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Comparison of microbial numbers in soils by using various culture media and temperatures

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Summary

The influence of different media and incubation temperatures on the quantification of microbial populations in sorghum, eucalyptus and forest soils was evaluated. Microbial growth was compared by using complex (tryptone soybean agar, TSA, casein-starch, CS, and Martin) and saline (Thorton, M3, Czapeck) media and incubation temperatures of 25 and 30° C. Higher numbers of total bacterial and fungal colony-forming units (CFU) were observed in sorghum soils, and of spore-forming and Gram-negative bacteria in forest soils than other soils. Actinomycetes counts were highest in forest soil when using CS medium at 30 °C and in sorghum soil at 25 °C in M3 medium. Microorganism counts were dependent on the media and incubation temperatures. The counts at temperatures of 30 °C were significantly higher than at 25 °C. Microbial quantification was best when using TSA medium for total and spore-forming bacteria, Thorton for Gram-negative bacteria, M3 for actinomycetes, and Martin for fungi.

Introduction

Bacteria and fungi, among soil organisms, actively participate in organic matter decomposition liberating chemical nutrients and furthering plant growth. Microorganism numbers vary in and between different soil types and conditions, with bacteria being the most numerous. Bacterial counts in different soils ranged from 4×10^6 to $2 \times 10^9\,g^{-1}$ dry soil (Whitman et al., 1998).

Growth of microbial populations and their action on soils are dependent on the interaction between plant species and soil (Grayston et al., 1998). According to Marschner et al. (2001), bacterial community composition results from the interaction between soil type, plant species and its rhizosphere localization. In a comparison of counts in corn, soybean and bare soils, fungal but not bacterial populations were influenced by the soil type. Nevertheless, both groups of microorganisms

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were affected by plant type (Buyer et al., 2002). Similar reports have shown that the size and structure of microbial populations are affected by soil type and plant species (Wieland et al., 2001; Pinto and Nahas, 2002). However, a study made on three soils in England, showed that the primary determinant of composition in bacterial communities was the soil type (Girvan et al., 2003).

Soil microorganisms have been isolated and quantified using several selective and non-selective culture media (Sorheim et al., 1989; Buyer 1995; Tabacchioni et al., 2000) that have some advantages over non-culturable methods, allowing for taxonomic, genetic and functional studies in the isolated microorganisms.

Different growth rates are often seen in microbial populations from different soils, which have different nutrient requisites that are not supplied by the media (Kennedy and Gewin, 1997). Thus, many media containing different nutritional requirements have been suggested for the estimation of bacterial diversity (Balestra and Misaghi, 1997), which include saline media prepared using a chemically defined mixture of salts and a carbon source, as well as complex media, rich in polypeptides, amino acids and vitamins or minerals (Seeley et al., 1991).

Soil or rhizosphere organisms include total, spore-forming and Gram-negative bacteria, actinomycetes and fungi, but only a small percentage (1%) are culturable, even when using of a set of media (Bakken, 1997). The results relating to group distributions and densities are thus affected by the choice of the media (Buyer, 1995). Furthermore, different incubation temperatures have been reported for the optimal growth of bacteria and fungi. Considering the soil type, culture media, and the time and temperature of incubation, the counting results may be different between authors (Devliegher and Verstraete, 1995; Buyer and Kaufman 1996; Cattelan et al., 1998; Grayston et al., 1998; Scott and Knudsen, 1999; Taylor et al., 2002).

The aim of this study was to compare the growth of different groups of microorganisms using complex and saline culture media at two incubation temperatures, 25 and 30 °C, and to evaluate factors which allow maximal microbial counts in soils with different plants.

Materials and methods

Soils

Sub-samples (20) from an Oxisol were randomly collected from the superficial layers (1–20 cm) of forest (tropical rain forest), sorghum and eucalyptus soils and pooled to form composite sample from each soil. These samples were homogenized and spread in trays to be cleaned of extraneous materials (pieces of root, leaves, small stems, etc.) followed by drying and storing in plastic containers. When used for microbial counts, the samples were sifted with 2-mm mesh sieves, hydrated to $\frac{3}{4}$ of the field capacity and incubated for 5 days. This was a standard procedure whenever a new group of organisms was studied.

Microbial counts

Ten grams of each soil sample were added to 95 mL of 0.1% (w/v) solution of sodium pyrophosphate. After homogenization for 30 min, this solution was decimally diluted (10^{-1} to 10^{-7}) and aliquots of the resulting solutions plated on appropriate culture media. After incubation at 25 or 30 °C, for up to 10 days, the colony forming units (CFU) were counted.

