BROWN, M. E. (1958). *J. gen. Microbiol.* **18**, 239-247

Preliminary Studies on the Inoculation of Selected Micro-Organisms into Partially-Sterilized Soils

BY MARGARET E. BROWN

Soil Microbiology Department, Rothamsted Experimental Station, Harpenden, Hertfordshire

SUMMARY: Samples of Rothamsted and Woburn soil were either partially sterilized, totally sterilized or untreated. Representative samples of each treatment were inoculated with a pure culture of *Nocardia cellulans* and other samples, uninoculated, were kept as controls. The change in numbers of nocardias/g. soil was followed at intervals for one year. In partially-sterilized soil wide fluctuations occurred during the first month, followed by a steadying in count, and at the end of the year *N. cellulans* was still present in high numbers. The numbers of bacteria in such a soil were depressed in comparison with the uninoculated control. *N. cellulans* disappeared from an untreated soil in 6 months. The wide fluctuations in numbers in partially sterilized soil were correlated with a regular cyclical morphological development of *N. cellulans*. This was demonstrated by morphological observations and a soil-shaking method. Peak counts corresponded to the rod form and troughs to the mycelial form and the steadying in count to the rod form.

After soil is partially sterilized by heat or fumigation the number of micro-organisms decreases rapidly for the first few days, then increases during the next few weeks to a number exceeding that in an untreated soil and finally falls again to a number similar to that in control soils (Hall & Clegg, 1949). Usually such partially-sterilized soils are at first very favourable to colonization by fungi, including root-disease fungi, because nutrients are liberated and competition is temporarily limited. With further colonization, however, such soils may become less favourable to parasitic activity than the original unsterilized soils, depending on the type of microflora that establishes itself after soil treatment (Garrett, 1956). Ludwig & Henry (1943) found that *Ophiobolus graminis* re-introduced into a steamed soil was less virulent than when introduced into untreated soil. They attributed this to the activity of *Trichoderma viride* which had become established in the steamed soil.

Inoculation of partially-sterilized soils with micro-organisms selected for their antagonistic action towards plant pathogens was recommended by van Luijk (1938; quoted in Waksman, 1947, p. 306) as a method for controlling plant disease. Hartley (1921) also suggested that partially-sterilized soil could be re-inoculated with a mixture of saprophytic micro-organisms before using it as a seed-bed, so as to counterbalance the injurious effects of parasites. Such inoculations as these have only a temporary effect in changing the biological balance of the soil population. However, if the organisms could be permanently established, then the inoculation method might become a more effective means of controlling disease.

The present work was undertaken to elucidate some of the factors which

affect inoculation into partially-sterilized soils. To follow the changes in population numbers of an introduced culture, an organism which formed easily recognizable colonies was used. *Nocardia cellulans*, a member of the Actinomycetales, which was isolated from chalk soil, can decompose cellulose and can fix atmospheric nitrogen (Metcalf & Brown, 1957), was selected since as it forms bright yellow colonies on yeast agar.

METHODS

Soil samples. Samples of soil were collected from an area of permanent grassland at Rothamsted and from Stackyard field at Woburn Experimental Station. The Rothamsted soil is a heavy clay-loam mixed with flints (pH 6.8); the Woburn soil is a light sandy soil derived from the Lower Greensand (pH 5.6). The soils were passed through a 3 mm. sieve and known amounts added to previously weighed 500 ml. Erlenmeyer flasks. The flasks were then either partially sterilized by steaming for 1 hr., totally sterilized by autoclaving at 15 lb./sq.in. for 1 hr., or left untreated. Representative samples of soil of each treatment were inoculated with *Nocardia cellulans* and other samples maintained as controls. All samples were incubated at 25° and the moisture content kept constant at 25 % (on dry-weight basis) for Rothamsted soil and at 11 % for Woburn soil, by adding sterile distilled-water when necessary.

Preparation of Nocardia cellulans inoculum. The *N. cellulans* inoculum was prepared by adding 1 ml. of a heavy suspension in saline of a 24 hr. culture (grown on yeast agar; (% w/v): 1.5, yeast; 0.5, peptone; 2.0, agar, pH 7.0) to 1 g. sterilized Vermiculite (made by Pan Britannica Ltd. Waltham Abbey, Essex) in a screw-capped bottle. The bottle was shaken vigorously to coat the Vermiculite particles with the suspension and the Vermiculite then shaken on to the partially-sterilized soil and well mixed with it, thus ensuring an even distribution of inoculum.

