WHY ARE THE GRASSES GREEN IN ALPINE MEADOWS?[1]

by

S. N. Acharya, R. N. Coleman, M. E. Neuwirth, and M. Dalpe[2]

Abstract. Soil samples from the rhizosphere of Poa alpina and Trisetum spicatum collected on Lookout Mountain, Banff National Park, Alberta contained at least 10 times more bacteria than the soil 3 cm away. The number of bacteria counted on nitrogen free growth media ranged from 5 to 56% of the total heterotrophic count on a minimal (R2A) media. Consistantly more nitrogen fixing bacteria were isolated from the P. alpina rhizosphere compared with T. spicatum despite their close proximity (<1 m). The microorganisms that readily grew on nitrogen free medium after six successive transfers were identified to be Xanthobacter flavus, X. autotrophicus, Azotobacter beijerinckii, Azomonas macrocytogenes, Flavobacterium multivorum, Flavobacterium aquatile and Beijerinckia indica. P. alpina seedlings inoculated with nitrogen fixing bacteria in vitro remained green and healthy for a much longer time than the uninoculated seedlings when grown in nitrogen free medium. Scanning electron micrographs of the roots of these inoculated seedlings and bulbils planted in Lookout Mountain soil revealed their close association with the bacteria.

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Introduction

Grassy alpine meadows of the Canadian Rocky Mountains present a lush green scenery. On close examination of the grasses, there appears to be no Nitrogen deficiency, although the soil is very low in this element (Macyk, 1974). This poses the question as to how these grasses access a supply of Nitrogen? There is an abundance of literature on biological N_2-fixation in grassy plants and many N_2-fixing microorganisms have been found in the rhizosphere of grasses (Dobereiner, 1961; Dobereiner and Day, 1976; Hashtela et. al., 1983; McClung et. al., 1983; Reinhold et. al., 1988). Among the diazotrophic microorganisms that have been found associated with grass roots, the genus Azospirillum is most the intensively studied (Review in Dobereiner and Perdosa, 1987). The other genera of interest are Beijerinckia (Dobereiner,1961), Campylobacter (McClung and Patriquin, 1980; McClung et. al., 1983), Enterobacter and Klebsiella (Haahtela et. al., 1981; Ladha et. al., 1983), Bacillus (Seldin et. al., 1989), Herbaspirillum (Baldani et. al., 1986), Azotobacter and Derxia (Dobereiner and Pedrosa, 1987).

Some form of plant-bacteria interaction might exist to allow for a mutual relationship or an association. In the absence of gross nodule-like morphological structures and a variability among grass-associated bacteria, the identification of the N_2-fixing systems and of the mechanism involved becomes very difficult. However, considerable progress has been made in establishing such associations for cereals and forage grasses (Boddy and Dobereiner, 1984; Dart, 1986; Dobereiner and Defolli, 1980; Habbell and Gaskins, 1984; Okon, 1983; Reinhold et. al., 1988; Van Berkum and Bohlool, 1980). Dobereiner and Pedrosa (1987) reviewed the evidence of physical association between grass roots and N_2-fixing bacteria. The International Biological Program has helped in understanding the nitrogen cycle in arctic tundra. In cold tundra, bacterial N_2-fixation is believed to be more critical to plant productivity than in any other terrestrial system. Under alpine meadow conditions the grass-bacterial interactions will be similar to that of the tundra ecosystem. However, such interactions have not been extensively studied in the alpine ecosystem. Studies conducted at Niwot Ridge, Colorado (Wojciechowski and Heinbrook, 1984) suggested the strong influence of soil moisture on N_2-fixation. Higher levels of bacterial N_2-fixation were observed in Deschampsia grassy meadows than Xeric kobresia meadow and fellfield. Alpine conditions characterized by low soil temperatures and restricted organic decomposition make biological N_2-fixation significant for the survival of plants in these environments (Alexander et. al., 1978; Alexander, 1974; Wojciechowski and Heinbrook, 1984).