Culture media

The media selected were among the most frequently cited in the literature, such as the complex media: Tryptone Soya Agar (TSA, Oxoid, Basingstoke, Hampshire, England), Martin (1950) and casein-starch (Kuster and Williams, 1964) and the saline media: Thorton (Sorheim et al., 1989), M3 (Rowbotham and Cross, 1977) and Czapeck (Acea and Carballas, 1990). TSA and Thorton media were utilized for the counting of total, Gram-negative and aerobic spore-forming bacteria. To count Gram-negative bacteria, 5 $\mu g\,mL^{-1}$ crystal violet and 100 $\mu g\,mL^{-1}$ cycloheximide were added to these media. Spore-forming bacteria were counted after being placed in serial dilution tubes at 80 °C for 10 min and then cooled to room temperature prior to the inoculation of the media. Martin and Czapeck media were used for fungal growth and casein-starch and M3 for actinomycetes growth after the addition of 50 $\mu g\,mL^{-1}$ nystatin, 50 $\mu g\,mL^{-1}$ cycloheximide, 5 $\mu g\,mL^{-1}$ polymixin B sulfate and 1 $\mu g\,mL^{-1}$ sodium penicillin (Williams and Davies, 1965).

Statistical analysis

The SAS statistical package (SAS Institute, Cary, NC, USA) was used for data analysis. When a significant *F* value was detected, Tukey's estimates of honest significant differences (HSD) were calculated from the ANOVA analysis. The level of

statistical significance was set at 0.05 and 0.01. Counts were calculated as $y = \log (x+1)$ where x was the number of CFU g^{-1} dry soil.

Results

The soils selected for this study had different physical structures and pH values, and contained different plant species, nutrient concentrations and organic matter (Table 1). CFU counts for total bacteria were, on average, 1.9-fold higher in sorghum soils, in comparison with the forest and eucalyptus soils (Fig. 1). Both incubation temperature and media showed a significant influence only in sorghum soils counts. Thus, total bacterial numbers increased 1.3 to 1.6-fold when the incubation was at 30 °C in comparison with 25 °C and was 1.4-fold higher when grown in TSA in relation to Thorton.

Spore-forming bacteria revealed significantly higher numbers (1.9-fold) in forest soils as compared to other soils (Fig. 2). However, the effect of temperature and media on the counts was significant only in forest soils incubated in TSA at 30 °C.

The growth of Gram-negative bacteria was, on average, 2.1-fold higher in forest soils as compared to the other soils (Fig. 3). The counts, influenced both by the incubation temperature and the media, were higher in Thorton (2.4 to 4.4-fold) and at 30 °C (1.3 to 1.6-fold) when compared to the other conditions (Fig. 4).

The actinomycetes counts showed small variations in different soil types ranging from 79.6 to 88.0 $\times$ 10^6 CFU g^{-1} dry soil (Fig. 5). However, counts were significantly higher, 1.8-fold, in the M3 medium in relation to casein-starch medium. In forest and eucalyptus soils, higher numbers of actinomycetes were found when incubated at 30 °C rather than 25 °C.

Fungi counts were 1.2 to 1.8-fold higher in sorghum and eucalyptus soils, respectively, when compared to forest soils (Fig. 5). Growth was better in Martin as compared to Czapeck (an increase of 1.3 to 1.7-fold) and incubation temperature only affected counts in forest soils (an increase of 1.6-fold).

Table 1. Chemical and physical properties of the soils selected for study

	Sorghum	Eucalyptus	Forest
P resin (mg dm^{-3})	112	47	32
Organic matter (g dm^{-3})	26	22	69
K ($mmol_c$ dm^{-3})	6.1	3.7	5.8
Ca ($mmol_c$ dm^{-3})	35	37	180
Mg ($mmol_c$ dm^{-3})	12	22	120
H+Al ($mmol_c$ dm^{-3})	34	18	15
Cation exchange capacity ($mmol_c$ dm^{-3})	87.1	80.7	320.8
pH ($CaCl_2$)	5.4	5.9	6.8
Clay (%)	49	46	44
Silt (%)	26	4	30
Sand (%)	25	50	26

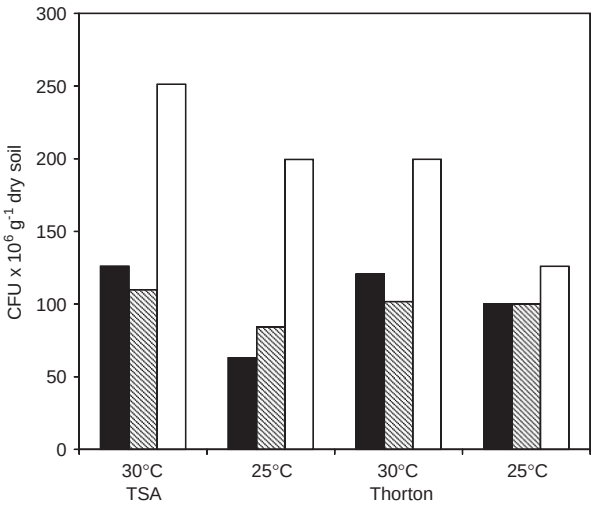

Figure 1. Effect of culture media and incubation temperature on the numbers of total bacteria from forest (■), eucalyptus (▧) and sorghum (□) soils. CFU is colony-forming units.

