Factors influencing endotoxin concentrations on cotton grown in hot, humid environments: a two year study

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ABSTRACT Cotton leaf, bract, fibre from opened bolls, and soil samples were collected weekly during two growing seasons (1984, 1985). Total and Gram negative bacterial populations were determined for each sample. Representative bacterial isolates were identified and endotoxin concentrations determined. For both years total and Gram negative bacterial populations on all sample types remained relatively stable until plant senescence. Afterwards, until plant death by frost, counts for all samples increased dramatically. Enterobacter agglomerans was the predominant species on leaf and bract, whereas the "all other" Gram negative bacterial species classification was the most common on fibre, with E. agglomerans a close second. Senescence affected the occurrence of the species isolated. Statistical analysis partitioned by sample type showed strong correlations between endotoxin concentrations and certain bacteriological and environmental variables. The data suggest that in hot, humid environments the concentration of endotoxin on cotton leaf, bract, and fibre may be predicted by total and Gram negative bacterial counts, daily high temperature, and week after plant germination.

Mill workers inhaling cotton, flax, or hemp dusts may develop byssinosis which is characterised by "mill" fever, chest tightness, inflammation of the lungs, and chronic bronchitis.1 Previous reports have presented data which suggest that endotoxin has a strong correlation with the acute pulmonary response to inhaled cotton dust.2-4 Gram negative bacteria, which produce the endotoxin (lipopolysaccharide) as part of their cell wall, are ubiquitous on agricultural dusts5 and on cotton leaf, bract, and fibre.6-9 Since bract and also leaf particles comprise most of the trash in raw cotton,10-11 we were interested in the possibility that the plant itself is the source of the endotoxin in raw cotton. Therefore, one objective of the present research was to study, during two growing seasons, the epiphytic bacteriological parameters and endotoxin concentrations on the cotton plant. Published reports indicated that the locality in which cotton is grown influences the concentrations of endotoxin and Gram negative bacilli on bracts but not on lints.7 12 In view of these findings our second objective was to determine whether any bacteriological, environmental, or time related variables could be used to predict endotoxin concentrations on cotton plants in hot, humid environments.

Materials and methods

Randomly collected cotton leaves, bract, and fibre from opened bolls and soil were collected weekly (between 0830 and 0900) during the growing seasons of 1984 and 1985 (early May to late October) from an experimental plot in the grounds of the Southern Regional Research Center. The plot consisted of two rows of Deltapine 61 variety without border rows. Each growing season encompassed the period when the first cotyledonary leaves appeared to the period when 90% of the cotton bolls were fully opened. The plant canopy was dense and rose to a height of about six feet. The randomly collected samples were placed in plastic bags and assayed on return to the laboratory.

Cotton leaves were cut with a sterile cork borer into 5 cm2 sections. For bacterial analysis, 20 g were placed in 200 ml of sterile 0·1 M phosphate buffer containing 0·1% peptone and shaken for two hours6 on a wrist action shaker. Afterwards, each sample was serially diluted (in the aforementioned diluent) and the appropriate dilution spread on triplicate sets of tryptone glucose yeast extract agar (TGY; tryptone, 5·0 g; glucose, 1 g; yeast extract, 5·0 g; agar, 15·0 g; distilled water, 1 l), and endo agar (Difco, Detroit, MI). TGY medium was used to study the total viable bacterial...
population whereas endo agar was used as a selective medium for the growth of Gram negative bacteria. Inoculated plates were incubated for 48 hours at 30°C.

Samples of surface soil were collected from the rows to study the relation, if any, between plant and soil bacteria. Soil, bract, and boll samples were weighed (1-0, 0-5, and 0-5 g, respectively) and placed in sterile diluent solution to give an initial dilution of 1:10. After repeated vortex mixing the appropriate dilutions were made and placed on the agar plates.

After incubation the bacterial colonies were counted, subcultured on to triple sugar iron agar slants, and incubated for 24 hours at 30°C. Gram negative isolates, as determined by Gram staining, were inoculated on to either Enterotube II or Oxi/Ferm Tubes (Roche Diagnostics, Nutley, NJ), depending on their carbohydrate reactions on triple sugar iron slants. Cultures that produced acid from glucose on these slants were inoculated into Enterotube II whereas non-glucose using isolates were inoculated on to Oxi/Ferm Tubes. Isolates were identified according to the numerical index generated by the biochemical reactions in these tubes.