The purpose of this study was to determine the presence of a potential N_2-fixing grass-bacteria association in alpine conditions in the Rocky Mountains of Alberta. This paper will deal with preliminary studies that were conducted with the following objectives; (1) determine presence of a rhizosphere effect in two alpine grasses, (2) identify the most abundant N_2-fixing bacteria in the rhizosphere, and (3) observe grass-bacteria association under natural and artificial environments.

Materials and Methods

Rhizosphere effect

Poa alpina and T. spicatum tussocks with soil clump (5 cm in diameter) were collected from the Lookout Mountain top (2600 m) in two consecutive years. Soil was also sampled from Window Mountain. Soil, shaken free from the outer edges of

these clumps, was referred to as non-rhizosphere soil. Approximately 10 g wet weight of this soil was transferred to a milk dilution bottle containing 90 ml dilution buffer (Standard Methods for the Examination of Water and Wastewater, 1985). Similar quantities of rhizosphere soil immediately surrounding the roots in each tussock were collected and treated in a similar manner. Ten fold dilutions of these initial dilutions were made into fresh buffer. Remaining soil was desiccated at 105°C for 16 h and the moisture content determined. This was used to adjust the bacterial count to a soil dry weight basis. Bacterial counts were determined by dispensing 0.1 ml amounts of the appropriate dilution to R2A medium (Difco, Michigan, U.S.A.) and nitrogen free agar medium (NFAM) (Pramer and Smith, 1965). To prepare NFAM, a solution consisting of K_2HPO_4, 1.0 g; $MgSO_4$, 0.5 g; $FeCl_2.H_2O$, 0.1 g; $CaCO_3$, 2.0 g; Noble agar, 15 g and H_2O, 900 ml was prepared and sterilized by autoclave. A second solution consisting of D-glucose, 20g; $Na_2MoO_4.2H_2O$, 2.52 mg; and H_2O, 100 ml was filter sterilized (pore size, 0.22 µm) and added to the first solution after it had cooled to 55°C. The mixed NFAM was then dispensed to sterile Petri plates. Two sets of plates were inoculated in duplicate from each dilution. One set of plates was incubated under aerobic and the other under microaerophilic conditions (Campypack, BBL microbiology system, Becton Dickenson and Company, MD, U.S.A.). After seven days incubation at 20°C bacterial colonies were enumerated.

Bacterial Identification

Major colonies were transferred 5-6 times on NFAM before species identifications were performed by using the computerized identification service at the Alberta Environmental Centre (Coleman, 1981).

Root Bacteria Association

Three experiments were conducted to establish physical association of N_2-fixing bacteria with P. alpina and T. spicatum roots.

Experiment I: The roots of P. alpina and T. spicatum tussocks collected from Lookout Mountain were washed carefully and cut into small 1-2 mm pieces. These were then placed into a vial containing fixative (1% paraformaldehyde and 1.5% glutaraldehyde in 0.15 M cacodylate buffer, pH 7.4) under slight vacuum until they sank. The pieces were fixed overnight, washed in 0.12 M cacodylate buffer, pH 7.2 and dehydrated in a graded series of ethanol. While in 100% ethanol, the pieces were transferred to wire baskets making sure to keep them wet. The ethanol was replaced by amyl acetate and the specimens were critical point dried in a SEEVAC critical point drier, mounted on aluminum stubs with silver paint and coated with gold using an Edwards sputter coater. The coated specimens were examined and photographed with a Hitachi S 510 scanning electron microscope (SEM) at the Northern Forestry Centre, Edmonton, Alberta.

Experiment II: Viviparous bulbils (vegetative propagules produced on the spike) of P. alpina were planted into rhizosphere soil collected from the Lookout Mountain and greenhouse potting mix (2: LOAM, 1: SAND; 2: PEAT; 1:VERMICULITE). These plants were nurtured in growth cabinets set to a day/night cycle of 15/8°C. After five months, root tips of these plants were prepared and photographed with the SEM as described above.