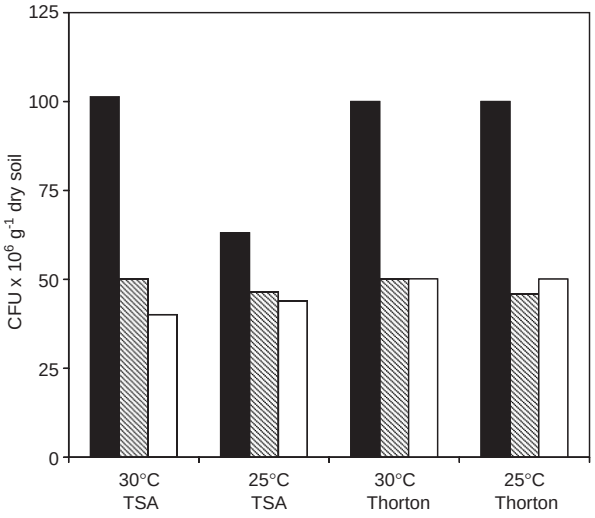

Figure 2. Effect of culture media and incubation temperature on the numbers of spore-forming bacteria from forest (■), eucalyptus (▧) and sorghum (□) soils. CFU is colony-forming units.

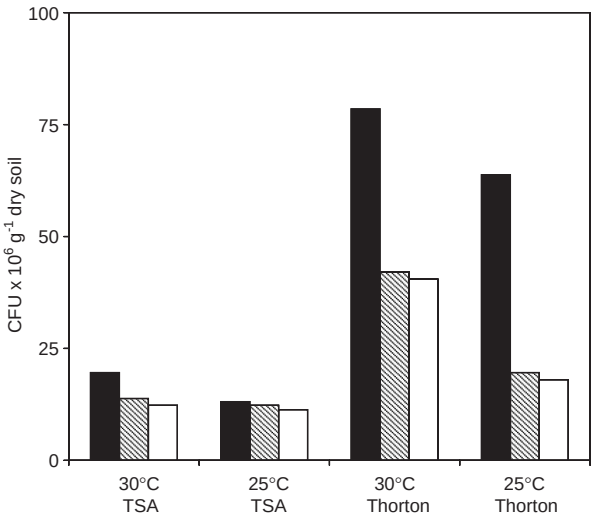

Figure 3. Effect of culture media and incubation temperature on the numbers of Gram-negative bacteria from forest (■), eucalyptus (▧) and sorghum (□) soils. CFU is colony-forming units.

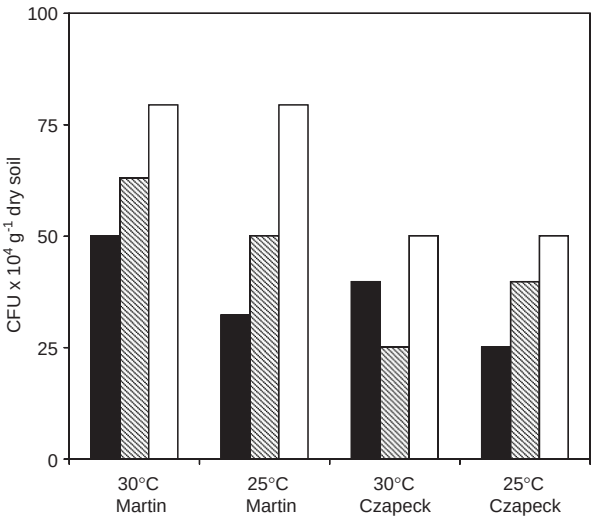

Figure 5. Effect of culture media and incubation temperature on the numbers of total fungi from forest (■), eucalyptus (▧) and sorghum (□) soils. CFU is colony-forming units.

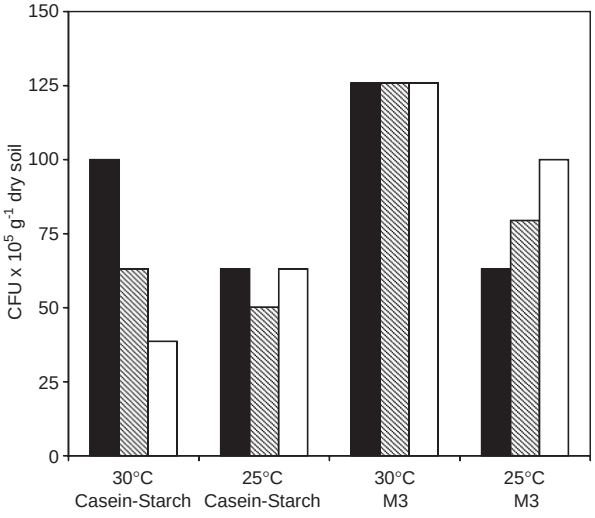

Figure 4. Effect of culture media and incubation temperature on the numbers of actinomycetes from forest (■), eucalyptus (▧) and sorghum (□) soils. CFU is colony-forming units.

