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竹林土壤內의 *Azotobacter* 生態

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Ecology of *Azotobacter* in Bamboo Forest Soil

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ABSTRACT

This experiment was designed to elucidate the environmental factors in rhizosphere of bamboo forest that affect the distribution and the population size of *Azotobacter*, and also to estimate the annual productivities of nitrogen fixed by *Azotobacter* species. The results of this experiment can be summarized as follows;

The rhizosphere of bamboo forest contained high free sugars as of 3—8 times more than non-rhizosphere (Bacon, 1968), and the contents of organic matter and amino acids of that are relatively higher than this. Because of high content of potassium, average of soil *p*H is near at 7.0.

As above-mentioned environmental factors, the population sizes of Actinomycetes, general fungi, general bacteria and *Azotobacters* are larger than those of non-rhizosphere and of other rhizosphere. The dominant group in the rhizosphere is Actinomycetes and the followings are general fungi and general bacteria by turns.

In the rhizosphere of bamboo forest, it is considered that the population size of *Azotobacter* is dependent upon the antagonistic Actinomycetes.

The main carbon source for *Azotobacter* in nitrogn fixation at the rhizosphere was glucose and minors were fructose, maltose and sucrose by turns.

Annual gains of nitrogen by *Azotobacters* in soil of bamboo forest within 10cm from surface are estimated as of 88.94 kg/ha at site A, 60.4 kg/ha at site B and 67.38 kg/ha at site C, respectively.

INTRODUCTION

Azotobacter belongs taxonomically to order Eubacteriales, family Azotobacteriaceae, genus *Azotobacter* and up to now 6 species were reported. The natural habitat of 6 species are soil or water (Norris, 1968) and their ecological role in nature is the fixation of dinitrogen (N_2) gas and the synthesis of nitrogen compounds. And also in the soil environment, they synthesize indole, therefore produce the growth hormone of plants (Vancura, 1961, Patel, 1969), and eventually are beneficial to the plants.

Azotobacters are asymbiotic nitrogen-fixing bacteria and their cell size is relatively large(5um). They have the highest respiration rate among the present living cells (Brock, 1970) and also are able to fix the largest amount of nitrogen as long as a suitable carbon

sources are present.

It is considered that the major factor affecting to the horizontal distribution of *Azotobacter* in nature are *p*H and the amount of carbon sources: they have large population, only when their habitat have neutral condition, above *p*H 6.0 (Anderson, 1958) and contain a lots of available sugar. On the other hand, the environmental factors affecting to the vertical distribution of *Azotobacters* are known to the rate of aeration in their habitat(Waksman, 1952, Pshenin 1970), that is, because *Azotobacters* are the obligate aerobic microbes, and then they live mostly on the surface of water in the aquatic environment and within 10cm deep from the surface within well-aerated soil.

Recently, Rovira(1956 a, b, c) and Strzelczyk(1961 a) reported that as the micro-environment, with the above mentioned environmental factors, rhizosphere is the most favorable habitat for the general soil microbes as well as for the *Azotobacter*, and the population size of the soil microbes were generally large.

The author has carried out the related experiments, since 1971, in order to isolate and identify *Azotobacter* from the soil and sea water in Korea (Hong & Choi 1974 a, b). In case of Korea, it was confirmed that rhizosphere has also large population size with respect to the distribution of *Azotobacter*. As the result of examination, it was pointed out that the rhizosphere of corn, legume, onion, oak, grass and bamboo forest have relatively large population size. Because author sustained that, of such rhizosphere that of bamboo forest has the largest population size, this experiment has been performed to find out what the environmental factor affecting to the distribution of *Azotobacter*, in the rhizosphere of bamboo forest is, and which annual productivity of nitrogen fixation under such condition can be expected by *Azotobacter*.

MATERIALS and METHODS

As the studied area of this experiment, the bamboo forest(*Phyllostachys reticulata* Koch), at the region of Ka-San ri, Dam-Yang Gun, Chun-La Nam Do, a well-known place of production of bamboo work, was plotted.

Each soil sample of rhizosphere, progressed 1 yr(site A), 5yr (site B), and 50yr (site C) after plotting of bamboo forest, was collected 4 times seasonally.

Soil samples(outer rhizosphere embracing the immediately adjacent soil) were collected from the surface and inside 10 cm deep with sterile scope, in the regions, at random in the above 20 places in each sites. They are packed with vinyl envelopes, mixed in it, and thereafter carried to the laboratory. The method of this experiment was divided for convenience' sake into the following 2 directions.

1. Examination of Environmental Factors in the Rhizosphere of Bamboo forest Soil

(1) Microflora in the rhizosphere of bamboo forest:

The population of soil microbes, that is, general bacteria, general fungi, Actinomycetes and *Azotobacter* in the soil(silty loam) of bamboo forest was counted seasonally. The method of isolation and colony count and the selective media were based upon the method of Hong *et*

al (1974 a, b). General bacteria were counted as the number of colony on the cultures of nutrient agar medium, general fungi as on the Czapek's agar medium (cultures of 7 days), Actinomycetes as on the Jensen's agar medium (cultures of 7 days) and *Azotobacter* on the colony of Brown's medium (1962).