Experiment III: Seeds of P. alpina plants originating from the Lookout Mountain site were surface sterilized (5% sodium hypochlorite for 2 min) and planted into a semisolid (0.25% Noble agar) NFAM contained in test tubes. These test tubes were then

placed in a growth cabinet set at 22/15°C day(16 h)/night cycle. After a week of seed germination one third of the test tubes were inoculated with a drop of fresh actively growing cultures (grown in the same liquid medium as used in the experimental plant growth medium) of Azotobacter chroococcum ATCC 9043 and a third with Azospirillum lipoferum ATCC 29707. The rest of the test tubes (not inoculated) were treated as controls. The plants were examined for N-deficiency symptoms for eight weeks. At the end of this period their roots were prepared and photographed using the SEM.

Soil Analysis

Soil samples collected from the field were air dried and ground to 2 mm or less. They were then analyzed for several chemical properties in duplicate using standard methods (McKeague, 1978). Organic matter was determined by oxidation (Walkley and Black, 1934).

Results and Discussion

Rhizosphere Effect

Soil samples taken immediately adjacent to the plant roots (rhizosphere) contained higher numbers of bacteria than soil from 3 cm distance (Table 1). The rhizosphere effect resulted in at least a ten fold increase in bacterial counts for both grass species tested. The proportion of N_2-fixing bacteria in the total populations were similar (69% and 68%) in rhizosphere and non-rhizosphere samples.

Bacterial Identification

During 1986, approximately 30 bacterial isolates obtained from the rhizosphere samples were tested for their ability to grow on NFAM after four successive transfers. Most of the microorganisms were identified as belonging to the genus Xanthobacter.

TABLE 1. Bacterial Counts on Rhizosphere and Non-Rhizosphere soils Collected from Lookout and Hindow Mountain in Alberta

	CFU/g[a] x10^6				
	R2A[b]		Eugon[c]	NFAM[d]	
Soil Sample	Aerobic	Micro	Micro	Aerobic	Micro
Poa alpina, Lookout Mountain					
Root and Rhizosphere 1986 (n=12)	3,800	ND[e]	830	1,200	ND
Root and Rhizosphere 1987 (n=8)	2,000	1,700	260	470	290
Root and Rhizosphere 1988 (n=8)	540	230	ND	18	16
Non-Rhizosphere soil 1987 (n=4)	110	750	ND	75	56
Non-Rhizosphere soil 1988 (n=8)	19	16	ND	12	12
Poa alpina, Hindow Mountain					
Root and Rhizosphere 1988 (n=8)	160	63	ND	46	49
Non-Rhizosphere soil 1988 (n=8)	16	9	ND	7	7
Trisetum spicatum, Lookout Mountain					
Root and Rhizosphere 1986 (n=12)	2,500	ND	660	920	ND
Root and Rhizosphere 1987 (n=8)	1,300	630	460	510	450
Non-Rhizosphere soil 1987 (n=4)	250	110	ND	87	77

[a] Colony Forming Units/g dried soil
[b] R2A Growth Medium-Total Heterotrophic Count incubated aerobically and microaerophilically
[c] Eugon Agar incubated microaerophilically
[d] Nitrogen Free Medium incubated aerobically and microaerophilically
[e] Not Determined

Identification of these isolates to species level, plus those identified as Azospirillum and Rhizobium species, was not possible according to the characteristics listed in Bergey's Manual of Systematic Bacteriology. Alpine conditions may have favoured other bacterial species than those typically found associated with lower elevation agricultural soils. Also, alpine soil bacterial populations may well have not yet been classified to species level. Bacterial isolates from P. alpina and T. spicatum rhizospheres were identified as belonging to five and four genera, respectively (Table 2). Among these, well known N_2-fixing bacteria such as

Xanthobacter, Rhizobium and Bacillus were found to be common to both plant species.

