Project: Bacterial Populations and Diversity within New USGA Putting Greens

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The overall objective of this project is to develop baseline data concerning bacterial composition (population and diversity) of new USGA putting greens, both during and after construction. In order to accomplish this task, it was necessary to ascertain the best methods for achieving this goal. In regards to enumeration of bacteria, various diluents and media for each specific group of organisms were evaluated.

The best overall diluent to use, across all media, was 0.1% sodium pyrophosphate with 1% glycerol. The following media will be used for enumeration:

a) S-1 medium for fluorescent pseudomonads
b) selective medium for *Stenotrophomonas maltophilia*, formerly *Xathomonas maltophilia*
c) reduced arginine soluble starch medium for actinomycetes
d) solidified 1/10 strength tryptic soy broth for total bacterial counts
e) Azide Blood Base Agar for gram-positive bacterial counts
f) Crystal Violet Agar for gram-negative bacterial counts
g) dilutions heated for 10 minutes at 80 C followed by plating on solidified 1/10 strength tryptic soy broth for heat tolerant bacteria such as *Bacillus* spp.

A protocol has been established that will be followed by all the cooperators in this project - myself, Dr. Beth Guertal at Auburn University and Dr. Horace Skipper at Clemson University.

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INTRODUCTION

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MATERIALS AND METHODS

Media used for comparison was dependent on the bacterial group to be isolated. All media were supplemented with cycloheximide and nystatin to inhibit fungal growth, unless specified below. *Xanthomonas maltophilia* was enumerated using a selective medium but was not compared to another medium.

To isolate and enumerate actinomycetes, the media included reduced arginine soluble salts (RASS), Difco actinomycete selective medium, solidified 1/10 strength tryptic soy broth (1/10 TSBA), humic acid vitamin agar (HAVA) and soil extract agar using a root-zone mix of sand and sphagnum peat moss (80:20; v:v) as the soil.

For general or total aerobic bacterial counts, the media compared were 1/10 TSBA, 1/10 TSBA with 1% glycerol, solidified 1/10 strength Luria Bertani broth (1/10 LBA), 1/10 LBA with 1% glycerol, 1/5 strength tryptone glucose extract agar (1/5 TGEA) and 1/5 TGEA with 1% glycerol. Heat tolerant bacteria were enumerated by placing dilution series tubes in an 80 C water bath for 15 minutes. Dilutions were then plated on 1/10 TSBA.

For fluorescent pseudomonads, the media compared included S1, Difco Pseudomonas Agar F (King's B), King's B amended with (per liter) 75,000 units penicillin G, 25 mg novobiocin and 5 mg chloramphenicol. These media were amended with antifungal antibiotics. These three media were compared with unamended S1.

Two media were compared for enumeration of gram positive bacteria, Bacto azide blood agar base and methyl red agar. The methyl red agar consisted of 1/10 TSBA amended with 20 g NaCl and 150 mg methyl red per liter. Two media were compared for enumeration of gram negative bacteria, solidified Bacto GN Broth, Hajna and crystal violet agar. The latter medium consisted of 1/10 TSBA amended with 5 mg crystal violet (C.I. 42555) per liter.

To determine if the bacteria isolated on these four media were gram negative or gram positive, four representative colonies from each dilution

series were selected and transferred to 1/10 TSBA. A total of 96 colonies were selected from each medium. After 24-48 hr growth, gram stain reactions were determined for each isolate.

All inoculated media were incubated at 28 C for 14 days, if practical. Colony counts were obtained at the appropriate time for each group of media. Fluorescent pseudomonad counts were made after 24-48 hr incubation. Other counts were made at 3, 7 and 14 days.

Six diluents were compared within each group of media. They were 0.1%, sodium pyrophosphate (pH=7.0), a saline phosphate buffer and a soil extract solution. Each diluent was made with or without a 1% glycerol solution (v/v). The soil extract solution was prepared using an 80:20 root-zone mix. One kg root-zone mix was placed in 1 liter deionized water, autoclaved 90 minutes, cooled and filtered through several layers of cheesecloth. Deionized water was added to the filtrate to obtain a 1 liter volume.

The SAS statistical package was used for analysis of data. Waller-Duncan k-ratio t test was used to compare means of diluents across each media/bacterial group and to compare means of media/bacterial group across all diluents, except for the Gram-Positive and Gram Negative groups which were compared using t-test (LSD) since there were only two media to compare.

RESULTS

Actinomycetes: There was no significant differences (Pr>F=0.9154) among diluents across all media. The HAVA and RASS media were significantly (Pr>F=0.0001) better in enumerating actinomycetes than 1/10 TSBA, Difco actinomycete selective agar and soil extract agar. However, they were not significantly different from each other. Not only were we able to enumerate more colonies, but there was less background contamination, especially mucoid spreading types, on these two media. On HAVA, background bacteria were very small, usually pinpoint in size, and no mucoid types were present at all.

