Growth of *Bacillus subtilis* and Spore Germination in Soil Observed by a Fluorescent-antibody Technique

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**SUMMARY**

Vegetative bacteria of *Bacillus subtilis* placed in contact with an acid forest soil initially declined in number but grew after the development of fungal hyphae. Growth did not occur in sterile soil, nor in alkaline forest soil unless fungal growth was stimulated. Spores of *B. subtilis* would not germinate in the same acid forest soil unless fungal growth took place, and hardly germinated at all in the alkaline soil. Roots of seedlings of *Pinus sylvestris* inhibited both vegetative growth and spore germination. These results are consistent with the observed distribution of vegetative bacteria and spores of *B. subtilis* in forest soils.

**INTRODUCTION**

Siala, Hill & Gray (1974) have shown that *Bacillus subtilis* is present mainly in the vegetative form in the acid horizon of a forest soil and as spores in the alkaline horizon. Direct observation of soil particles stained with fluorescent antisera suggested that vegetative bacilli were largely associated with fragments of organic matter. The difficulty of observing relatively small numbers of bacteria spread over the extensive surface area of opaque soil particles makes it impossible to follow changes in form with time and so to investigate this, we have placed bacteria in the soil on a readily recoverable glass slide which we could observe from time to time. We have been able to follow spore formation, spore germination and vegetative growth of *B. subtilis* in soil containing a complete microflora and in sterile soil, and have suggested reasons for the observed distribution of bacteria outlined above.

**METHODS**

*Preparation of soil.* Large roots and twigs were removed from samples of *A* and *C* horizon soil from a pine forest at Freshfield, Lancashire (Siala et al. 1974). The moisture content was adjusted to 5% (w/w) oven-dried soil, a level which is favourable to bacterial growth (Shameemullah, Parkinson & Burges, 1971) and which approximates to the water content of field soil. Approximately 40 g of this soil was placed in each of several Petri dishes.

For the preparation of sterile soil, the moisture content was first adjusted to 10% (w/w) of oven-dried soil and then autoclaved for 30 min at 121 °C on two occasions, incubating at 25 °C in between. After the second autoclaving plates were cooled to room temperature, by which time the water content had fallen to 5%.

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The water content of all dishes was maintained throughout the experiment by further addition of water as necessary to restore the dishes to their original weight.

Preparation of Bacillus inoculum. Bacillus subtilis (NCTC3610) was grown in 250 ml nutrient broth in shaken Erlenmeyer flasks at 25 °C for 14 h. The vegetative bacilli were harvested by centrifugation, washed four times in sterile deionized water and resuspended in 25 ml deionized water. After dispersion of aggregates by magnetic stirring, the suspension was diluted to contain 1.0 x 10^7 bacilli/ml.

A spore suspension of Bacillus subtilis was prepared by growing the organism on nutrient agar slopes in 250 ml medical flats for 14 days at 30 °C. The spores were harvested in deionized water, centrifuged, and washed twice in deionized water. The suspension was incubated for 4 days at 37 °C in a 1:20000 solution of thiomersal (Delpy & Chamsy, 1949; Norris & Wolf, 1961) to remove all traces of vegetative bacilli. The spores were centrifuged, washed twice, and resuspended in deionized water to give a concentration of 1.0 x 10^7 spores/ml.

Introduction of Bacillus subtilis into soil. Clean, grease-free microscope slides were sterilized and inoculated by smearing 0.01 ml of vegetative bacilli or spores over one side of the slide with a flame-sterilized glass rod. The slides were allowed to dry in sterile Petri dishes and then placed on the surface of soil samples with the inoculum in contact with the soil. This resulted in an inoculum of about 30 cells/microscope field of view under the ×100 oil-immersion objective.