In 1987, about 120 isolates were tested for their ability to grow on NFAM, 39 of them continued to grow under microaerophilic conditions after six successive transfers. Most of the microorganisms were identified as Xanthobacter. Bacterial isolates from rhizospheres belonged to four genera (Table 2). The reason(s) for the difference in microbial populations between the two years is not clear. More N_2-fixing bacteria were isolated from, P. alpina than from T. spicatum rhizosphere (Table 3).

TABLE 2. Bacteria Isolated from Poa alpina and Trisetum spicatum Rhizospheres in 1986 and 1987 from Nitrogen Free Medium

Poa alpina	
1986	1987
Xanthobacter sp.	Xanthobacter
Xanthomonas sp.*	Xanthobacter flavus
Azospirillum sp.	Azotobacter beijerinckii
Rhizobium sp.	Azomonas macrocytogenes
Bacillus sp.*	Flavobacterium aquatile*

Trisetum spicatum	
1986	1987
Xanthobacter sp.	Xanthobacter autotrophicus
Rhizobium sp.	Flavobacterium multivorum*
Bacillus sp.*	Beijerinckia indica
Nocardia sp.*	Zoogloea ramigera*

* Not known to fix Nitrogen

TABLE 3. Abundance of Nitrogen Fixing Bacteria Isolated from Rhizosphere Samples of the Alpine Plant Species in 1987

Plant Species	Incubation Conditions	# of Isolates	# of N_2 Fixing Bacteria Identified	Genera of N_2 Fixing Bacteria
Poa alpina	Aerobic	16	6	Xanthobacter sp. (25%) Azomonas sp. (6%) Azotobacter sp. (4%)
	Micro*	22	3	Xanthobacter sp. (9%) Azotobacter sp. (4%)
Trisetum spicatum	Aerobic	34	0	-
	Micro	12	3	Xanthobacter sp. (17%) Beijerinckia sp. (8%)

* Microaerophilic

Root-Bacteria Association

Root samples of P. alpina and T. spicatum collected from alpine sites were found to contain bacteria, especially in the cavities and injured areas (Fig. 1a and 1b). Root tips collected from plants grown in the mountain soils carried bacteria, whereas, the control plants (grown in greenhouse potting soil) did not (Fig. 2a and 2b). The vegetative propagule grown in the greenhouse potting soil grew vigorously and showed nitrogen deficiency symptoms. In the mountain soil, on the other hand, the bulbils grew slowly without any N_2-deficiency symptoms (Fig. 3). Seedlings grown in NFAM exhibited N_2-deficiency within two weeks of their germination, whereas, seedlings inoculated with Azospirillum or Azotobacter species rarely showed N_2-deficiency symptoms. Colonization of the mucilaginous sheath surrounding the roots and root hairs in these cases occurred rapidly and was profuse. The spread of the colonies along the roots could be observed using a light microscope during the first week after inoculation. The inoculated roots were heavily infected with bacteria (Fig. 4 and 5). Under higher magnification these bacteria appeared to be attached to each other and to the root walls by capsular material (Fig. 4b and Fig. 5b). These experiments indicate there is an association between the N_2-fixing bacteria and grass roots and the grasses appear to benefit from such an association. A cold alpine environment, with slow organic matter accumulation and degradation, probably forces the two organisms to cohabit for mutual benefit.