(2) *p*H, temperature and moisture content of soil;

*p*H of soil was measured twice, meanly, in the sampling site with the soil *p*H meter, and in the laboratory, with electrode, after filtrating the suspension (soil; distilled water=1 : 1). Soil temperature was also measured with a mercury thermometer at each sampling site and the average of soil temperature in each season was depended upon the data provided from the Central Meteorological Observatory. Moisture content of soil was determined by the following procedure: Ten gram of soil sample was weighed, dried 5hrs in 110—130°C of drying oven, and each dried soil was allowed to stand overnight in desicator. And dry weight of each sample was determined and expressed by means of percentage.

(3) Chemical components of bamboo forest soil:

Soil samples were first sifted with the sieve, 2mm in diameter. Ten gram of the sieved soil was added to 50 m*l* of Morgan's extracting solution (Grewelling, 1960) and 2.5 gr of active carbon was added. The mixtures were shaked 30 minutes and filtered. The filtrate was treated and measured by the following method.

Mg: Magnesium was measured by Melich's method by using spectrophotometer(525mμ).

K: The content of potassium was measured by sodium cobalt nitrite method by spectrophotometer(505mμ).

NO_3: Nitrate was measured by Noll's method by spectrophotometer(470mμ).

PO_4: Phosphorous was measured by ammonium molybdate blue method by spectrophotometer (660mμ).

Fe: The content of iron in soil was measured by o-phenanthroline method by spectrophotometer (525mμ).

SiO_2: The soil extract was treated with 20% ammonium molybdate and measured by spectrophotometer at 350mμ.

Ca: The soil extract was treated with ammonium citrate solution and soap solution, and compared with the control series with respect to turbidity.

Total organic matter: Ten gram of soil sample was added to 25 m*l* of 1 N potassium dichromate and 50 m*l* of conc. H_2SO_4 was added to digest in wet condition. The digested solution was diluted with distilled water and allowed to stand overnight, the supernatant was measured by spectrophotometer at 625mμ.

Soluble sugar: Ten gram of soil sample was taken. Free sugar was extracted with 80% ethanol from it and then its total amount was measured by means of Anthron test at 625mμ.

Kind of sugars: Sugars were extracted with 80% ethanol from 10 gr of soil sample, passed through cation resin, and then concentrated to 3—5m*l* at 90°C. In the solvent system of 3 parts of isopropyl alcohol to 2 parts of water the spot was expanded.

As the result of development with aniline diphenylamine, kinds of sugars were determined(running temperature =room temperature, paper=Wattman

No. 1)

Composition of amino acids:

Contents and composition of amino acid were determined by means of ion-exchange column chromatography. Thirty m*l* of 6N HCl was added to 10 gr of soil sample, hydrolyzed in vacua at 110°C, for 24 hrs, and also in vacua dried. After that the remaining solid part was dissolved in 10 m*l* of 0.2M sodium citrate buffer(*p*H 2.2) and each fraction of amino acids was analyzed in accordance with elusion time by Hitachi liquid chromatograph Model 034.

2. Physiological experiments of *Azotobacter*

(1) Culture of Isolates:

The isolated species from the soil of bamboo forest, *Azotobacter insignis*, *Azotobacter beijerinckii* and *Azotobacter chroococcum*, and the control species, *Azotobacter vinelandii*, IAM 1195 were all inoculated in the media, which were, respectively, added with glucose, sucrose, fructose, and maltose to the inorganic part of Norris medium(1968) and which were adjusted to 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, in initial *p*H.

The changes of *p*H and population were observed, which the microbes were incubated 4 weeks at 25°C.

The basal composition of nitrogen-free medium is as follow:

K_2HPO_4	1g
$MgSO_4 \cdot 7H_2O$	0.2
$CaCO_3$	1.0
NaCl	0.2
$Na_2MoO_4 \cdot 2H_2O$	0.005
Sugar(glucose, fructose, sucrose, or maltose)	10
Distilled water	1000m*l*

Norris medium of the above-mentioned composition was treated with 1N HCl, to adjust to *p*H 4.0, with 1N KH_2PO_4 to adjust to *p*H 5.0 and 6.0 and 1N K_2HPO_4 to adjust to *p*H 7.0, 8.0 and 9.0. And then the population changes were measured weekly by heamacytometer and the changes of *p*H by *p*H meter. To compare with the culture phase in Norris medium, the isolates and control species were inoculated in the sterilized soil of bamboo forest and the changes of population and *p*H were measured weekly.

(2) Nitrogen Fixation in accordance with different carbon sources:

Glucose, fructose, sucrose, and maltose were added to the inorganic parts of Norris medium, respectively and also various conditions of initial *p*H were adjusted.

The isolates and control species were inoculated in each conditioned medium and incubated 4 weeks at 25°C. One gr of mixed powder of K_2SO_4, and $CuSO_4$, and 10 m*l* of conc. H_2SO_4 were added to 3m*l* of 4 weeks cultures of each medium. After digestion, the amount of nitrogen fixation was determined twice with the micro-Kjeldahl apparatus.