Recovery, staining and observation of slides. Five replicate slides were removed from each type after different periods of incubation. The slides were heat-fixed and stained with a 1:1 mixture of FITC-conjugated vegetative ‘O’ antiserum and RB200-conjugated spore antiserum prepared against Bacillus subtilis (NCTC3610) (Hill & Gray, 1967). The slides were stained in a moist chamber for 30 min at room temperature, washed for 10 min in phosphate-buffered saline (0.01 M, pH 7.2) and rinsed in deionized water for 10 min. The bacteria were covered with buffered glycerol (pH 9.0) and a cover glass and observed with a Leitz Ortholux microscope (×10 wide-field eyepiece, ×100 oil-immersion achromat objective, HBO 200 mercury lamp with a 3 mm BG 12 exciting filter). Vegetative bacilli fluoresced green, while spores fluoresced an orange-yellow colour. Fifty randomly selected fields of view were examined on each replicate slide.

RESULTS

Growth of vegetative bacilli in sterile and non-sterile A1 and C horizon soil

Slides smeared with vegetative bacilli were recovered at 0, 1, 2, 3, 5, 7, 9 and 11 weeks after placing them in contact with A1 horizon soil. Numbers of vegetative bacilli and spores that had appeared on the slides were counted and the changes of number are recorded in Fig. 1. The number of vegetative bacilli fell rapidly in both sterile and non-sterile soils during the first week. In the sterile soil, numbers continued to fall to a very low level after 5 weeks, but in the non-sterile soil, growth took place for a period of 2 weeks. Vegetative bacterial numbers then fell to the same level as sterile soil after 11 weeks.

During the same period, some bacilli sporulated in both sterile and non-sterile soils. Numbers of spores stabilized in the sterile soil after 3 weeks but continued to rise until the 9th week in the non-sterile soil.

The observations on growth of vegetative bacilli in non-sterile soil in the first 3 weeks were confirmed by recovering a further set of slides at 2-day intervals for 20 days. The same pattern was observed.
**B. subtilis growth and germination in soil**

When a similar experiment was carried out using C horizon soil, growth of vegetative bacilli in non-sterile soil did not occur, while spore numbers stabilized after 3 to 5 weeks (Fig. 2).

Observation of the slides from the A1 horizon throughout the incubation period showed that the period of growth corresponded with the appearance of fungal hyphae on the slide: growth took place adjacent to the hyphae in most cases. In the alkaline C horizon soil, there were fewer fungal hyphae (see below).

**Germination of spores in sterile and non-sterile A1 and C horizon soils**

The localization of vegetative bacilli around fungal hyphae might have been because of stimulation of their growth by the hyphae or attraction by physical forces. It was decided to examine the fate of spores placed in contact with soil, since stimulation of spores might lead to germination while physical attraction of spores to hyphae, by itself, would not.

Slides with spores were recovered as described above and the results are shown in Fig. 3 and 4. Spore numbers fell only slightly when placed in contact with sterile A1 horizon soil and very few vegetative bacilli were observed. However, in non-sterile A1 horizon soil, spore numbers dropped and then recovered to give a constant number after 7 weeks. At the same time, germination took place and the numbers of vegetative bacilli rose for 3 weeks before
Fig. 3. Changes in numbers of *Bacillus subtilis* spores and vegetative bacteria originating from spores introduced into the acid A horizon soil. Key as in Fig. 1.

Fig. 4. Changes in numbers of *Bacillus subtilis* spores and vegetative bacteria originating from spores introduced into the alkaline C horizon soil. Key as in Fig. 1.

decreasing to the same level as in the sterile soil after 11 weeks. The appearance and decline of vegetative bacilli resembled closely that observed in the previous experiment. When spores were placed in the C horizon soil, spore numbers fell only slightly in sterile or non-sterile soils and almost no spore germination took place.

_Mycelial growth of fungi in the A and C horizon soils_

Slides placed in contact with soil were recovered at 4-day intervals for 4 weeks, stained with aqueous methyl violet for 5 min and washed in deionized water. The length of stained fungal hyphae that had developed in each of 20 randomly selected fields of view were determined by use of a camera lucida technique and a map-measuring device (Thomas, Nicholas & Parkinson, 1965). Fig. 5 shows the rate of development of the fungal hyphae on the slides. Clearly a larger quantity of mycelium developed on slides in contact with A horizon soil and in a shorter time than on slides in contact with C horizon soil. Fungal growth in the A horizon soil ceased at approximately the same time that growth of bacilli stopped, i.e. 3 weeks.