Soil Analysis

Analysis of soil from Lookout Mountain and Window Mountain area

revealed low levels of N, P and K (Table 4). The soils were found to be low in salt and slightly alkaline. The alkalinity may explain, in part, the abundance of Gram negative bacteria such as Xanthobacter. The N and Organic Matter (OM) content of the Lookout Mountain soil was about half of that found in the soil collected from Window Mountain. This bias may be due to the fact that the soil from the latter was collected from areas of thick plant cover in order to get the upper range of N, P and K soil levels. Compared to prairie agricultural soil, however, the N content of Window Mountain soil was only 20%. The P and K levels from both alpine soils were also in the range considered extremely deficient for plant growth. Lack of apparent deficiency in the grasses growing in these alpine areas may be due to their inherent low nutrient requirement for growth and reproduction. This lower nitrogen requirement of these plants may be satisfied through their association with N_2-fixing microbial population present in the soil. Inoculation of alpine grass selections with appropriate N_2-fixing bacteria, therefore, may have a positive effect on their establishment and survival, particularly, in areas devoid of the necessary soil microorganisms.

TABLE 4. Mean and Standard Deviation (in parenthesis) of Various Physical and Chemical Parameters of the Alpine Soils*

Sample	pH	EC ($mScm^{-1}$)	Nitrogen(ppm) NO_3/NO_2	Nitrogen(ppm) NH_4	P (ppm)	K (ppm)	OM (%)
Lookout Mountain (July, 1987)	7.8 (0.1)	0.16 (0.01)	0.5 (0.07)	6.5 (0.78)	9.4 (0.85)	12.1 (0.57)	2.7 (0.14)
Lookout Mountain (Aug, 1987)	7.8 (0.14)	0.12 (0.00)	0.5 (0.07)	6.6 (1.06)	9.5 (0.64)	7.0 (0.00)	2.6 (0.14)
Lookout Mountain (Aug, 1988)	7.8 (0.07)	0.20 (0.00)	0.3 (0.07)	4.6 (0.07)	8.1 (1.06)	4.1 (0.28)	3.2 (0.07)
Phillips Pass (Aug, 1988)	8.0 (0.07)	0.22 (0.00)	5.2 (0.14)	10.8 (0.07)	9.6 (1.13)	16.0 (0.57)	5.7 (0.21)

* Parameters measured were pH, Conductivity(EC), Nitrogen, Phosphorous, Potassium and Organic Matter.

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Figure Legends

Fig. 1. Bacterial association of (a) Poa alpina and (b) Trisetum spicatum roots collected from Lookout Mountain.

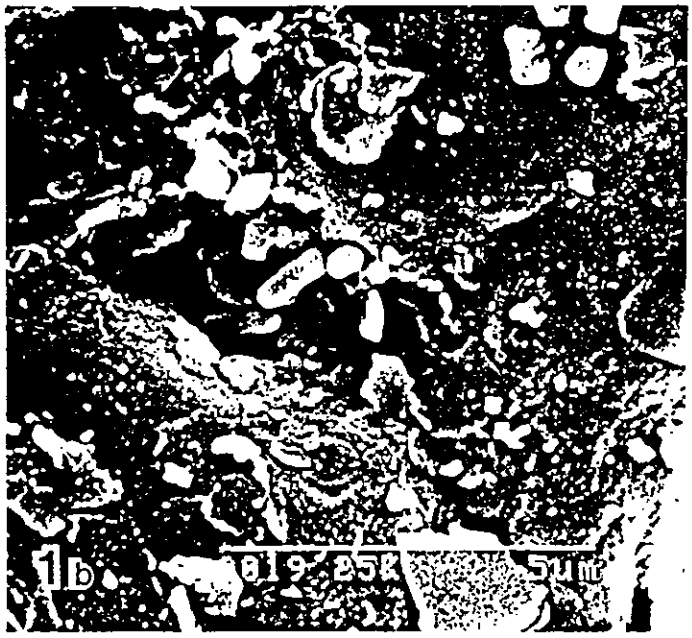
Fig. 2. (a) Bacterial association of Poa alpina roots when grown in Lookout Mountain soil, (b) Absence of bacteria in P. alpina roots grown in sterilized greenhouse soil mix.

Fig. 3. Presence and absence of nitrogen deficiency symptoms in plants growing in Lookout Mountain soil and greenhouse soil mix.