Method of counting Nocardia cellulans in the inoculated soil samples. The numbers of *N. cellulans*/g. soil were estimated from twofold dilution series, to 10^{-6} , of the soil made in sterile tap-water. Counts were made by inoculating 16 replicate drops (each of 0.01 ml.) of a suitable soil dilution on to the surface of a yeast agar plate previously dried for 1 hr. The number of colonies which developed within each drop-area was counted after 4 days of incubation at 25°; thus 16 replicate counts were obtained for each dilution. These counts followed a Poisson distribution. The number of *N. cellulans*/g. soil with the standard error was estimated from the total of the 16 counts. Counts were done immediately after inoculation of the soil and at regular intervals during one month, followed by less frequent estimations during one year. In all the experiments significance of differences between numbers of organisms/g. soil was taken at the 5 % level. In addition, counts were made, from the yeast agar plates, of bacteria present in the partially-sterilized soils, with and without the inoculum of *N. cellulans*.

RESULTS

Establishment of Nocardia cellulans in partially-sterilized soils

Separate experiments were made on three samples of Rothamsted soil and one with Woburn soil. With Rothamsted soil in Expt. 1, the following sets of soil samples were examined: partially sterilized, inoculated with *Nocardia cellulans* and uninoculated; autoclaved, inoculated; untreated, inoculated. In Expts. 2 and 3 partially-sterilized and inoculated soils were used. With Woburn soil in Expt. 4 partially-sterilized and inoculated soil samples were used.

Following inoculation into partially sterilized Rothamsted soil (Expts. 1-3) the count of *Nocardia cellulans*/g. soil rose steadily for 8-12 days and then fell (Fig. 1). Further wide fluctuations occurred for the next 18 days and over the one-year period the number varied significantly, but never to the same extent as during the first month. At the end of one year counts were still high (Expt. 1: 2.5×10^8 /g. soil; Expt. 2: 1.7×10^8 /g.; Expt. 3: 2.7×10^7 /g.) indicating that *N. cellulans* was competing successfully for available nutrients with the rest of the microbial population in the partially-sterilized soil. These counts were compared with those of *N. cellulans* inoculated into autoclaved and untreated soil (Expt. 1). Similar fluctuations were observed at first in both soils, but in the totally-sterilized soil the count was much higher than in that partially sterilized reaching 5.0×10^9 /g. soil in 7 days and a maximum of 9.3×10^9 /g. in 12 days. After 16 days in the untreated soil the count gradually decreased to 4.0×10^6 /g., a value below that of the initial inoculum of 1.2×10^7 /g. (Fig. 1). After 6 months, at a dilution of 1/4000 *N. cellulans* could not be counted in this soil, nor could it be re-isolated either by enrichment of the soil with cellulose or inoculation of the soil on to a cellulose medium. It was presumed, therefore, that the organism had been killed out.

A similar pattern of events was followed in inoculated partially-sterilized Woburn soil (Expt. 4). There were six wide fluctuations in 32 days before the count steadied at 1.0×10^7 /g. soil. Following inoculation the count rose from 6.0×10^6 /g. to a maximum of 4.7×10^8 /g. soil. A count was made at 76 and 107 days of the total bacterial population (as appearing on yeast agar) in partially sterilized, uninoculated Rothamsted soil (Expt. 1). This population was greater (9.5×10^7 /g. and 4.9×10^7 /g., respectively) than that in the similarly treated inoculated soil (2.1×10^7 /g. and 3.0×10^7 /g., respectively). Thus, *Nocardia cellulans* appeared to have a depressing effect on the numbers of other bacteria. No antagonistic reaction between *N. cellulans* and the other bacteria was noted on the plates.

These experiments showed that *Nocardia cellulans* could be established in two different soil types, provided that the soils were partially or totally sterilized. The organism could not compete successfully with the microbial population in an untreated soil, and after a few weeks it disappeared.

Life cycle of Nocardia cellulans in relation to the fluctuating numbers in the soil

From previous observations on the morphology of *Nocardia cellulans* on different nutrient media (Metcalf & Brown, 1957) it is known that the organism passes through a regular life cycle, from rod to mycelium to rod, remaining in this last form until re-inoculated to fresh media. It seemed possible that the

Fig. 1. Changes in the numbers of *Nocardia cellulans* inoculated into partially-sterilized soil and untreated Rothamsted soil. Exp. 1: ●, partially-sterilized soil; ○, untreated soil. Expt. 2: △, partially-sterilized soil. Expt. 3: ▲, partially-sterilized soil.

marked fluctuations in numbers of *N. cellulans* in the soil, especially during the first month, might be correlated with this cycle. To see whether the cycle actually took place in the mass of the soil, morphological observations were made and the soil-shaking technique developed by Skinner (1951) used.