(3) Nitrogen fixation in soil incubation:

Fifty gr of bamboo forest soil was put into petri-dish and sterilized. 15.13×10^4 cells of isolates were inoculated and incubated 4 weeks, respectively at 4°C, 15°C, 20°C, and 25°C. And thereafter, the amount of nitrogen fixation were measured by micro-Kjeldahl method, based upon this data, seasonal amount and annual amount of nitrogen fixation, expectable by the ability of

the *Azotobacter* in the bamboo forest soil were calculated.

(4) Colonization in soil:

To elucidate the some effects on the colonization of isolated *Azotobacter* in soil, the relationship between 10 kinds of antibiotics and 9 kinds of amino acids were examined respectively by replica plating technique (Stotzky, 1965), as shown in figure 32 and 33.

Thirty gr of bamboo forest soil and 6 m*l* of Norris medium were poured into petri-dish, sterilized and each soil was treated with 0.2m*l* of antibiotics solution, 4mg/10m*l* in concentration (Pinck *et al*, 1960).

In case of amino acids, of the 15 amino acids measured as the result of examination of environmental factors, 9 quantitatively dominants were conducted, and 0.2 m*l* of solution, at the concentration of the soil level, was added. After that, the isolates and control were inoculated at 25°C. The colonization were observed by the replica plate in a week. Antibiotics and amino acids were treated in soil as Fig. 32 and 33.

Ⅳ. RESULTS

1. Analysis of Environmental Factors

(1) Microflora in the Rhizosphere of bamboo forest Soil.

Microflora and population of soil microbes in rhizosphere of bamboo forest soil are as shown in Table 1. According to the ages of bamboo forest, there are some differences in the population size. In the case of site A, *Azotobacter* had the large population in the summer and its population was tend to be reduced in order of autumn, spring, and winter. Either site B or C were also similar to the result of the former. The population size of general bacteria was measured that it was larger than that of *Azotobacter*, but smaller than that of Actinomycetes, and the seasonal variation of general bacteria was similar to that of *Azotobacter*.

The population size of general fungi was similar to that of general bacteria, smaller than that of Actinomycetes. It was pointed out that in the rhizosphere of

Table 1. Microflora in the Rhizosphere of Bamboo forest Soil.

Site & Microbes \ Season		Spring	Summer	Autumn	Winter
Site A	Azotobacter(10^4)	6.73	8.6	8.1	3.6
	Gen. Bacteria(10^5)	29.1	86.2	80.3	36.0
	Gen. Fungi(10^5)	87.3	132.0	122.0	34.0
	Actinomycetes(10^5)	120.2	304.0	422.0	123.0
Site B	Azotobacter(10^4)	3.0	6.0	5.9	4.1
	Gen. bacteria(10^5)	45.5	67.1	65.6	45.0
	Gen. fungi(10^5)	29.1	91.6	60.0	26.0
	Actinomycetes(10^5)	48.1	341.0	438.0	46.0
Site C	Azotobacter(10^4)	4.1	6.8	5.9	7.2
	Gen. bacteria(10^5)	32.9	112.2	101.4	94.0
	Gen. fungi(10^5)	30.7	120.1	109.0	26.0
	Actinomycetes(10^5)	94.0	338.0	643.0	32.0

Microbial population was measured in the soil of bamboo forest soil within 10cm from surface. The unit is equivalent to multplication of 10^4 or 10^5 per dried soil gram.

Table 2. Temperature, Moisture contents and *p*H of Bamboo forest soil.

Site	Season	Spring (April)	Summer (July)	Autumn (Oct.)	Winter (Jan.)	*p*H
Site A	Soil Temperature(°C)	16.5	26.5	22.4	4.2	
	Moisture Contents(%)	19.95	22.5	20.1	17.06	6.73
Site B	Soil Temperature(°C)	12.5	25.4	22.2	4.1	
	Moisture Contents(%)	18.0	24.4	22.7	17.18	6.70
Site C	Soil Temperature(°C)	12.0	24.8	22.0	4.0	
	Moisture Contents(%)	27.39	34.5	22.9	30.2	7.0

the bamboo forest, Actinomycetes have the largest population size. They showed common phenomena in all sites, with respect to the seasonal variation that the population size was the largest in autumn and the smallest in winter.

(2) Temperature, Moisture contents, and *p*H of soil:

Table 2 shows the changes of seasonal soil temperature, moisture contents, and *p*H in the bamboo forest. The soil temperature measured in spring was ranged of 12—16.5°C, in summer the range of 24.8—26.5°C, in autumn the range of 22.0—22.4°C, and in winter the range of 4.0—4.2°C. Moisture contents of the surface soil, inside of 10cm, were maximum, 22.5—34.5% in summer, 20.1—22.9% in autumn, 18.0—27.39% in spring, and 17.06—30.2% in winter. It is the special case that site C (50 yrs old soil) was 30.2% moisture content in the winter. The soil *p*H of the 3 sites were 6.73 in site A, 6.70 in site B, and 7.0, neutral in site C.