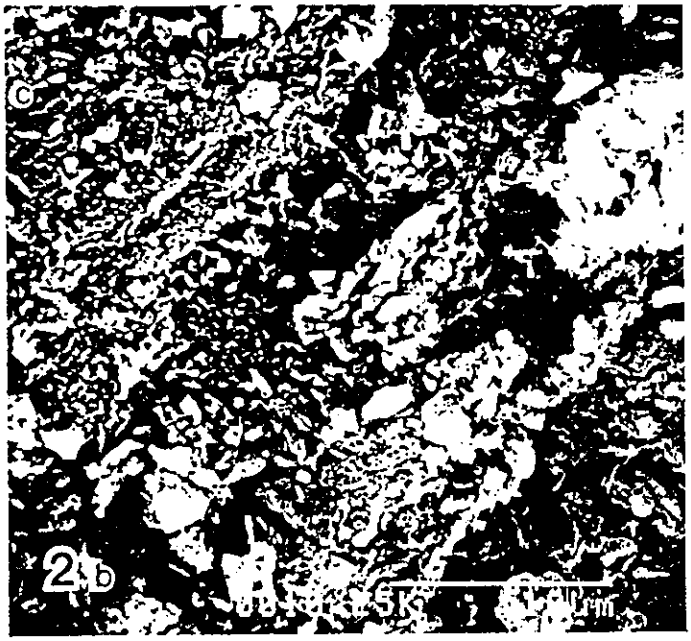
Fig. 4. Poa alpina roots infected with Azospirillum sp. (a) Lower magnification, (b) Higher magnification.

Fig. 5. Azospirillum sp. infected Poa alpina roots recovered from NFAM. (a) Lower magnification, (b) Higher magnification.