Endotoxin analysis was performed by using the limulus amoebocyte lysate (LAL) test (Associates of Cape Cod, Woods Hole, MA). One gram of soil or 0-5 g of leaf, fibre, or bract were serially diluted twofold in sterile, pyrogen free water. All glassware was depyrogenated by heating at 180°C for three hours. After the appropriate dilutions were inoculated into LAL tubes, the tubes (with positive and negative controls) were incubated for one hour in a 37°C water bath. The LAL sensitivity was 0-03 EU/ml of endotoxin which was checked against Escherichia coli control standard endotoxin (Associates of Cape Cod). To obtain the amount of endotoxin (ng) present per milligram of sample each test result was divided by five. After incubation, the endpoint clot method was used to determine endotoxin concentration as prescribed by the manufacturer (Associates of Cape Cod).

Ambient air temperature, relative humidity, and dew point measurements were obtained from the National Weather Service (about 10 km from the sample sites). Precipitation was measured on site. These environmental and biological data were subjected to simple correlation, split plot analysis of covariance, and multiple regression using SAS software. To establish a balanced data set, means of the duplicate weekly 1984 data were computed for each sample type (since duplicate samples were not taken weekly during 1985). Before analysis, seven severe outliers (which falsely inflated correlations involving toxin and bacterial count) were omitted from a total of 163 observations. Furthermore, the data for endotoxin, total bacteria, and Gram negative bacteria were log-normally distributed and thus required log transformation to meet the criteria for parametric analysis. The data were first subjected to a split plot analysis of covariance with year in the subplot and log Gram negative bacteria as the covariable. The main effect for year as well as interactions involving year were not significant. Consequently, data from the two years were pooled together for simple correlation analysis.

**Results**

Figures 1a and 1b show the respective bacterial populations for the development of the first cotyledonary leaves to plant death from frost. The changes over time of the total bacterial counts (fig 1a) were similar to those of the Gram negative populations (fig 1b). Relatively stationary population levels were observed on leaf and bract until senescence began (week 16). An increase in bacterial counts was associated with senescence. A second, more dramatic increase occurred
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The data in Table 1 show the percentage of total isolates from cotton leaves, bract, fibre, and soil during the growing seasons of 1984 and 1985. Numbers in parentheses equal numbers of isolates per species.

<table>
<thead>
<tr>
<th>Bacterial species</th>
<th>Leaves</th>
<th>Bract</th>
<th>Fibre</th>
<th>Soil</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enterobacter agglomerans</td>
<td>18-2 (105)</td>
<td>25-4 (85)</td>
<td>22-9 (25)</td>
<td>2-6 (8)</td>
</tr>
<tr>
<td>Other Enterobacter sp</td>
<td>9-0 (52)</td>
<td>3-6 (12)</td>
<td>0-0 (0)</td>
<td>0-0 (0)</td>
</tr>
<tr>
<td>Klebsiella sp</td>
<td>1-0 (6)</td>
<td>1-2 (6)</td>
<td>0-9 (1)</td>
<td>0-6 (2)</td>
</tr>
<tr>
<td>Serratia sp</td>
<td>12-3 (71)</td>
<td>12-2 (41)</td>
<td>6-4 (7)</td>
<td>0-6 (2)</td>
</tr>
<tr>
<td>Citrobacter sp</td>
<td>9-2 (53)</td>
<td>0-9 (3)</td>
<td>0-9 (1)</td>
<td>0-6 (2)</td>
</tr>
<tr>
<td>Pseudomonas sp</td>
<td>15-3 (88)</td>
<td>17-9 (60)</td>
<td>22-0 (24)</td>
<td>14-4 (44)</td>
</tr>
<tr>
<td>Acinetobacter sp</td>
<td>5-9 (34)</td>
<td>4-8 (16)</td>
<td>6-4 (7)</td>
<td>1-9 (6)</td>
</tr>
<tr>
<td>All other Gram negative bacterial sp</td>
<td>15-3 (88)</td>
<td>15-5 (52)</td>
<td>23-8 (26)</td>
<td>10-8 (33)</td>
</tr>
<tr>
<td>All other Gram positive bacteria</td>
<td>13-7 (79)</td>
<td>17-9 (60)</td>
<td>16-5 (18)</td>
<td>68-4 (208)</td>
</tr>
</tbody>
</table>

*Total isolates number: leaves (576), bract (335), fibre (109), and soil (304).