(3) Chemical compnents of bamboo forest soil:

The data of chemical analysis for the bamboo forest soil are described in Table 3. Available silicate(SiO_2) in the surface

Table 3. Chemical Components of Bamboo Forest Soil

Components / Sites	Total Organic Matter (%)	SiO_2 (ppm)	Ca (ppm)	Mg (ppm)	K (ppm)	NO_3 (ppm)	PO_4 (ppm)	Fe (ppm)	Soluble sugar (mg/g.)
Site A(U)	18.7	41.45	33.2	36.5	91.3	9.13	7.05	0.82	2.94
(L)	11.6	84.1	67.2	26.0	trace	1.428	2.1	0.81	
Site B(U)	17.4	62.1	66.4	34.9	161.85	1.66	5.81	0.65	4.95
(L)	8.13	32.01	34.0	37.4	73.1	2.12	8.07	1.00	
Site C(U)	16.18	48.54	84.0	29.1	161.0	6.3	6.3	1.40	8.55
(L)	14.2	30.45	26.0	35.4	107.1	10.74	9.07	0.86	

soil was the range of 41.45—62.1 ppm, lower than 78 ppm paddy soil in Korea (Kim, 1971).

Its content below 10 cm from the soil surface was the range of 30.45—84.1 ppm. The content of calcium ranged 33.2—84 ppm in surface soil, lower value than the analytical data, 125—320 ppm, obtained from A horizon of Cheju-Do by Park *et al.*(1971) and also the range was 26.0—67.2 ppm in the bellow 10cm.

The content of magnesium in the rhizosphere soil was the range of 29.1—36.5 ppm, slightly lower value than the analytical data, 40—105 ppm, by Park *et al*(1971), and the range of 26.0—35.4 ppm in the lower layer (below 10cm). The content of potassium was the range of 91.3—161.85 ppm and it is above 5 times higher than 16—24 ppm of general paddy soil in Korea, 20—38 ppm obtained from A horizon of Cheju-Do (Park *et al* 1971). The content of nitrate was 1.66—9.13ppm, low compared with 0.45% reported by Hong *et al* (1969), and 1.42—10.74 ppm in the lower layer. And the content of available PO_4 and Fe were confirmed to the low level.

On the other hand, the content of total organic matter was 16.18—18.7% in surface soil, 6—7 times more than 2.47% reported from the white pine forest by Chung *et al*(1971), and in the low layer organic matter was also contained, 8.13—14.2%. The content of soluble sugar were 2.94 mg/g soil in site A, 4.95 mg/g soil in site B, and 8.55 mg/g soil in site C, and its content were 3—8 times as much as the 1.03 mg/g soil of soluble sugar measured from loam soil by Bacon (1968).

To determine the kind of soluble sugar, soil sample was extracted with 80% ethanol, and as the result, 4 kinds of sugars-glucose (Rf. 0.391), fructose (Rf. 0.609), sucrose (Rf. 0.477), and maltose (Rf. 0.32)—were discerned and the size of spot of glucose was the maximum.

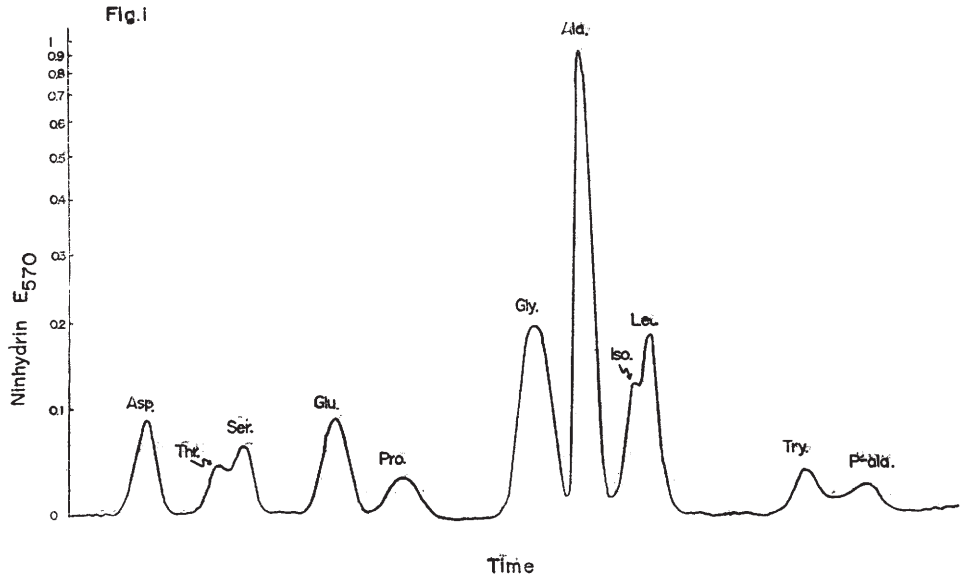


Fig. 1—2. The figures represent the chromatographic seperation of amino acids of bamboo forest soil at site A. Ninhydrin color intensity was plotted against the time of elution. Thirteen kinds of amino acids were discerned in the hydrolysates of the soil.

