar to tight locks caused by *Fusarium moniliforme*. Tight locks from *Fusarium semitectum* and *Curvularia lunata* were more extensively rotted and often stuck to the carpel walls. *V. nigrescens* often caused tight locks when inoculated into young or old bolls, but caused brown spots on partially fluffed locks when inoculated into middle-aged bolls. Latent infections apparently were not activated by boll age with this fungus, whereas latent infections by *Phoma* again became active in the aging boll.

Bacterial infections of bolls less than 14 days old often caused extensive disintegration of the locks leaving only a small black hard mummy or sooty black residue. The sutures over the inoculated locks often did not separate, keeping the diseased lock partially concealed. Bolls less that 6 days old at inoculation often were aborted, cavitated, or split prematurely exposing the rot. Locks from bolls inoculated after 14 days of age often show dark spots or stripes which developed immediately under the suture.

## Conclusions

Seed rot in 'Acala Maxxa' bolls from South Carolina was caused by a variety of fungal and bacterial pathogens, all of which require wounds for initial infection and which finally cause boll rots. The *Pantoea* species found in discolored seed were unusually virulent for these species and may have unique genetic characteristics. The possible basis for seed rot caused by *Pantoea* and *Agrobacterium* is being studied.

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Table 1. Pathogens isolated from discolored seed: Fungi

| **Fungal Species** | **No. of Bolls Affected*** |
|---|---|
| *Phoma exigua* | 20 |
| *Verticillium nigrescens* | 13 |
| *Alternaria alternata* | 7 |
| *Fusarium semitectum* | 1 |
| *Curvularia lunata* | 1 |

*Seed from Ca. 60 bolls observed.

Table 2. Pathogens isolated from discolored seed: Bacteria

| **Bacterial Species** | **No. of Bolls Affected*** |
|---|---|
| *Pantoea agglomerans* | 10 |
| *Pantoea stewartii* | 4 |
| *Agrobacterium tumefaciens* | 2 |

*A single discolored seed from each of 20 locks, each from separate bolls, was tested.

Table 3. Effect of inoculation technique on infection by *Pantoea agglomerans*.

| | **Infected Bolls/Total Bolls*** | | |
|---|---|---|---|
| **Inoculation Technique** | **TAMCOT** | **ACALA** | **COKER** |
| Placed on Stigma | 0/3 | 0/3 | 0/3 |
| Placed on Stigma Scar | 0/16 | 0/3 | 0/3 |
| Placed on Boll Surface | 0/3 | 0/3 | 0/3 |
| Placed on Boll Nectaries | 0/4 | 0/4 | 0/4 |
| Boll Wall Puncture | 2/3 | 3/3 | 3/3 |
| Boll Suture Puncture | 5/5 | 4/4 | 2/2 |
| Peduncle Puncture | 0/4 | 0/4 | 0/4 |

*Evaluated 2 weeks after inoculation.

Table 4. Variation in seed rot severity caused by fungal species and isolates.

| | **Disease Grade (1-5)*** | | |
|---|---|---|---|
| **Species** | **2** | **2-4** | **5** |
| | (No. isolates in grade) | | |
| *F. semitectum* | 0 | 0 | 1 |
| *C. lunata* | 0 | 0 | 1 |
| *A. alternata* | 0 | 0 | 2 |
| *P. exigua* | 1 | 7 | 10 |
| *V. nigrescens* | 0 | 2 | 0 |

*Scored 14 days after suture-puncture inoculation of 15-18 day old bolls; 1=about 20% of lock discolored and 5=entire lock discolored.

Table 5. Variation in virulence among *Pantoea* species and isolates.

| Species | Disease Grade (1-5)* | | |
|---|---|---|---|
| | <2 | 2-3 | >3 |
| | (No. of isolates) | | |
| *P. agglomerans* | 3 | 23 | 3 |
| *P. stewartii* | 0 | 9 | 2 |

*15-18 day-old bolls inoculated by suture puncture. Seed rot rated 14 days later.

Table 6. Seed rot severity caused by boll and root isolates of *Agrobacterium*

| ***A. tumefaciens* Isolate** | **Source** | **Disease Grade (1-5)*** |
|---|---|---|
| 25A | Root | 5.0 |
| 14C | Root | 4.3 |
| 1A | Root | 4.0 |
| SC2 | Boll | 3.7 |
| 26A | Root | 3.3 |
| 34B | Root | 2.0 |
| SC2 | Boll | 1.7 |
| 7A | Root | 0.1 |

*Mean of 3 bolls.

Table 7. Effect of boll age and bacterial species on severity and spread of seed rot.

| Bacterial Species | Boll Age at Inoculation (Days from Anthesis) | | | | |
|---|---|---|---|---|---|
| | 1-4 | 5-8 | 9-11 | 12-14 | 15-18 |
| | (Grade, 1-5, & Spread*) | | | | |
| *P. agglomerans* | Abort | 5* | 4-5* | 2-4 | 1-2 |
| *P. stewartii* | Abort | 5* | 4-5* | 3-5* | 1-3 |
| *A. tumefaciens* | Abort | 5* | 4-5* | 2-3 | 1-2 |

Rated 2 weeks after puncture inoculation of boll suture. Ranges of responses of 5 bolls are given. Spread in some bolls indicated by asterisks.

Table 8. Symptoms from seed borne pathogens at boll opening.

| Pathogen | Symptoms |
|---|---|
| *F. semitectum* | Completely rotted, tan-brown colorBoth tight and matted locks |
| *A. alternata* | Tight locks, tan-gray color |
| *P. exigua* | Tight locks, tan-gray color |
| *C. lunata* | Completely rotted and matted, dark gray color |
| *V. nigrescens* | Tight locks, tan color; and dark spots on partially loose white locks |
| All Bacteria | Tan to dark brown spots and streaks on mostly loose white locks |

Table 9. Spread of seed borne pathogens to other locks at boll opening*.

| | Frequency of bolls with infections confined to | | |
|---|---|---|---|
| **Pathogen** | **1 lock** | **2-3 locks** | **All locks** |
| Fungi: | | | |
| *F. semitectum* | 5 | 4 | 4 |
| *A. alternata* | 12 | 6 | 1 |
| *P. exigua* | 17 | 2 | 0 |
| *C. lunata* | 6 | 2 | 0 |
| *V. nigrescens* | 17 | 1 | 0 |
| Bacteria: | | | |
| *P. agglomerans* | 19 | 0 | 0 |
| *P. stewartii* | 16 | 0 | 0 |
| *A. tumefaciens* | 11 | 0 | 0 |

*Bolls 17-34 days old when inoculated.