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Development of a microbial community on cellulose buried in waterlogged soil

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Summary. The development of a microbial community on cellulose (cellophane film and filter paper) buried in waterlogged soil was observed under a microscope. Throughout the decomposition of the cellulose, the biomass, immobilized N, ATP and gas metabolism of the microbial community were examined. As cellulose decomposition progressed, a microbial succession was recognized. This succession was divided into two stages. In the first stage, a few types of cellulolytic microorganisms predominated on the cellulose. Vigorous decomposition of the cellulose was accompanied by a rapid increase in microbial biomass, and H_2 was evolved from the microbial community on the cellulose. In the second stage, the rate of cellulose decomposition was slow. The cellulose remaining was thickly covered with various types of microoganisms. The H_2 produced was consumed by the microorganisms closely adhering to the remaining cellulose. In addition, non-cellular organic N accumulated on the remaining cellulose. A large part of the microorganisms seemed to be dormant in this stage. The trends in this microbial succession were similar to those found in ecosystem successions.

Key words: Cellulose decomposition – H_2 transfer – Microbial biomass – Microbial succession – Community structure – Wetland rice soil

In all terrestrial ecosystems the bulk of photosynthesized organic matter finally enters the soil as organic remains or detritus. It is well known that the decomposition of organic remains is carried out mainly by soil microorganisms and that this process is essential to nutrient cycling in ecosystems. Some attention has been paid to the microbial aspect of the decomposition processes (Swift et al. 1979). However, only a few studies have been made on waterlogged rice soil (Wada 1980).

In wetland rice soils a large part of the soil organic matter occurs in the form of organic remains, because the soils are kept under flooded conditions. Under these conditions some components of plant remains are relatively resistant to decomposition. To clarify the characteristics of organic remains in wetland rice soils a series of experiments have been conducted (Wada and Kanazawa 1970; Wada 1975; Wada et al. 1978; Kanazawa 1979; Wada 1984). The results show that organic remains, isolated intact from the soils, are covered with a dense population of microorganisms. The examinations also indicated that organic remains are the main microhabitat for microorganisms. Furthermore, direct microscopic observation of waterlogged soil revealed that the microsites surrounding individual pieces of organic remains, which were heterogeneously distributed in the soil, constituted micro-subsystems of the entire waterlogged soil system. Wada (1975) proposed that these micro-subsystems be denoted the "sphere of organic debris", which is comparable to the rhizosphere in its importance in soil microbial processes.

To understand this concept, we must clarify the principles governing the microbial processes associated with the decomposition of organic remains in the sphere of organic debris. Since organic remains are too complex in both organization and chemical composition, they are unsuitable for detailed investigation. Therefore, in the present experiments we used cellulosic material (cellophane film and filter paper) instead of plant remains. The cellulose sheets were buried in two waterlogged soils by a modification of Tribe's method (1957), and the microbial processes in the micro-subsystems surrounding these substrates were investigated.

Materials and methods

Soils

Soil samples were collected from the ploughed layer (0–15 cm depth) of wetland rice fields at the Agricultural Experimental Stations in

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Nagano and Kohnosu cities, Japan. The material was passed through a 2-mm sieve with or without previous air-drying. Both of these soils are Haplaquepts. For more than 50 years, only inorganic fertilizer for rice cultivation had been applied. Some properties of these soils are as follows. Nagano: clay loam, pH(H_2O) 5.6, organic C content 1.69%, total N content 0.20%; Kohnosu: light clay, pH(H_2O) 5.6, organic C content 2.73%, total N content 0.24%.

Experiment 1

Soil samples were placed in beakers (diameter 4 cm, height 10 cm) and kept waterlogged. The floodwater was 2 cm deep. The method of Tribe (1957) was modified for application to waterlogged soil as follows (Saito et al. 1977a, b). A piece of cellophane film (1×1 cm; Sawai Paper Co., Japan) was fixed on a coverslip with water, or a piece of filter paper (2×3 cm; No. 51, Toyo Roshi Co., Japan) was enclosed in a bag of saran mesh (Tokyo Screen Co., Japan; opening width 0.5 mm). The coverslips and bags were buried in the water-logged soils and incubated at 30 °C in a dark room. Cellulose films were collected from the soil every 5–7 days, washed gently with water to remove adhering soil particles, and stained with lactophenol cotton blue, phenol rose bengal or nitro-blue tetrazolium chloride (Wada et al. 1978). They were examined under a bright light microscope (Olympus Co., type BHB, Japan).