Fig 2  Endotoxin concentrations on cotton plant parts and soil along with daily high temperature.

(weeks 24 and 25) after the first killing frost (week 24). Fibre showed a similar trend of increasing total and Gram negative bacterial populations beginning with the first sampling date (week 16) to week 25. This was not the pattern followed by the soil bacterial populations, however, which remained relatively constant throughout the study. Presumably the stability of the bacterial counts in the soils resulted from the bacterial types present or the soil acted as a buffer against deleterious environmental factors.

Figure 2 shows daily high temperature and endotoxin values of the samples from development of the first cotyledonal leaves to plant death from frost. Endotoxin values varied greatly through time on all four sample types. Despite this some general conclusions may be drawn. Until senescence, soil usually yielded the highest endotoxin values, which ranged from 0-03 to 3-4 ng/mg. Later, soil endotoxin concent-

Table 2  Average weekly population percentage during 1984 and 1985 growing season of the bacterial isolates from cotton leaves and bract before and after senescence. (Numbers in parentheses equals number of isolates per species)

<table>
<thead>
<tr>
<th>Bacterial isolates</th>
<th>Leaf*</th>
<th>Bract*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before senescence</td>
<td>After senescence</td>
<td>Before senescence</td>
</tr>
<tr>
<td>Enterobacter agglomerans</td>
<td>18-7 (82)</td>
<td>16-6 (23)</td>
</tr>
<tr>
<td>Other Enterobacter sp</td>
<td>10-7 (47)</td>
<td>3-6 (5)</td>
</tr>
<tr>
<td>Klebsiella sp</td>
<td>1-3 (6)</td>
<td>0-0 (0)</td>
</tr>
<tr>
<td>Serratia sp</td>
<td>11-8 (52)</td>
<td>13-7 (19)</td>
</tr>
<tr>
<td>Citrobacter sp</td>
<td>11-4 (50)</td>
<td>2-1 (3)</td>
</tr>
<tr>
<td>Pseudomonas sp</td>
<td>13-0 (57)</td>
<td>22-4 (31)</td>
</tr>
<tr>
<td>Acinetobacter sp</td>
<td>6-3 (28)</td>
<td>4-3 (6)</td>
</tr>
<tr>
<td>All other Gram negative bacterial sp</td>
<td>14-1 (62)</td>
<td>18-8 (26)</td>
</tr>
<tr>
<td>All other Gram positive bacterial sp</td>
<td>12-3 (54)</td>
<td>18-1 (25)</td>
</tr>
<tr>
<td>Total isolates</td>
<td>438</td>
<td>138</td>
</tr>
</tbody>
</table>

*Total bacterial count: leaf, 576; bract, 335.

trations ranged up to 33-0 ng/mg. After senescence, there was a trend of increasing endotoxin concentrations on all tissue types with values equaling or exceeding that of soil. Bract endotoxin concentrations became the highest at this time, and ranged from 0-04 to 33-0 ng/mg. Cotton fibre contained relatively low endotoxin concentrations (0-0004 to 6-5 ng/mg) whereas leaf, overall, and postsenescence had endotoxin concentrations of 0-0006 to 8-0 ng/mg, with one sampling (week 24) having 64-0 ng/mg. Two sampling dates, weeks 24 and 25, occurred after the first killing frost during both years. Except for cotton fibre, all samples increased in endotoxin at this time.

A total of 1324 epiphytic bacterial isolates were obtained from the different sample types. The greatest number of isolates was obtained from leaves (43.5%) followed by bract (25.3%), soil (23.0%), and fibre (8.2%), respectively (table 1). Fibre contained the fewest isolates, largely because they were not exposed (opened) until late in the study. For leaf and bract Enterobacter agglomerans was the most common epiphytic bacterial type, whereas the “all other” Gram negative bacterial species classification was the most common on fibre, with E agglomerans a close second. As might be expected, the flora isolated from the soil were most distinct, differing primarily in the number of Gram positive bacterial species (68.4% of the soil total) which were much less common in other sample types.

Table 2 shows the percentage of the various bacterial isolates from leaves and bract, presenesence and postsenesence. On the leaf, E agglomerans had about the same population before and after senescence whereas Pseudomonas sp showed substantial increases.
after senescence. The other species either experienced large decreases or little change. The bacterial species found on the bract samples followed the same trends.