While HAVA provides the "cleaner" plates, it is certainly more difficult to prepare. It requires boiling urea and sucrose in 1N hydrochloric acid for 6 hours in a closed vessel, numerous washes, followed by precipitation of the solid material using sodium hydroxide.

Fluorescent Pseudomonads: There were significant differences (Pr>F=0.0052) among diluents across all media. The diluent which resulted in the highest colony counts was the soil extract solution with glycerol. Significant differences were observed among media across all diluents (Pr>F=0.0001). The S1 media had significantly better counts than the King's B media and reduced the bacterial background contamination also making it much easier to conduct the counts.

Total Bacterial Counts: There were significant differences (Pr>F=0.0125) among diluents across all media. The diluents which resulted in the highest colony counts were 0.1% sodium pyrophosphate, with or without glycerol. There were no significant differences (Pr>F=0.6686) among media across all diluents.

Since there were no differences, 1/10 TSBA was selected for enumeration since this medium is used for enumeration of heat-tolerant bacteria such as *Bacillus* spp.

Gram-Positive Counts: There were no significant differences (Pr>F=0.8815) among diluents across both media but the methyl red agar did result in significantly greater counts than the Bacto azide blood agar base (Pr>F=0.0001). However, when gram stain reactions were conducted on 96 colonies from each media, only 18% of the colonies on the methyl red agar were gram-positive whereas 70% of the colonies on the azide blood agar base were gram-positive.

Gram-Negative Counts: There were no significant differences (Pr>F=0.9778) among diluents across both media, but the crystal violet agar did result in significantly greater counts than the GN Broth, Hajna, agar (Pr>F=0.0001). Gram stain reactions conducted on 96 colonies from each media resulted in 95% gram-negative reactions from the crystal violet agar and 87% gram-negative reactions from the GN broth, Hajna, agar.

Heat Tolerant Bacterial Counts: There were significant differences (Pr>F=0.0164) among diluents. There were significant differences (Pr>F=0.0001) between counts before and after treating the dilution series with 80 C heat, with greater numbers before heating. This indicates that the heat treatment is effective in reducing non-heat tolerant bacterial numbers.

CONCLUSION

The best overall diluent to use, across all media, was 0.1% sodium pyrophosphate with 1% glycerol. The following media will be used for enumeration:

a) S-1 medium for fluorescent pseudomonads
b) selective medium for *Stenotrophomonas maltophilia*, formerly *Xathomonas maltophilia*
c) reduced arginine soluble starch medium for actinomycetes
d) solidified 1/10 strength tryptic soy broth for total bacterial counts
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A protocol has been established that will be followed by all the cooperators in this project - myself, Dr. Beth Guertal at Auburn University and Dr. Horace Skipper at Clemson University. Attached is the protocol that will be followed (modifications are sure to occur as the project progresses) and an outline of the experiments that will be initiated shortly here at the FLREC.

Comparison of the six diluents across all media/bacterial groups.

Diluent	$\log_{10}$ CFU ml^{-1}					
	Total bacteria	Heat-tolerant bacteria	Fluorescent pseudomonads	Actinomycetes	Gram-negative bacteria	Gram positive bacteria
0.1% $Na_4P_2O_7$	8.9 ab	7.1 ab	5.3 abc	5.4	6.7	6.0
0.1% $Na_4P_2O_7$ + glycerol	9.0 a	7.1 ab	5.4 ab	5.7	6.6	5.9
saline KPO_4	8.8 bc	6.9 b	4.8 bc	5.8	6.6	6.1
saline KPO_4 + glycerol	8.8 bc	7.0 b	4.4 c	5.7	6.5	5.7
soil extract solution	8.8 bc	7.2 a	5.4 ab	5.6	6.7	5.9
soil extract solution + glycerol	8.8 bc	7.0 b	6.2 a	5.6	6.7	5.9
Pr>F	0.0125	0.0164	0.0052	0.9154	0.9778	0.8815
MSD	0.1	0.2	1.0	----	----	----

Comparison of media across all diluents

Total bacteria		Actinomycetes		Fluorescent pseudomonads		Gram-positive bacteria		Gram-negative bacteria	
Medium	$\log_{10}$ CFU ml^{-1}	Medium	$\log_{10}$ CFU ml^{-1}	Medium	$\log_{10}$ CFU ml^{-1}	Medium	$\log_{10}$ CFU ml^{-1}	Medium	$\log_{10}$ CFU ml^{-1}
1/10 TSBA	8.8	RASS	6.5 a	S1 +	6.2 a	Methyl Red	6.6 a	Crystal Violet	7.1 a
1/10 TSBA/glycerol	8.8	HAVA	6.4 a	S1 -	6.2 a	Azide blood	5.2 b	GN, Hanja	6.2 b
1/10 LBA	8.8	1/10 TSBA	6.2 b	King's B	4.6 b				
1/10 LBA/glycerol	8.8	ASM	6.1 b	King's B+	4.0 b				
1/5 TGEA	8.9	SEA	3.0 c						
1/5 TGEA/glycerol	8.8								
Pr>F	0.6686	Pr>F	0.0001	Pr>F	0.0001	Pr>F	0.0001	Pr>F	0.0001
MSD	------	MSD	0.2	MSD	0.6	LSD	0.1	LSD	0.2

Microbial Population Sampling Protocol for USGA Project

1. From each plot, remove 10 cores (3/8 inch diameter x 4 inches deep). A plot equals a replicate. For Beth and Monica, a plot/replicate is one container. For Skip, a plot/replicate equals one green; but same area on green will be sampled each time.