Fig. 3—4. The figures also represent the chromatographic seperation of amino acids of the soil at site C. Ten kinds of amino acids were discerned.

Fig.3

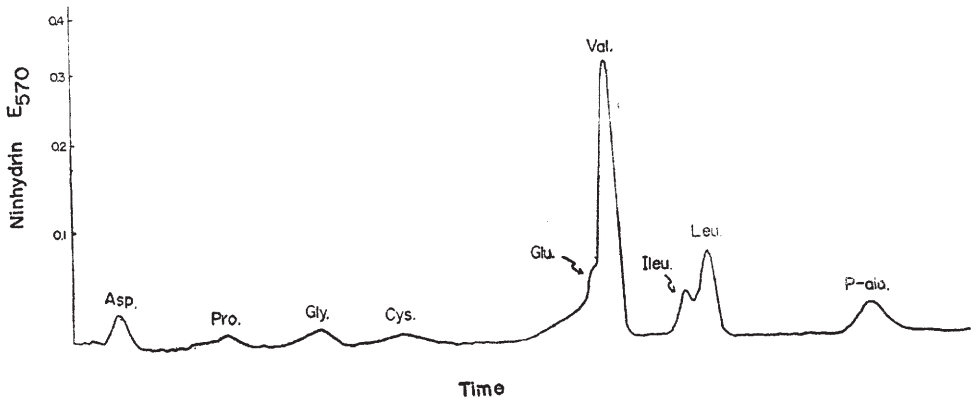


Fig.2

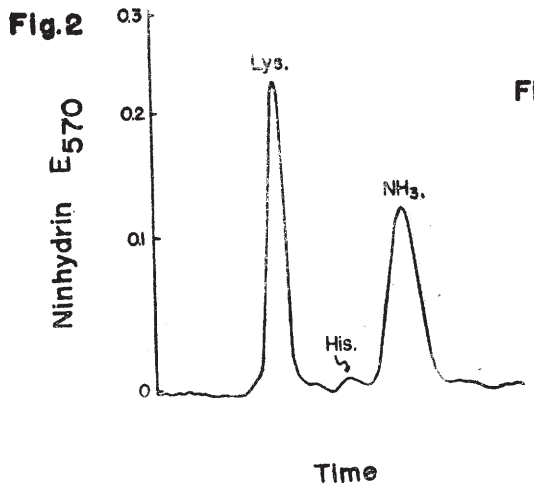


Fig.4

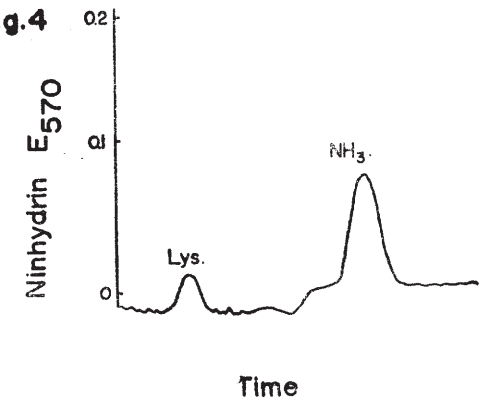


Fig. 1 and Fig. 2 are the chromatographic separation of amino acids shown in the analysis of soil of site A, and 3 and 4 are the analytical chart of amino acids in site C. Table 4 is the contents of amino acids of the sites A and C, expressed as micromole. Site A contained total 13 kinds of amino acids and site C contained 10 kinds of amino acids.

Table 4. Amino Acids in Bamboo Forest Soil

(Site) (Amino acids)	site A	site C	(Micro-mole)/10g soil
Aspartic acid	0.097	0.021	
Threonine	0.043	—	
Serine	0.055	—	
Glutamic acid	0.087	0.026	
Proline	0.053	0.035	
Glycine	0.278	0.021	
Alanine	0.506	—	
iso-Leucine	0.071	0.020	
Leucine	0.092	0.036	
Tyrosine	0.045	—	
Phenylalanine	0.056	0.033	
Lysine	0.070	0.009	
Histidine	0.003	—	
Valine	—	0.119	
Cysteine	—	0.051	

Chromatographic seperation of the amino acids of hydrolysates of bamboo forest soil.

Stevenson(1954) and Sowden(1955) analyzed total 19 kinds of amino acids in soil, and Schmidt(1959) reported that of them, the quantitatively dominant amino acids were aspartic acid, glutamic acid, valine, leucine, and so on.

2. Physiological test of *Azotobacter*

(1) Cultural experiment

As the result of 4 weeks-culture of 4 species in the media with various sugars (glucose, fructose, sucrose, maltose), total 32 tables(16 tables of the changes of *p*H and 16 tables of the population) could be obtained. But this paper dealt with the results as Figures based on the record of all tables, because the tables are too numerous to describe. Fig. 5—8 represent the changes of initial *p*H measured in cultures of 4 species of *Azotobacter*, when glucose as carbon source is added to Norris medium.