Discussion

Plant and soil effects

The highest phosphorus content was found in sorghum soil (Table 1). This soil has been utilized for several yearly crops (besides sorghum, corn and soybean) and it is periodically fertilized (data not included). The soil effects on the number of CFU of total bacteria and fungi decreased in the following order: sorghum > eucalyptus > forest, while the counts of spore-forming and Gram-negative bacteria decreased from forest > eucalyptus > sorghum.

The number of actinomycetes was higher in forest soil than in other soils when casein-starch medium was used; in medium M3, the counts were the highest in sorghum soil. It is possible, therefore, that a selective effect was responsible for the variation in microbial counts where the populations of microorganisms were adapted to an agroecosystem modified by different plants and types of soil in a short time (Atlas and Bartha, 1987). Eucalyptus soil showed an intermediary number of counts when compared to other soils, reinforcing the concept that, besides soil type, plant species have an effect on the size and composition of the microbial populations. The interactions between plant species, soil and microbial communities have been reported in the literature (Marschner et al., 2001). The type of soil was considered a determinant factor in the composition of microbial populations in cultivable soils (Damastri et al., 1999; Girvan et al., 2003). However, other authors (Miethling et al., 2000; Pinto and Nahas, 2002; Sanomiya and Nahas, 2003) have shown that in the same soil type different plant species influence microbial populations distribution.

Chemical fertilizer or manure enhanced the number of total and spore-forming bacteria, actinomycetes and fungi in soil (Kanazawa et al., 1988). Belay et al. (2002) also reported a direct effect of NPK on the number of bacteria, actinomycetes and fungi in a maize cultivated soil. Therefore, the results in this study suggest that

microbial population variation in sorghum soils might be attributed to the effect of chemical fertilization (Gryndler et al., 2003).

Temperature and culture media effects

Incubation temperatures reported in the literature usually vary between 25 and 35 °C (Alexander, 1977). Our results show that larger numbers of CFU were found at 30 than at 25 °C for all microorganisms and soils, although the differences were not always statistically significant ($P < 0.05$). A possible explanation is that the majority of soil microorganisms are mesophilic with maximal growth temperatures between 25 and 35 °C (Brock et al., 1994) and may be better adapted to the higher temperatures in the regions where the soils were collected, which were between 27.8 and 32.2 °C at a depth of 20 cm, from 2000 to 2002 (Agroclimatic Station/UNESP, Jaboticabal, SP. Brazil). However, some discrepancies were also found; actinomycetes counts of sorghum soils growing in casein-starch and eucalyptus soil fungi growing in Czapeck were higher at 25 °C than at 30 °C, though not significantly.

When complex media (TSA and Martin) were used, the numbers of total bacteria in sorghum soils, spore-forming bacteria in eucalyptus soils and fungi in all three soils were higher compared to the same in saline media (Thorton and Czapeck). In general, the growth rate was also faster in complex than in saline media (data not included). Confirming our findings, Brendecke et al. (1993) showed that bacterial counts in cotton soil were 1.9 higher in peptone-yeast agar than in soil extract agar.

Johnson and Manka (1961) did not find significant differences in the microbial counts between Czapeck and Martin media, but Czapeck was supplemented with 0.5% yeast extract. According to Taylor et al. (2002), nutritional requirements and microbial growth influenced the number of microbial counts. Possibly larger peptide molecules or a greater variety of chemical compounds will allow the growth of microorganisms which are nutritionally more selective (Tsoraeva and Zhurbenko, 2000).

However, numbers of Gram-negative bacteria in all three soils and of actinomycetes in eucalyptus soil were significantly higher in saline media as compared to the complex media. According to Holding (1960), Gram-negative bacteria grow best in saline media due to preferences for mineral nitrogen sources. Our results show that the number of actinomycetes were higher in M3 than in casein-starch. Elliott and Des Jardin (1999) also reported that actinomycetes populations were higher in reduced arginine-soluble starch agar medium (RAAS) than in the other complex media tested.

In conclusion these results suggest that while bacterial and fungal counts were higher in agricultural soil (sorghum), spore-forming and Gram-negative bacteria numbers were higher in forest soil than in the other soils. The highest counts were found when microbial groups were incubated at a temperature of 30 °C in complex media as compared to incubations at 25 °C in saline media, respectively.

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