Experiment 2

Three pieces of filter paper (approximately 0.5 g, 2×5 cm; No. 514, Toyo Roshi Co., Japan), whose oven-dry weight had previously been determined, were enclosed in a bag of saran mesh. The bag was buried in 50 g air-dried Nagano soil and incubated as described for experiment 1. Soil without the addition of filter paper was incubated in the same way. After 10, 20, 30, 40, and 60 days' incubation, the following analyses were made.

Weight loss

The bag of saran mesh containing the partially decomposed filter paper was collected and gently washed. The paper was analysed for its oven-dry weight and ash content. The weight loss of cellulose due to decomposition was calculated by correcting for the amount of adhering soil particles.

Immobilized N

The NH_4^+-N content in the soil was determined by Bremner's method (1965). The amount of N immobilized in the partially decomposed filter paper was estimated from the difference between the amount of NH_4^+-N in soil incubated with filter paper and that in soil without it.

Microbial biomass

Five grams or the partially decomposed filter paper of the soil were homogenized in 45 ml membrane-filtered water in a blender (Sakuma Co., Japan) for 5 min. After the mixture had been diluted 200–400 times, melted agar at a temperature of approximately 60 °C was added so that the final agar concentration was brought to 0.015%. This suspension was sonicated for 5 min at 80 W. One hundred microliters of the solution was smeared over a 4-cm^2 area on a glass slide. The smear was stained with phenol rose bengal (1.0% rose bengal and 0.05% $CaCl_2$ in 5% aqueous phenol). Two smears were prepared for each sample and 50 fields of each smear were examined at a magnification of 1500× for bacteria and 300× for fungi. The bacteria were classified into 24 size classes (cocci: 6 diameter-size classes; rods; 3 width classes ×6 length classes) and the numbers in each class were counted. Fungal hyphae were classified into three diameter classes and the hyphal length was measured in each class by the intersection method (Newmann 1966). The total biovolume of the microorganisms was calculated from the bacterial number, the hyphal length, and the mean biovolume for each size class. The total microbial biomass was calculated by multiplying the total biovolume with the following conversion factors: bacteria 0.8 g dry weight/cm, fungi 0.3 g dry weight/cm (van Veen and Paul 1979).

ATP

ATP was extracted from the partially decomposed filter paper or the soil with $CHCl_3$ and $NaHCO_3$ (pH 8.5, 0.5 *M*) by the method of Paul and Johnson (1977). Their method was modified to avoid the interference of other nucleotide triphosphates such as GTP. Instead of the enzymic solution recommended in the original paper, a vial of firefly lantern extract (Sigma FLE-50) was dissolved in 5 ml TRIS buffer (0.02 *M*, pH 7.6). This mixed solution was left for 3–4 h at 30 °C and then centrifuged; 4 ml of the supernatant was added to 4 ml D-luciferin aqueous solution (250 g/ml), and 100 µl of this solution was added to 200 µl of the sample using an automatic dispenser. The light emitted for the first 5 s was integrated with a Chemi-Grow photometer (Amino Co., USA). The interference of the sample extract with light emission was corrected by means of the internal standard.

Experiment 3

Two pieces of filter paper (approximately 0.2 g, 2×2.5 cm; No. 514) were enclosed in a bag of saran mesh, buried in 20 g air-dried Nagano soil, and incubated as described previously. After 10, 20, and 40 days' incubation, the bags were removed from the soil. Some bags were washed gently with water to remove the adhering soil particles (washed treatment). Others were not washed (unwashed treatment). Each bag was placed in a 30-ml Erlenmyer flask with a gas-tight rubber cap. The atmosphere of the flask was replaced with Ar in three evacuations. After the replacement of the atmosphere, the flask was incubated at 30 °C. The gaseous products from the bag were analysed over 24 h at 3 to 6-h intervals by gas chromatography (Saito and Wada 1984).

Results

Experiment 1

The microbial population appearing on these cellulose substrates was essentially the same, irrespective of soil type, type of substrate, and air-drying pretreatment of the soil. Therefore these factors are not taken separately in the following discussion.

In the course of several weeks' incubation, a succession was recognized in the microbial population on the cellulose. After 7–10 days' incubation, when the soil was not yet strongly reduced, a fungus belonging Chytridales appeared on the cellulose. Along with the fungus, a few kinds of rod-shaped bacteria were growing on the cellulose. The fungus and the bacteria gradually disappeared with the development of the reductive state in the soil, probably because of intolerance to strongly reduced conditions. After about 2 weeks' incubation, rod-shaped bacteria (width 0.2–0.4×length 5–15 µm) with a large terminal endospore (diameter 2–2.5 µm) predominated on the decomposed cellulose. They appeared to decompose the cellulose vigorously. In morphology (Fig. 1), these bacteria resembled *Clostridium dissolvens*, which were isolated by Khouvine (1923).

