GREENHOUSE AND MICROPLOT STUDIES ON IDENTIFICATION OF A CASUAL AGENT OF SEED ROT

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Abstract

First observed in 1999, seed rot has now been reported in all cotton producing counties in South Carolina. Microbial isolations made from both symptomatic and asymptomatic bolls have revealed a diverse microbial community present in healthy and symptomatic cotton boll tissue. Commonly isolated microorganisms include members of the following genera, *Enterobacter, Pseudomonas, Bacillus, Pantoea*, and *Cedecea*. Twenty-four, single-colony purified strains isolated from symptomatic boll tissue were screened under greenhouse conditions for their ability to induce the seed rot symptoms observed under field conditions. Sixteen flower or soil-inoculated isolates induced at least twice the level of seed rot as observed for water-treated controls. Twelve seed-rot-positive isolates tested in the greenhouse, along with 29 additional isolates, were screened in microplot studies in the field. Nine isolates that induced seed rot under greenhouse conditions were unable to do so under field microplot conditions. Two isolates, *Enterobacter agglomerans* 33 and *Cedecea neteri* 86 induced seed rot symptoms under both greenhouse and microplot conditions at twice, or greater than, the levels observed in the water-treated controls. The involvement of a microbial agent in the occurrence of seed rot will be discussed.

Introduction

First observed in 1999, seed rot has now been reported from each of the 23 cotton producing counties in South Carolina. Yield losses of up to 21% were documented in South Carolina in 1999 and up to 15% in 2000. In addition, seed rot has been detected in GA, LA, MS, NC and TX in 2000. Symptoms of seed rot are first visible in bolls approximately three-weeks old. Early symptoms include discolored seed coats of one or more seeds/locule. Later stages include dead and necrotic embryos, pink colored integumants, dark and thickened seed coats, and hollow seeds. Seeds that were cut open often appeared to be slightly moist, superficially resembling soft rot. These initial observations led to the term seed rot and the hypothesis that the cause maybe bacterial in origin. At maturity, bolls exhibiting these symptoms did not fluff properly and remained hard/tight locked significantly reducing yields.

Although important, we have been unable to correlate such abiotic parameters as weather and nutrient levels, with the incidence of seed rot in South Carolina. Consequently, we have concentrated our efforts into exploring the possible involvement of a microbial agent in the cause of this new malady of cotton. Here we report our initial efforts into the characterization of the microbial community in boll tissue and subsequent greenhouse and microplot testing of "boll" isolates for their ability to induce seed rot.

Materials and Methods

Intact bolls with no detectable cracks in the locule sutures were surface sterilized by immersing the bolls in 70% ethanol for 10 min, followed by 10 min. in 20% Clorox and 10 min in 70% ethanol. After this three-step sterilization procedure the bolls were rinsed three times in sterile distilled water. Under aseptic conditions the bolls were sliced transversely exposing the seeds in each locule for sampling. Samples were removed from both symptomatic and asymptomatic seeds, necrotic boll tissue and placed onto TSB agar (Becton Dickinson, Cockeysville, MD) and incubated at 28C for 24 to 72 hours. Unique colony types were removed from the isolation plates and passed through two single colony isolations and then suspended in liquid TSBA ammended with 20% glycerol and frozen at -80C. Microbial identification of purified isolates was performed as described by MIDI Inc. using microbial fatty acid extraction and gas chromatographic analysis (Microbial ID Inc. 1992).

Bacteria used in either greenhouse or microplot pathogenicity tests were removed from frozen stocks and cultured in trypticase soybroth media over night at 28C on a gyrotory shaker at 250 rpm. The cells were then pelleted, resuspended in sterile distilled H_2O and adjusted to approximately 10^8 cfu/ml. In the greenhouse, plants were inoculated using two methods. Inoculation at the time of planting was accomplished by applying a 1 ml solution of 10^8 cells/ml directly onto the seed at the time of planting. Flower inoculations were accomplished by teasing apart the petals of an unopened flower on a 45 day-old plant and injecting 200 µl of a 10^8 cfu/ml suspension of the strain being tested. All inoculated flowers were tagged and the resulting boll examined 30 days later for the incidence of seed rot. Plants were grown under supplemental lighting in the greenhouse.

Microplots consisted of 55-gallon barrels cut in half and drilled into the soil profile leaving 10cm of the barrel above the soil line. Microplots were cultivated and then planted with 4 seeds per microplot which were inoculated at the time of planting as described above. At early bloom, "lower" position unopened flowers were inoculated with the same strain as was used to inoculate the seeds at planting. Bolls were rated for seed rot 30 days after inoculation on August 24 and September 15. On both dates, all bolls 21-days old or older were harvested.

To rate the bolls, two transverse cuts were made in each boll resulting in 3 approximately equal width sections. The number of locules containing one or more seeds exhibiting seed rot symptoms was recorded. The lint and seeds were removed from each affected locule and the presence or absence of insect feeding (punctures or warts on the locule wall) recorded. Seed rot was not reported for any locule rated positive for insect feeding.

Results

The results from the microbial isolates screened in the greenhouse are shown in Table 2. The results from the microbial isolates tested in microplots are shown in Table 1. A total of 20 bacterial isolates induced seed rot equal to or greater than the incidence observed in the water treated checks in microplots. These data are shown in Table 1. Twenty-one isolates exhibited an incidence of seed rot that was less than the water treated controls in the same set of microplot tests (data not shown). Two isolates, *Enterobacter agglomerans* (33) and *Cedecea neteri* (86) induced seed rot under both greenhouse and microplot conditions at levels that were equal to or greater than 2X the levels found in the water treated controls.