2. Remove green leaves/stolons and half of thatch present; discard.

3. Remaining soil and root material for each plug will be placed in a Glad-lock brand zipper sandwich bag. Thoroughly break-up and mix materials thoroughly together in the bag.

4. Randomly remove 10 grams (wet weight) from a bag and place in a 250 ml flask with 90 ml of sterile diluent. Place flask on a rotary shaker (200 rpm) for 30 min.

5. Complete a 10-fold dilution series using this suspension and the sterile diluent. We normally remove 0.5 ml (500 ul) and place in 4.5 ml sterile diluent.

6. 0.1 ml (100 ul) aliquots of the dilutions are spread on the following media for enumeration:
   a) S-1 medium for fluorescent pseudomonads
   b) selective medium for Stenotrophomonas maltophilia, formerly Xathomonas maltophilia
   c) RASS medium for actinomycetes
   d) solidified 1/10 strength tryptic soy broth for total bacterial counts
   e) Azide Blood Base Agar for gram-positive bacterial counts
   f) Crystal Violet Agar for gram-negative bacterial counts

7. The dilutions are then placed in an 80^{o}C water bath for 10 minutes. Aliquots (100 ul) of the dilutions are spread on solidified 1/10 strength tryptic soy broth to enumerate bacteria which produce heat tolerant spores, e.g. Bacillus.

8. Plates are incubated at 28^{o}C and microbial colonies counted at the appropriate time. The greatest counts for each bacterial group for each medium are used for statistical analysis. In other words, only actinomycetes are counted on RASS; only fluorescent colonies are counted on S-1; all colonies are counted on 1/10 TSBA, etc.

9. Select 40 colonies from the 1/10 TSBA plate for each sample. Use the 1/10 TSBA plate spread prior to the 80 C water bath treatment, i.e. the total bacterial count plate. Do not select from the countable plate, but from the plate that is 2 dilutions back from the countable plate. Divide the plate

into 4 quadrants and randomly select 10 colonies from each quadrant. Transfer colonies to 1/10 TSBA to obtain clean cultures.

10. After pure culture obtained, transfer to sterile cryogenic tubes (3 or 4 per isolate) containing sterile deionized, distilled water. Seal with Parafilm. Store at room temperature. Send one tube to Kloepper's lab at Auburn.

    Univeristy Code: AU=Auburn University
    CU=Clemson University
    UF=University of Florida

    Note: Each lab can select and store as many isolates as they may want for future use, but the minimum for this project will be 40 per sample each time. This way we should have the minimum of 35 suggested by Kloepper's staff.

DILUENT: 0.1% sodium pyrophosphate/1% glycerol

Experiment I at FLREC

Root-zone Mix: Quartz sand plus sphagnum peat moss; percentage based on site located by SC group

Fumigant: methyl bromide

Cultivar: 'Tifdwarf' - source dependent on SC source

Longevity: 4 years; maintained as close as possible to actual green (<3/16 inch); locate on blank spot on new green

Reps: 4 Lerio tree pots

Special Considerations: permanent location; extra root-zone mix to use for top-dressing each year?; hand weed; non-toxic insecticides when possible

Sampling Schedule: November, February, May, August of each year

Experiment II at FLREC

Root-zone Mix:
a. quartz sand plus sphagnum peat moss
b. quartz sand plus Dakota reed sedge peat

Fumigant:
a. methyl bromide
b. metam sodium
c. dazomet

Treatments:
1. peat moss x methyl bromide
2. peat moss x metam sodium
3. peat moss x dazomet
4. reed sedge peat x methyl bromide
5. reed sedge peat x metam sodium
6. reed sedge peat x dazomet

Replicates: 4 per treatment; total of 24 Lerio tree pots

Cultivar: 'Tifdwarf'

Longevity: approximately 7 months in pots; greens level maintenance for grown-in

Special Considerations: extra root-zone mix to use for top-dressing; hand weed; no insecticides except Bt if necessary

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Bacterial

18 November 1996: sample individual root-zone components

2 December 1996: sample root-zone mixes before placement in pots

13 January 1997: sample immediately prior to fumigation

3 February 1997: sample 14 days after fumigation, when plastic removed

3 March 1997: sample sprigs immediately prior to sprigging grass

31 March 1997: sample roots/soil 1 month after sprigging

~18 August 1997: sample roots/soil 6 months after sprigging