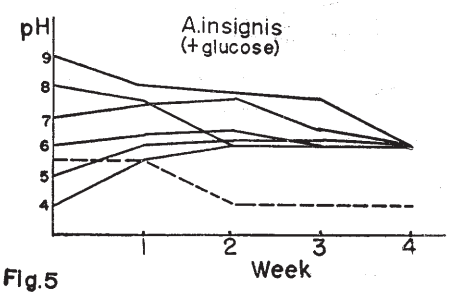


Fig.5

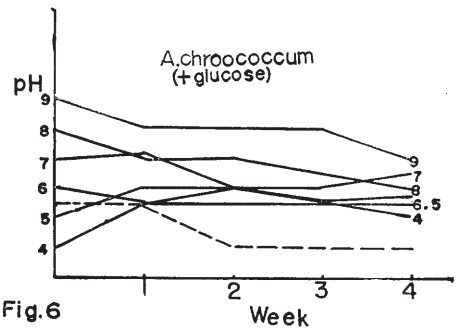


Fig.6

Fig. 5. *p*H changes in cultures of *Azotobacter insignis* were measured week intervals for 4 weeks. The medium was added with glucose as carbon source to inorganic part of Norris medium. Dotted line means the incubation (at 25°C) of soil. The medium treated with glucose were adjusted initial *p*H 4.0—9.0.

Fig. 6. *p*H changes in culture of *Azotobacter chroococcum* were measured at same condition as in Fig. 5.

Fig. 7. *p*H changes in culture of *Azotobacter beijerinckii* were measured week intervals at same conditions as in Fig. 5 and 6.

Fig. 8. *p*H changes in cultures of control species, *Azotobacter vinelandii* IAM 1195 were measured to compare with those of 3 isolates.

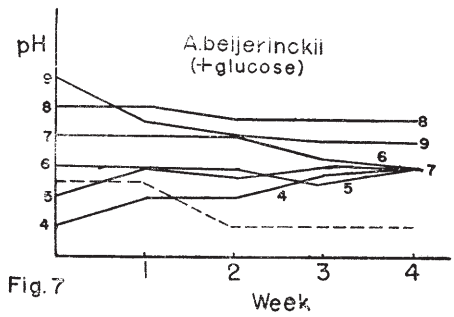


Fig. 7

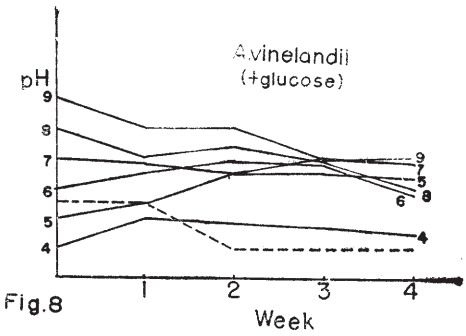


Fig. 8

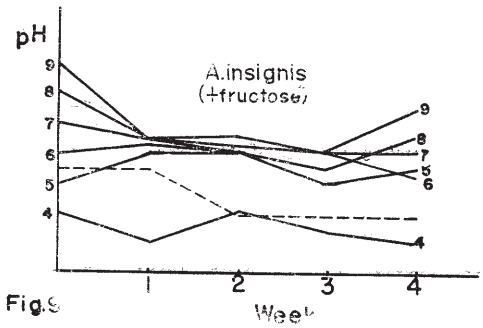


Fig. 9

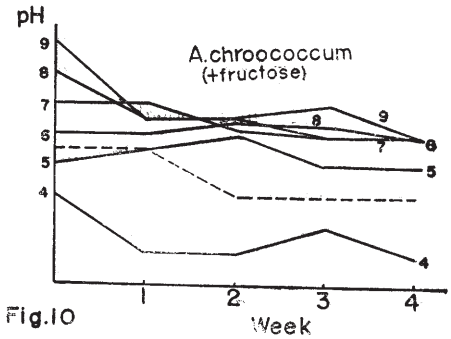


Fig. 10

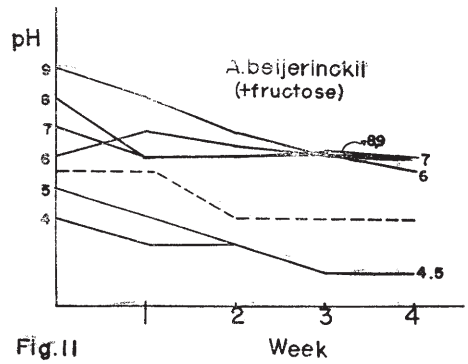


Fig. 11

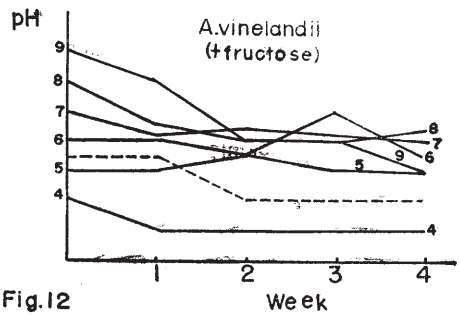


Fig. 12

Fig. 9. The figure represent the *p*H changes in cultures of *Azotobacter insignis*. The medium was added with fructose as carbon source. Initial *p*H and incubation temperature were conditioned as in case of glucose medium. Dotted line means the results of soil incubation.