With the advance of decomposition, cellulose fibres became further disintegrated and so thickly covered with a mass of bacteria that they looked like aggregates of bacterial cells. Filamentous bacteria threaded through the bacterial aggregate, and other bacteria formed small colonies on the bacterial mat adhering to the cellulose fibres. The microorganisms not adhering to the cellulose fibres were considered to be non-cellulolytic.

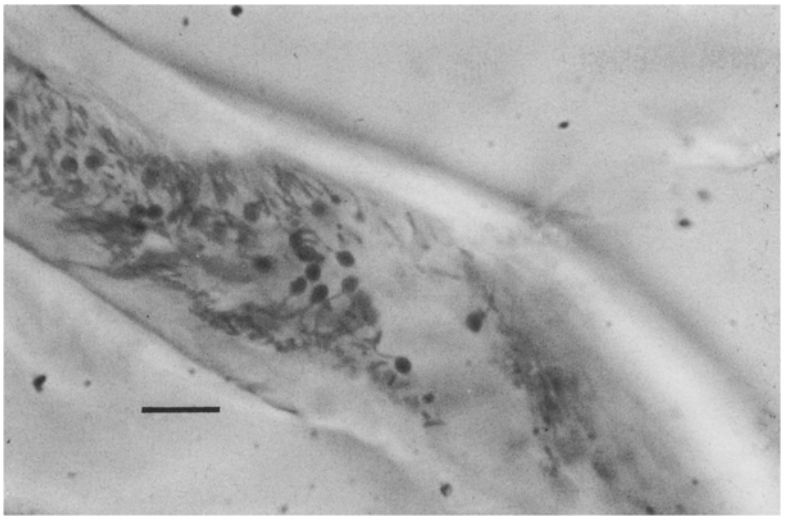

Fig. 1. Long rods with large terminal endospore growing inside cellulose fibre. *Bar* indicates 10 μm

Experiment 2

The results are shown in Fig. 2. After a lag phase of several days, the cellulose substrate lost weight rapidly until the 20th day of incubation and slowly thereafter. N immobilization was very active from the 10th until the 20th day of incubation and became less active in the later period of incubation. This parallel progression of weight loss and N immobilization suggests that mineralized N was taken up and used mainly by the microorganisms that decomposed the cellulose.

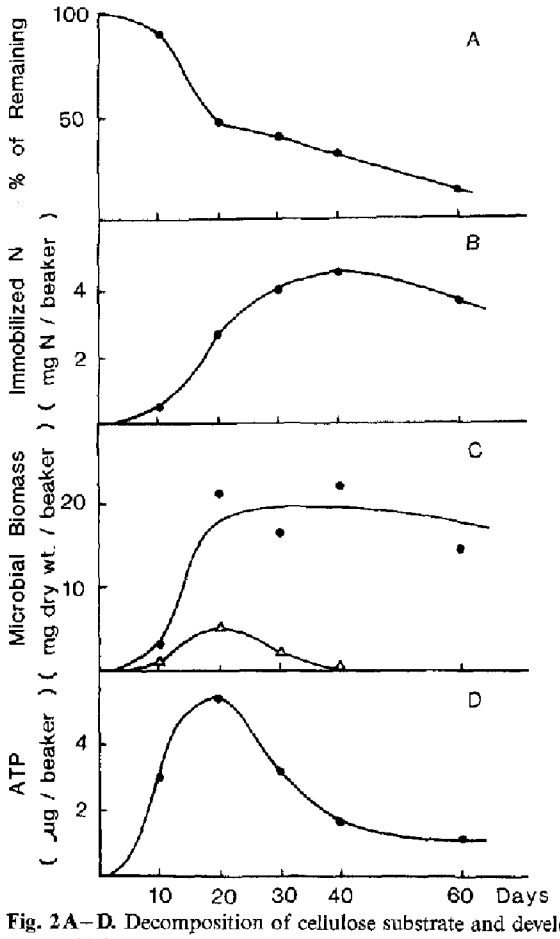

Fig. 2A–D. Decomposition of cellulose substrate and development of a microbial community on it. All data are expressed per beaker. **A** Percentage of the cellulose substrate remaining; **B** the amount of immobilized N; **C** microbial biomass; ●, total microbial biomass; △, biomass of fungal hyphae. **D** the ATP level

The time trends of the microbial biomass were also similar to those of immobilized N. The amount of N contained in the microbial biomass was calculated by assuming that the N content of microbial cells was 10% of their dry weights. Despite this rough assumption, the calculated amount of microbial cellular N was similar to the measured value (the amount of immobilized N) in the early period of incubation. However, the calculated microbial N became much smaller than the measured immobilized N in the later period of incubation. This fact indicates that in the later period nitrogenous non-cellular organic matter accumulated on the partially decomposed cellulose substrate. Autolysed cells and extracellular substances may have been the main source of non-cellular nitrogenous organic matter.