Preparation of material for morphological observations. Square 1 in. coverslips were coated on one side with plain 2% (w/v) agar and drops of a tap-water suspension of a 24 hr. *Nocardia cellulans* culture put at the centres of the coverslips, which were then placed agar side down on to the surface of partially-sterilized and untreated Rothamsted soil (Expt. 5). Slides were prepared and treated in a similar way. A plate of plain agar was inoculated as control. The morphological changes in *N. cellulans* were observed daily by

removing a slide and coverslip from the soil and examining these with a phase-contrast microscope. Slides were then replaced and re-examined the following day. The agar plate was also examined daily.

Examination of the coverslips in contact with the soil showed that within one day, on both treated and untreated soils, *Nocardia cellulans* had produced long mycelial threads radiating from the inoculation point. These threads began to segment, and by 5 days were divided along their length into short rods. By 7 days, new mycelial threads radiating towards the edges of the coverslips were produced from the tips of the old divided mycelia; these new threads then segmented. This process was repeated twice, by which time the threads had reached the edges of the coverslips and no further mycelium was produced. A summary of the changes in morphology during 26 days of observation is given in Table 1, since this shows that *N. cellulans* went through 4 complete cycles on both types of soils. These cycles were completed in 21 days on partially sterilized soil and in 17 days on untreated soil.

Table 1. *Changes in morphology of Nocardia cellulans during 26 days of observations made on inoculated coverslips placed in contact with partially sterilized and untreated soils.*

Day	Partially-sterilized soil	Untreated soil
1	1 Germinating cells, long branching filaments	Same } 1
	2 Filaments beginning to segment	
	3 Segmentation continuing	
	5 Segmentation to short rods complete	
	6 Short rods	New unsegmented filaments produced from tips of divided mycelia
2	7 New unsegmented filaments produced from tips of divided mycelia	Segmentation of new filaments commencing } 2
	9 Segmentation of new filaments commencing	
	12 Segmentation complete. New mycelium at tips	Segmentation complete. Short rods
3	13 —	New mycelium produced at tips } 3
	14 Segmentation commencing	
	15 Segmentation complete. Short rods	Segmentation commencing
	16 Short rods	Segmentation complete. Short rods
4	17 New mycelium produced at tips, growing to edge of coverslip	New mycelium produced at tips, growing to edge of coverslip } 4
	18 —	
	19 Segmentation commencing	
	20 Segmentation continuing	
	21 Segmentation complete. Short rods	Segmentation commencing
22-26	Short rods	Segmentation complete. Short rods

The repetitive cycle was also observed on the slides. There were 6 cycles in 23 days but, although the mycelium had not reached to the edges of the slides, the *Nocardia cellulans* remained in the rod form and stayed as such for a further

32 days, this being the whole time of observation. On plain agar, after the first mycelial production followed by segmentation there was no further morphological change.

The results of these observations indicated that cyclical development of *Nocardia cellulans* could take place when the organism was in surface contact with the soil and that these cycles occurred at the same time as the wide fluctuations in numbers of *N. cellulans* were occurring in partially-sterilized soil, that is, during the first 3–4 weeks following inoculation. Also, on untreated soil the completion of the cycles in 17 days corresponds to the period of wide fluctuations in the numbers of *N. cellulans* found after inoculation into such a soil, as found in Expt. 1. This, however, is not proof that cyclical development occurs in the mass of the soil, but evidence of such was obtained from shaking experiments.

Fig. 2. Changes in numbers of *Nocardia cellulans* in a suspension of a pure culture shaken for 1 hr.

Technique for shaking soil samples. Skinner (1951) showed that when shaken in suspension, vegetative mycelium of *Streptomyces albidoflavus* broke up into a large number of viable fragments which were killed when shaking was prolonged; spores did not break up and were more resistant than vegetative fragments to shaking. He was able to detect very small quantities of mycelium and spores in a soil suspension by his technique. Shaking was performed by a reciprocating machine at 265 strokes/min. with a vertical movement of 9 cm. A preliminary experiment was performed with a pure culture of *Nocardia cellulans*, shakings being made daily for 4 days starting with a 24 hr. culture. A suspension (varying from 0.6 to 3.5×10^8 cells/ml.) of each culture was made in 10 ml. sterile tap-water and added to 5 g. fine sand in a sterile 1 oz. screw-capped bottle, which was shaken for 1 hr. on the reciprocating machine. The numbers of *N. cellulans*/ml. suspension were estimated from 0.5 ml. samples taken at zero time before shaking began, and thereafter at 10 min. intervals. Twofold dilution series were prepared and the same technique used for estimations as for the soil counts. The counts were compared with visual observations of the morphology at the beginning of each period of shaking. The results obtained are shown in Fig. 2. With a 24 hr. culture of germinating organisms and short filaments the count rose slightly in 10 min. and then fell steadily

(Fig. 2a). A 2-day culture composed of long branching filaments gave a rise in count in 40 min. followed by a sharp fall (Fig. 2b); 3- and 4-day cultures, composed of short rods, showed a steady decline in count (Fig. 2c, d). Thus the shaking method could be used to distinguish between the rod and mycelial stages of *N. cellulans* and was used for this purpose in the main experiments.