Fig. 10. *p*H changes in cultures of *Azotobacter chroococcum* were measured at same conditions as in Fig. 9.

Fig. 11. *p*H changes in cultures of *Azotobacter beijerinckii* were measured. Conditions of cultures were same as in Fig. 9 and 10.

Fig. 12. *p*H changes in cultures of control species, *Azotobacter vinelandii* IAM 1195 were measured to compare with those of 3 isolates (Fig. 9, 10, and 11).

Initial *p*H were generally decreased during incubation and final *p*H were tend to approach 6.0—6.5 in cultures, in which initial *p*H were 9.0, 8.0 and 7.0. However, in the culture, in which initial *p*H was 6.0 and below it, tendency were not constant, and in soil cultures initial *p*H was maintained up to a week after inoculation, and thereafter final *p*H is decreased to 4.0. Fig. 9—12 represent the change of *p*H measured in the culture, in which fructose as carbon source is added to Norris medium.

General tendency, that is, the cultures of initial *p*H 9.0, 8.0, 7.0, and 6.0 all approach to each other, with respect to final *p*H, were similar to the case of medium, to which glucose were added. And the changes of *p*H in the media added with sucrose, or maltose had also similarity to the case of media treated with glucose or fructose.

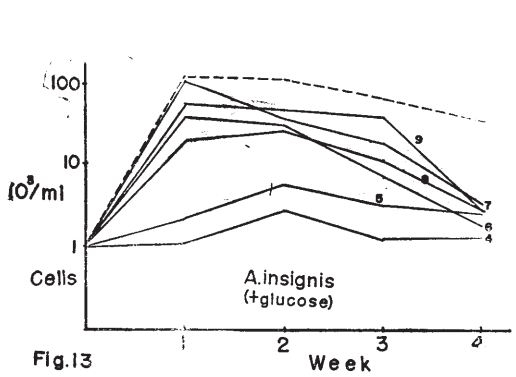


Fig.13

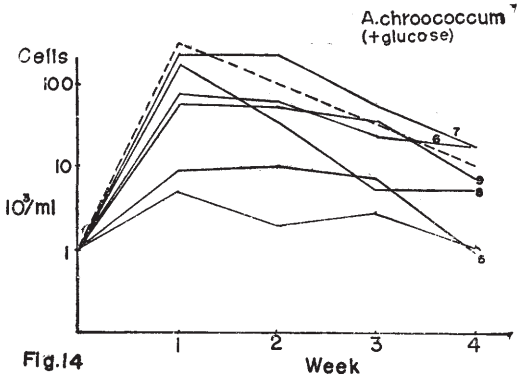


Fig.14

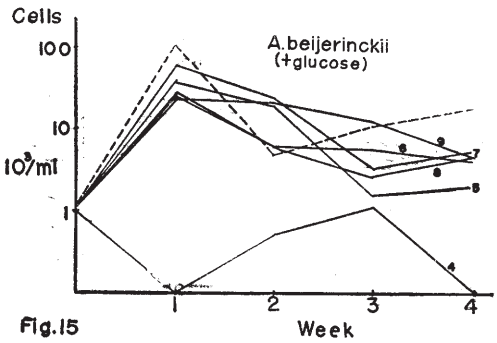


Fig.15

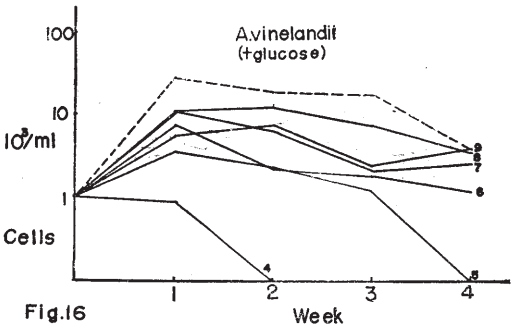


Fig.16

Fig. 13. The figure shows the population changes of *Azotobacter insignis* in culture medium treated with glucose as carbon source to the inorganic part of Norris medium. The media were adjusted initial *p*H 4.0—9.0, inoculated with the isolate as about 10^3 cells/m*l* and incubated 4 weeks at 25°C. Dotted line means the result of soil incubation.

Fig. 14—15. The figures represent the population changes of *Azotobacter chroococcum* and *Azotobacter beijerinckii* in glucose medium, respectively. Conditions of cultures were same as in Fig. 13.

Fig. 16. Population changes of control species, *Azotobacter vinelandii* IAM 1195, were counted to compare with those of 3 isolates.

Fig. 13—16 represent the changes of population in Norris medium, to which glucose as carbon source was added. The maximum population size in the cultures of 4 species rose immediately in a week after inoculation and after that, populat-

ion size were decreased according to the order of initial *p*H. And the culture, in which glucose was added to soil extract, was tend to have larger population than that of Norris medium.