The amount of ATP in the decomposed cellulose increased rapidly after the lag phase, reached a maximum on the 20th day of incubation, and decreased sharply thereafter. Since ATP occurs only in viable cells, this result suggests that the proportion of the viable and active microbial biomass to the total microbial biomass, which was directly estimated under the microscope, decreased in the later period of incubation, probably because many microorganisms became dormant or moribund in this later period.

Experiment 3

For both the washed and unwashed treatments the evolution of gaseous products was linear with time over 24 h, and the results are shown in Fig. 3. After 10 days' incubation there was active evolution of H_2 and CO_2 with both treatments. The evolution of gases was quite active with the unwashed treatment. In contrast, after 20 days of incubation, the amount of the gases evolved with the unwashed treatment, especially H_2, was much less than with the washed treatment. After 40 days, methanogenesis was observed, and little H_2 was evolved with either treatment.

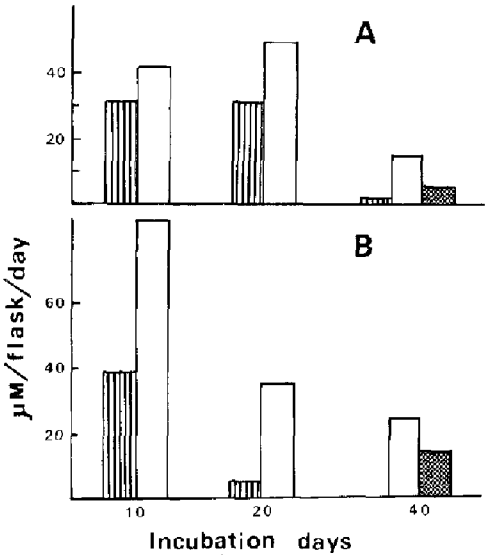

Fig. 3A–B. Gas evolution from microbial community on cellulose substrate. **A** Washed treatment; **B** Unwashed treatment. ▥, H_2; □, CO_2; ▩, CH_4

Discussion

In the present work microbial processes in the micro-subsystem surrounding cellulose substrates of waterlogged soils were studied. On the basis of the microscopic observation, the microbial succession associated with cellulose decomposition was divided into two stages. In the first stage, a few types of cellulolytic microorganisms predominated on the cellulose. In the second stage, numerous non-cellulolytic bacteria grew on the partially decomposed cellulose together with the cellulolytic microorganisms. Tribe (1957) investigated microbial successions on cellophane film buried in diverse soils under aerobic conditions, and found that the sucessions comprised two main phases. The first phase was characterized by the dominance of cellulolytic fungi. In the second phase dead fungal cells were decomposed by various bacteria and soil fauna. These observations suggest that irrespective of soil properties and of environmental conditions, there is a general trend in microbial successions on cellulose buried in soil; the dominance of cellulolytic microorganisms is followed by the growth of various non-cellulolytic secondary microorganisms. The dynamics of the microbial community found in experiment 2 (Fig. 2) supported the conclusions drawn from experiment 1.

In experiment 2, on the 20th day of incubation, more than 20% of the total microbial biomass was occupied by fungal hyphae (Fig. 2). Since the O_2 in the submerged soil must have been depleted before the 20th day, the hyphae must have been tolerant to anaerobic conditions. Active growth of certain fungi on plant remains has been observed in rumen (Bauchop 1979) and in wetland rice soil (Wada 1980). Therefore, some fungi may play a significant role even in the anaerobic decomposition of cellulose in the waterlogged soil, although the main decomposers are bacteria.