_Effect of fungal growth on bacilli in the C horizon soil_

Although there was less fungal mycelium in the alkaline C horizon soil compared with the acid A horizon soil, Parkinson & Balasooriya (1967) have shown that some fungi, e.g. Mortierella alpina, a chitinoclastic fungus, are confined to the C horizon. The effect of *M. alpina* on *Bacillus subtilis* has been investigated by growing the fungus in sterilized C horizon soil inoculated with *M. alpina*. The fungus was allowed to grow for a week, by
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Fig. 5. Lengths of fungal hyphae developing on slides buried in non-sterile \( A_1 \) and \( C \) horizon soils.
- ■ \( A_1 \) horizon soil
- □ \( C \) horizon soil

Fig. 6. Growth of *Bacillus subtilis* vegetative bacteria introduced into sterilized \( C \) horizon soil inoculated with *M. alpina*. ● Fluorescing vegetative bacteria.

which time slides placed in contact with the soil were colonized to the same degree as slides in contact with non-sterile \( A_1 \) horizon soil after one month. *Bacillus subtilis* vegetative bacteria were introduced on fresh slides and were observed clearly, again associated with fungal mycelium (Fig. 6). After 11 weeks’ incubation, bacterial numbers had dropped almost to zero.

**Effect of pine roots on Bacillus subtilis in \( A_1 \) and \( C \) horizon soil**

*Rhizosphere effect*. Growth and germination of spores around fungal hyphae might be because of the supply of nutrients by the fungus or the production of a local pH favourable to bacterial growth, or both. However, the growth of bacilli around hyphae even in the alkaline \( C \) horizon soil suggests that nutrient provision may have been the important factor. Nutrients are also supplied by growing plant roots which stimulate the growth of many bacteria, although it has been reported that *Bacillus* spp. are sometimes less common on the root surface than in the surrounding soil (Clark, 1940). Pinus roots, abundant in both soil horizons, are frequently colonized by fungal hyphae, and so it was decided to examine the rhizosphere effect on *Bacillus subtilis*.

Soil was placed in 9 cm diameter plastic pots and germinated seeds of *Pinus sylvestris* were placed on the soil surface adjacent to a microscope slide inserted vertically in the soil. These slides had been inoculated with either vegetative bacilli or spores.

The pots were placed in a greenhouse and watered regularly from the bottom to allow the seedling roots to grow down over the surface of the slide. Slides were removed at weekly
Fig. 7. Changes in numbers of *Bacillus subtilis* vegetative bacteria introduced in the rhizosphere of pine seedlings growing in the $A_1$ horizon soil. Changes measured at 1-00 mm distance from root surface. ●, Control soil with no roots; ○, 10 mm from soil surface; △, 20 mm from soil surface; □, 30 mm from soil surface.

Fig. 8. Changes in numbers of *Bacillus subtilis* vegetative bacteria introduced in the rhizosphere of pine seedlings growing in the $C$ horizon soil. Changes measured at 1-00 mm distance from root surface. Key as in Fig. 7.

Table 1. *Changes in the numbers of Bacillus subtilis bacteria and spores placed on the root surface of Pinus sylvestris seedlings*

<table>
<thead>
<tr>
<th>Days after replanting</th>
<th>No. of vegetative bacteria/mm² root surface</th>
<th>No. of spores/mm² root surface</th>
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<tbody>
<tr>
<td></td>
<td>$A_1$ horizon</td>
<td>$C$ horizon</td>
</tr>
<tr>
<td>1</td>
<td>145</td>
<td>135</td>
</tr>
<tr>
<td>5</td>
<td>7</td>
<td>4</td>
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<tr>
<td>10</td>
<td>0·1</td>
<td>0·3</td>
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<tr>
<td>15</td>
<td>0·05</td>
<td>0·01</td>
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intervals for 5 weeks and the position of the roots marked on the slide with a diamond. Ten replicate slides were examined at each sampling period and stained as previously described. Vegetative bacilli and spores were counted at 1·0 and 5·0 mm distances from the root at 10, 20 and 30 mm from the soil surface.