Fig. 3. Changes in the numbers of *Nocardia cellulans* in soil samples shaken for 1 hr. Soil from Expt. 2.

Two of the partially-sterilized Rothamsted soil samples, (Expts. 2 and 3), were examined by the shaking technique at intervals during 27 and 18 days respectively, the first examination being made immediately following inoculation. To do this small quantities of soil were taken from the flasks and suspended in 10 ml. sterile tap-water. This suspension was added to a bottle containing sand and sampled and shaken as before. The state of the *Nocardia cellulans* in the soil was deduced from the counts obtained.

The results obtained from shaking the sample at intervals for 27 days are shown in Fig. 3, but both experiments gave similar data. Following the observations with pure cultures an increase in the viable count with time of shaking, followed by a decrease was taken to indicate that *Nocardia cellulans*

was predominantly mycelial (Fig. 3*a*), and a gradual decline, that rods were predominant (Fig. 3*f, k*). An interesting feature sometimes observed was a sudden increase in count which occurred at the 30, 40 or 50 min. time of sampling (Fig. 3*c, g*); this might be interpreted as a fragmentation of young mycelium which occurred only after prolonged shaking. On any one day when the initial counts indicated a predominance of rods and an increase in count occurred with continued shaking, the counts for the following day showed that the mycelial stage was again predominant (Fig. 3*c, d*, or 3*g, h*). Thus the initial stages of mycelial production could be detected.

This repetitive life cycle can be related to the fluctuations in *Nocardia cellulans* numbers in the soil. The graph shown in Fig. 1, Expt. 2, is the daily count of *N. cellulans* in the soil at zero time before shaking the sample. This should be studied in conjunction with the graphs in Fig. 3, which show the results of shaking this same sample. It is seen that a peak count corresponds to a predominance of rods (Fig. 3*f, k, o*). The soil count decreases during the development of mycelium and the count rises again as the mycelium fragments. During the first 12 days, when the soil count is rising to a maximum, the *N. cellulans* apparently passes through two life cycles. This same phenomenon was noted in the second experiment (the 18-day shake) where there was a peak which occurred at 8 days associated with one life cycle, and a maximum peak at 18 days associated with two life cycles (Fig. 1*c*, Expt. 3). After the wide fluctuations which occurred during the first month the count became steadier and the *N. cellulans* remained in the rod form. A shaking experiment performed on a soil sample in which *N. cellulans* was well established, 315 days after inoculation, indicated that rods were predominant. It is thought that actinomycetes and fungi exist in the soil most of the time as rods or spores; *N. cellulans* is probably no exception and once established it remains as a rod. The behaviour of *N. cellulans* when inoculated into partially sterilized soils alien to that from which it was isolated would suggest the possibility of other organisms, such as streptomycetes, becoming established for an indefinite period in partially sterilized but alien soil. The establishment of such an organism selected for its antagonistic action towards plant pathogens would be a step towards an effective means of controlling some plant diseases.

I wish to thank Dr H. G. Thornton, For.Sec. R.S., for his interest in the work and for criticizing the manuscript, Miss Jill Royston for her technical assistance and the Statistics Department, Rothamsted, for their advice.

REFERENCES

- GARRETT, S. D. (1956). *Biology of Root-infecting Fungi*. Cambridge University Press.
HALL, N. M. & CLEGG, L. F. L. (1949). Microbiological aspects of the partial sterilization of soil by chemicals: a review. *Proc. Soc. appl. Bact.* 2, 105.
HARTLEY, C. (1921). Damping off in forest nurseries. *Bull. U.S. Dep. Agric.* 934, 1.

- LUDWIG, R. A. & HENRY, A. W. (1943). Studies on the microbiology of recontaminated sterilized soil in relation to its infestation with *Ophiobolus graminis*. *Canad. J. Res. C*, **21**, 343.
- METCALFE, G. & BROWN, MARGARET E. (1957). Nitrogen fixation by new species of *Nocardia*. *J. gen. Microbiol.* **17**, 567.
- SKINNER, F. A. (1951). A method for distinguishing between viable spores and mycelial fragments of actinomycetes in soils. *J. gen. Microbiol.* **5**, 159.
- WAKSMAN, S. A. (1947). *Microbial Antagonism and Antibiotic Substances*. New York: The Commonwealth Fund.

(Received 19 August 1957)