1a
5um


1b
5um


2a
0026 25K
5um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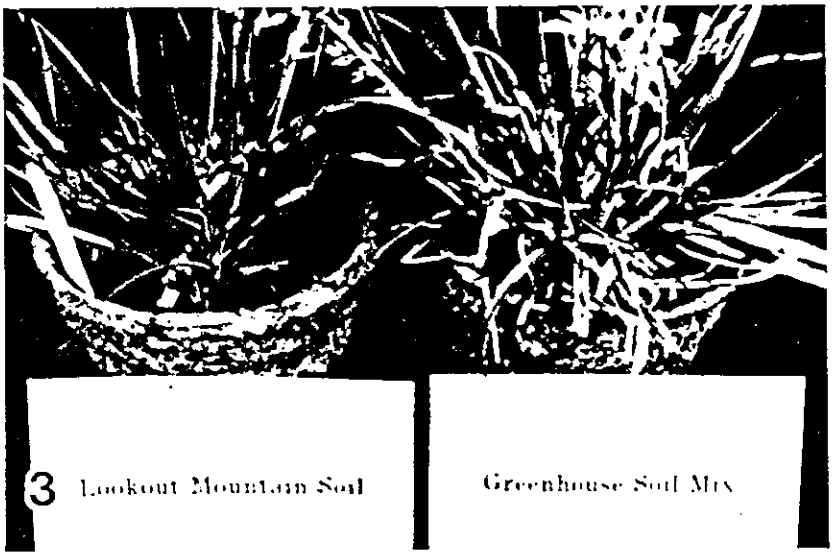
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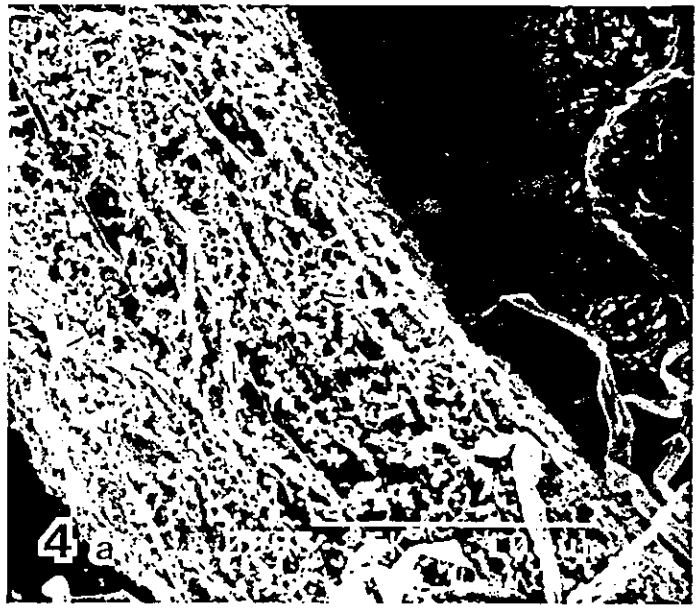
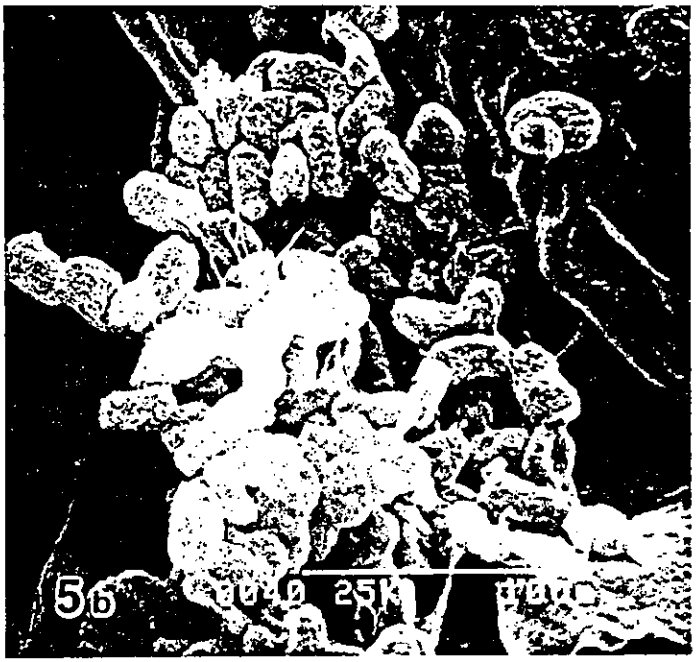
Lookout Mountain Soil
Greenhouse Soil Mix


4a


4b
25K
10µm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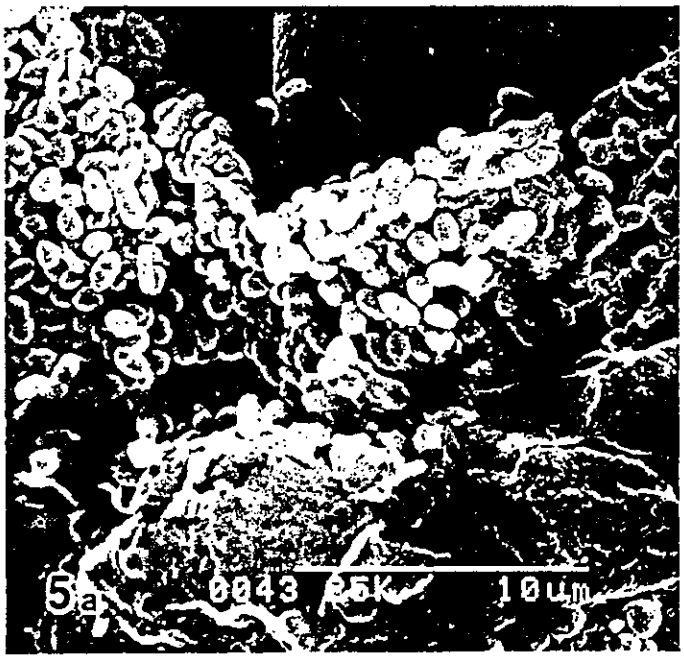
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