To obtain more specific information on contamination of fibres, bacterial populations from the boll were followed from the onset of lock opening to its completion (table 3). Lock opening is defined as the stage of cotton boll development when the wall of the valves dries up, turns brown, and the capillary sutures open up along the dehiscence zones of the carpels with cleaving also taking place along the placental axis.18 E agglomerans showed the greatest increase (0–0–24.5%) during this period whereas Pseudomonas sp experienced a pronounced reduction (71.4–18.6%) in total population percentage.

We were particularly interested in the dynamics of the bacterium, E agglomerans, because it is found at high levels in cotton mills.15 Since bract is the major component of botanical trash in unprocessed cotton10 11 we postulated that the high levels of E agglomerans encountered in cotton mills is due to this bacterium being on the cotton fibre and botanical trash. It may be seen from tables 2 and 3 that E agglomerans is the predominant species on fibre and bract after senescence.

Several interesting correlations with endotoxin value occurred for certain microbial and environmental variables (table 4). When the data were pooled as a single group, significant correlations existed between endotoxin concentration and total bacterial count, Gram negative bacterial count, daily high temperature, and week of sampling. When the data were partitioned by sample type, especially for leaf and bract, the correlations increased substantially. Endotoxin value was negatively correlated with daily high temperature and positively correlated with the week of sampling. Dew point was highly collinear with daily high temperature (r = 0.86, p = 0.001) and, therefore, this variable was excluded from the analysis. As with the microbial variables, the environmental variables appeared to be more closely associated with endotoxin on the leaf and bract samples than the fibre and soil samples. Precipitation was not significantly correlated with endotoxin concentration or bacterial counts for any of the sample types (p < 0.3 for all correlations).

A split plot analysis of covariance was performed to determine whether a significant year effect existed, and to separate the relative contribution (in terms of explained variation in endotoxin level) of sample type, Gram negative bacterial count, and the interactions among these and time. The model as a whole accounted for 54% of the variability in endotoxin value. Most of this variability was attributable to the covariable Gram negative bacterial count (producing f = 66.99, p = 0.001). However, sample type (producing f = 16.84, p = 0.001) was also highly significant, indicating the existence of four separate regression lines. The subplot effect of year was not significant, nor were interactions involving year.

### Discussion

The data showed strong correlations on leaf and bract between certain bacteriological, environmental, and time variables with the concentration of endotoxin. Overall, Gram negative bacterial count was the most accurate bacteriological predictor of endotoxin values. Nevertheless, multiple regression of endotoxin on daily high temperature and week (after cotyledonal leaf emergence) suggested that these relatively easily monitored variables may also adequately predict endotoxin concentrations. Indeed, the week of the growing season was superior to the Gram negative bacterial count on bract and fibre (but second to it in the pooled and leaf categories) in predicting endotoxin concentrations.

It is plausible that the significant correlations of...
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endotoxin concentrations with the environmental and time variables may simply be attributed to a corresponding release of biochemical constituents from the dying plant, which would provide nutrients for epiphytic bacterial multiplication. Presumably such an increase in bacterial—that is, Gram negative—population would in turn result in increased endotoxin concentrations.

Fischer and Jacobs found that endotoxin and Gram negative bacterial count concentrations on bracts were much higher on three cotton types grown in Mississippi than were found on cotton from Texas or California. Our data complements their work which found these high endotoxin and Gram negative bacterial count concentrations from a warm, humid region as opposed to those from presumably much less humid areas.

Our research indicated that predictable trends in endotoxin occurred during a two year study. More importantly, the trends over each year did not differ significantly, at least suggesting that these trends may be stable (broadly predictive). Consequently, it may be possible in hot, humid environments (such as that of the test plot) to use Gram negative bacterial counts or the more easily monitored variables (daily high temperature or the week of the growing season, or both) as predictors of endotoxin concentrations on leaf, bract, and fibre of cotton plants.

The data suggest that by monitoring the aforementioned variables cotton producers and cotton mills in hot, humid environments may be able to determine the best time to harvest cotton just before the dramatic increase in endotoxin concentrations. This could prevent high amounts of endotoxin from becoming airborne during processing, thus reducing the risk of byssinosis to the workers.

References

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