In experiment 3, gaseous metabolism was studied in relation to the development of a microbial community. In an anaerobic microbial community most microbial end-products are incompletely oxidized, and a sort of food web is built up among the microorganisms. H_2 is a key intermediate product in this food web and the interspecies transfer of H_2 limits the rate of various anaerobic metabolic processes (Winfrey et al. 1977; Abram and Nedwell 1978; Saito and Wada 1984). Furthermore, a direct interspecies H_2 transfer occurs between juxtapositioned microbial associations with flocs of sewage sludge (Conrad et al. 1985). In view of these reports, the present results suggest that the following processes took place. In the early stage of decomposition, H_2 was evolved through anaerobic cellulolysis in the saran bag. As decomposition progressed, the microorganisms able to use the H_2 gradually proliferated in the soil surrounding the bag or the cellulose. Finally these secondary microorganisms adhered closely to the cellulose while growing and used the H_2 more efficiently. Only CH_4 and CO_2, which are the final products of anaerobic decomposition, were evolved from the cellulose.

Consequently, the development of a microbial community on cellulose can be summarized as follows. In the first stage, a few types of cellulolytic microorganisms grow actively, taking up mineral N from outside the cellulose for cell production. The cellulose rapidly decreases in weight and becomes enriched with N, mainly in the form of viable microbial cells. Gases such as CO_2 and H_2 are evolved, suggesting anaerobic mineralization of the cellulose. In the second stage, numerous microorganisms with various physiological abilities grow and form bacterial aggregates on the residual cellulose fibre. However, most of the microbial cells are resting and moribund, and a large part of the immobilized N occurs in non-cellular materials. This allows the proliferation of secondary non-cellulolytic microorganisms. The metabolites are consumed efficiently within the community on the cellulose, and the community metabolism tends to be independent of that outside. These trends are schematically summarized in Fig. 4.

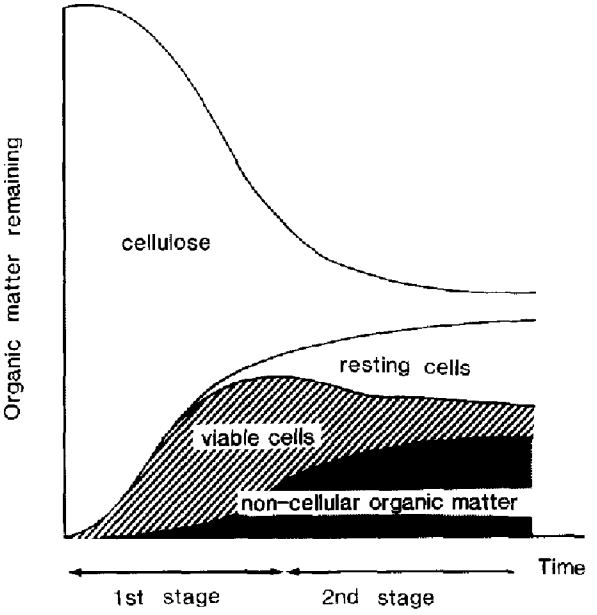

Fig. 4. Hypothetical scheme of the development of a microbial community on cellulose during cellulose decomposition in water-logged soils

Table 1. Development of a microbial community on cellulose buried in a waterlogged soil

Attributes	1st stage	2nd stage
Community structure	Simple	Complex
Flora	Predominance of cellulolytic microorganisms	Diverse
Non-cellular organic matter	Low	High
ATP/microbial biomass	High	Low
Community metabolism	Open, simple	Closed, complex
Gas metabolism	Evolution of H_2	Efficient use of H_2
Immobilized N/cellulose decomposition	High	Low (recycling of N)

Swift (1976, 1982) reviewed the structure of microbial communities associated with the decomposition of plant materials in soil and proposed the following hypothesis for the microbial succession during decomposition. Despite the decline in substrate availability as decomposition progresses, the physicochemical diversity of the system increases with time, resulting in a diversity of microbial niches, which allows the development of a secondary microflora with various physiological abilities. His hypothesis is compatible with our findings described above.

In soil microbiology it has often been emphasized that the nature of a microbial succession associated with the decomposition of organic remains is completely different from that of an ecosystem succession. It is said that in the ecosystem succession, the reserve of organic matter in the system increases as succession progresses until the ecosystem reaches a climax, when primary production and respiration are balanced, whereas in the microbial succession the reserve of organic matter decreases gradually until it disappears (Garrett 1963; Frankland 1981).

However, the progress of the ecosystem succession is characterized by an increase in both the complexity of community structure and the diversity of niches, and by the development of a closed nutrient cycle (Margalef 1968; Odum 1969). These trends were found in the present experiments (Table 1). This indicates that the intrinsic rules governing succession might be present whether a primary producer is involved in the system or not. We therefore propose that the trends found in a microbial succession associated with organic matter decomposition are essentially the same as those found in the ecosystem succession. This viewpoint may be more comprehensive than the former one. To confirm the hypothesis it is necessary to examine and compare microbial successions associated with organic matter decomposition in diverse environments.

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