Discussion/Summary

The vascular tissue and other internal parts of both symptomatic and asymptomatic plants can support a rich and diverse microbial community (Misaghi and Donndelinger. 1990). A few of these endophytic microbes are known plant pathogens while the majority are not known to cause plant disease (Ashworth, et al. 1970). Similary, we have found a wide diversity of microorganisms in bolls exhibiting seed rot symptoms. A total of 20 genera representing >30 species were isolated from both healthy and diseased boll tissue. Similarly others have reported the occurrence of endophytic populations of bacteria in cotton including seed, vascular and boll tissue Misaghi and Donndelinger, 1990; Hallmann, et al. 1998). Of the 600+ isolates identified from boll tissue we selected members from the five most commonly isolated genera which included, *Enterobacter, Pseudomonas, Cedecea*, *Pantoea*, and *Bacillus* and screened them for

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pathogenicity in bioassays in both the greenhouse and field microplots. Sixteen of the 24 isolates screened under greenhouse conditions induced seed rot symptoms in 6.7% to 37.5% of the locules examined. These levels were at least 2X the incidence found in the water-treated controls. Twelve isolates shown to induce seed rot in the greenhouse, along with 29 additional isolates were tested in the field under microplot conditions, where 20 isolates induced seed rot at levels >2X those found in the water treated checks. Interestingly, two isolates, *Enterobacter agglomerans* (33) and *Cedecea neteri* (86) were able to induce significant amounts of seed rot under both greenhouse and microplot conditions. These isolates are currently being retested under greenhouse conditions.

In addition to being isolated from diseased boll tissue, *Enterobacter, Cedecea, Pseudomonas* and *Bacillus* isolates also have been found to occur in/on cotton seeds at the time of planting. Upon germination these soil-borne microbes may infect the seed at a low levels which may account for the low frequency of seed rot in the water treated controls. It is also interesting to note that in nearly all the grower's fields that have been examined we typically observe 1-5% seed rot in the locules examined, which may represent the background level of infection due to the common occurrence of these microoganisms in/on seed and soil.

Here we report the initial steps towards satisfying Kochs' postulates which is a prerequsite to proving causality of seed rot. The next steps in this process involve genetically marking putative causative microbial agents prior to their introduction into the host followed by their re-isolation from diseased tissue. We are now preparing to conduct such experiments under both greenhouse and microplot conditions.

References

Ashworth Jr., L. J., D. C. Hildebrand, and M. N. Schroth. 1970. *Erwinia*-induced internal necrosis of immature cotton bolls. Phytopathology 60:602-607.

Hallmann, J., A. Quadt-Hallmann, R. Rodriguez-Kabana, J. W. Kloepper. 1998. Interactions between *Meloidogyne incognita* and endophytic bacteria in cotton and cucumber. Soil Biol. Biochem. 30:925-937.

Microbial ID Inc. 1992. Microbial Identification System Operating Manual, Version 4, Newark, DE.

Misaghi, I. J., and C. R. Donndelinger. 1990. Endophytic bacteria in symptom-free plants. Phytopathology 80:808-811.

Table 1. Field Microplot Screening of Selected Bacterial Isolates.

Genus species	Isolate #	Locules w/seed rot (%)
Bacillus megaterium	11	25.5
Cedecea lapagei	374	21.3
Enterobacter agglomerans	493	13.0
Cedecea lapagei	83	11.1
Cedecea lapagei	57	10.8
Enterobacter agglomerans	39	10.7
Cedecea lapagei	65	10.2
Enterobactor agglomerans	33	10.0
Cedecea neteri	86	8.8
Pseudomonas putida	139	8.0
Enterobacter agglomerans	60	7.9
Enterobacter agglomerans	36	7.0
Pseudomonas putida	41	6.8
Cedecea lapagei	35	6.2
Cedecea davisae	91	5.6
Stenotrophomonas maltophilia	50	5.5
Agrobacterium radiobacter	308	5.4
Bacillus cereus	7	5.0
Enterobacter agglomerans	34	4.8
Pseudomonas putida	61	4.6
Water Treated Control	**--**	**4.5**

Table 2. Greenhouse Screening of Selected Bacterial Isolates.

		Seed Rot by Locule (%)	
Genus species	**Isolate #**	**Flower**	**Soil**
Cedecea davisae	268	0.0	7.2
Cedecea davisae	269	3.5	14.3
Cedecea davisae	134	3.4	3.8
Cedecea daviase	2	16.0	37.5
Cedecea lapagei	3	2.5	0.0
	90	4.5	16.7
	333	4.6	0.0
	124	6.2	11.1
	42	8.5	0.0
	54	6.2	21.7
Cedecea neteri	86	5.0	10.6
	1	32.3	12.9
Enterobacter agglomerans	33	30.8	20.0
	34	22.0	8.8
Erwinia amylovora	318	1.8	3.9
	262	9.8	20.0
Erwinia rhapontici	267	9.8	0.0
Klebsiella pneumonia	259	3.1	0.0
Pantoea ananas	14	2.5	12.4
	5	9.8	12.5
	55	12.8	12.9
	127	9.5	6.7
Pseudomonas chlororaphis	253	24.0	23.4
Pseudomonas mendocina	272	7.6	0.0
Water Treated Control	---	12.2	2.9