Numbers of vegetative bacilli near roots in the $A_1$ horizon soil fell rapidly and did not show the growth observed on control slides with no roots. Exactly the same happened in the $C$ horizon, although here no growth occurred on the controls. There were no differences between the growth of bacilli at any of the sampling points near the roots (Figs. 7, 8). It was concluded that the roots were inhibiting the growth of vegetative bacilli. They also inhibited the germination of spores near the roots, for spore numbers remained nearly constant in both soil horizons at about 30/field of view.

*Rhizoplane effect.* The marked negative rhizosphere effect suggested that the root surface or rhizoplane of *Pinus* would be unfavourable for growth of *Bacillus*. Roots of young intact
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pine seedlings were dipped in suspensions of vegetative bacilli or spores \((1.0 \times 10^9\) propagules/ml) for 5 min, removed, allowed to dry, and then replanted in \(A_1\) or \(C\) horizon soil in pots. Seedlings were sampled at intervals and the loosely adhering soil particles removed by shaking. The roots were cut into 3 mm lengths and stained with the fluorescent antisera by the method of Hill & Gray (1967). Counts of vegetative bacilli and spores were made at weekly intervals and expressed as propagules/mm² of root surface. Both types of propagule decreased in number, although the majority of the spores were recoverable after 15 days (Table I). The drop in vegetative bacterial numbers was probably a result of the inhibition and death of the cells, while the drop in spore numbers may have been caused by the expansion of the root and subsequent redistribution of the original inoculum over the surface. Spore germination was not observed.

DISCUSSION

It is often assumed that one function of bacterial endospores in soil is to resist the toxic effects induced by acidity. However, in this study and that of Siala et al. (1974), endospore production was more characteristic of alkaline soils. The results suggest that lack of available nutrients causes the formation of endospores and that germination of the spores and growth of vegetative bacilli is stimulated by the development of fungal hyphae. Whether the bacteria use nutrients excreted by living hyphae or cause the death of the fungus and live off the products of lysis is not known. The possibility that fungi alter the pH of the soil by ammonification is not ruled out and is likely to be of some importance in view of the results obtained by Williams & Mayfield (1971) on the growth of Streptomyces and Lowe & Gray (1973) on the growth of Arthrobacter in these same soils. Mayfield (1969) has also shown that pine roots contain a leucoanthocyanin inhibitory to streptomycetes which might also have an effect on bacilli.

The results underline the importance of studying the ecology of soil organisms by following growth in the presence of the native soil microflora. Growth in sterile soil was entirely different and experiments carried out in such conditions would be misleading. The presence of a glass slide has been held to produce artificial conditions in which microbial growth is enhanced. However, a single Bacillus subtilis and its progeny would have to divide thirty times in order to cover a microscope slide. In the experiments reported here lasting 11 weeks, this would require a generation time of 2-57 days. Complete cover is never achieved and so it is possible that the growth observed is characteristic of that found on any new soil surface.

The development of Bacillus subtilis in non-sterile soil is consistent with its known distribution (Siala et al. 1974). They found that vegetative bacilli predominated in the \(A_1\) horizon soil while spores predominated in the \(C\) horizon soil. They were unable to detect bacilli on detached fungal hyphae but recorded considerable colonization of organic matter aggregates bound together with hyphae. Many detached hyphae in soil are known to be dead (Warcup, 1960) and might not support microbial growth.

These results contrast with those reported for the germination of fungal spores in contact with soil. Most workers have found that unsterilized soil has a fungistatic effect on spore germination which is removed by autoclaving (Dobbs & Hinson, 1953). This phenomenon has been explained by assuming that either a heat-labile, wide-spectrum toxic substance is present in most soils (Dobbs & Hinson, 1953) or that the nutrient supply is insufficient to promote germination (Lockwood, 1964). The experiments reported here are consistent with the second hypothesis and if toxins inhibiting spore germination do occur in soil, then it is
around roots rather than in root-free soil that they would be found. Recently, Brown (1973) has suggested that bacteria not stimulated by roots are not subject to bacteriostasis. Thus Bacillus ought not to be affected by bacteriostasis and should grow in contact with non-sterile soil, as found in our experiments. If non-rhizosphere bacteria resemble Bacillus and are stimulated by fungi rather than inhibited by them, this would account for some of the qualitative differences observed between root and soil microfloras.

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REFERENCES