Fig. 17—20 represent the changes of population size, when fructose as carbon source was added to Norris medium. Compared with the medium treated with glucose, the population size in the culture of high initial *p*H had such tendency that its size was about 1/10, and in the control, through all fructose media, population sizes were tend to be medium degree.

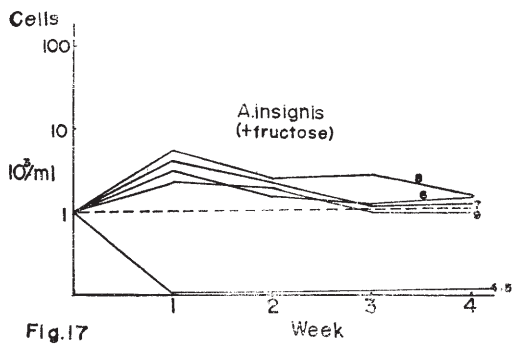


Fig.17

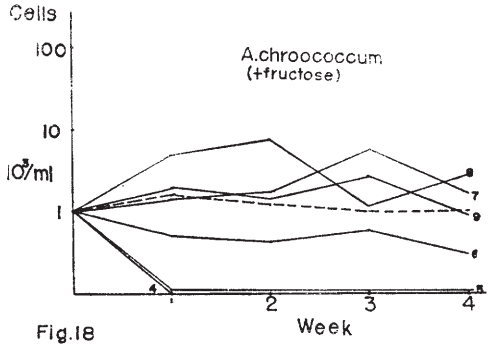


Fig.18

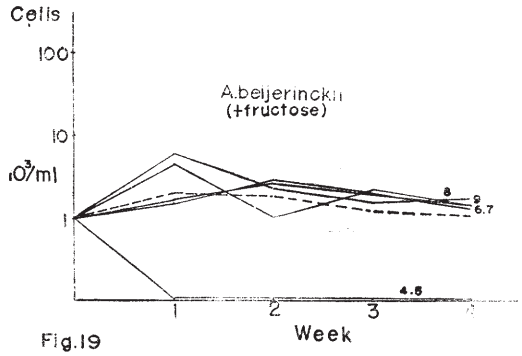


Fig.19

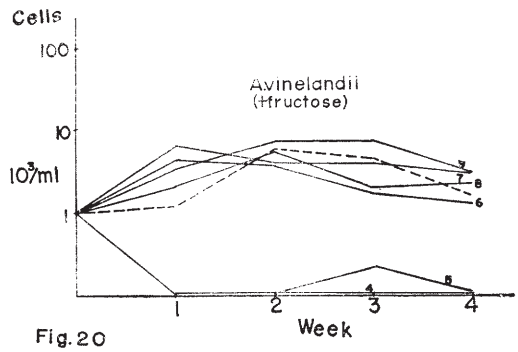


Fig.20

Fig. 17. The figure shows the population changes of *Azotobacter insignis* in culture medium treated with fructose as carbon source to the inorganic part of Norris medium. The conditions of cultures and incubation time are same as in case of glucose medium.

Fig. 18—19. The figures represent the population changes of *Azotobacter chroococcum* and *Azotobacter beijerinckii* in fructose medium, respectively.

Fig. 20. Population changes of control species, *Azotobacter vinelandii* IAM 1195, were counted to compare with those of 3 isolates.

Fig. 21—24 represent the changes of population, when sucrose as carbon source was added to Norris medium. Population has high peak in 2—3 weeks after inoculation, population size were smaller than that of medium added with fructose, and control had irregular phases.

Fig. 21—24. The figure 21 shows the population changes of *Azotobacter insignis* in culture medium treated with sucrose as carbon source. And the figure 22 and 23 represent the results of *Azotobacter chroococcum* and *Azotobacter beijerinckii*, respectively. To compare with those of control species, *Azotobacter vinelandii* IAM 1195 were incubated at same condition as in glucose medium.

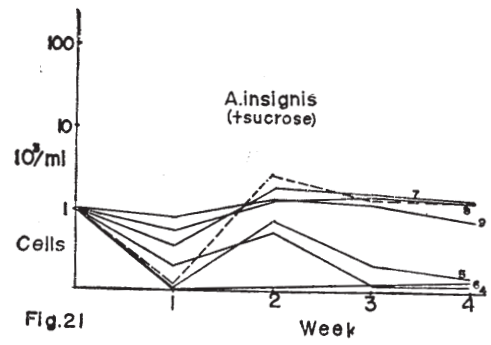


Fig.21

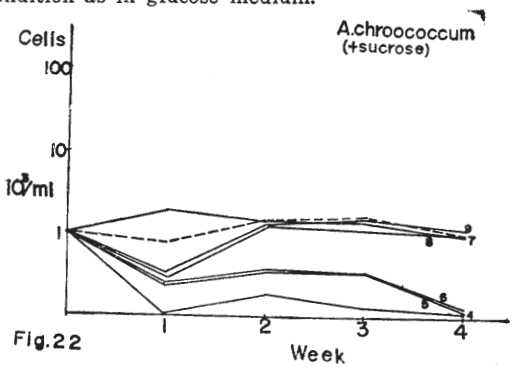


Fig.22

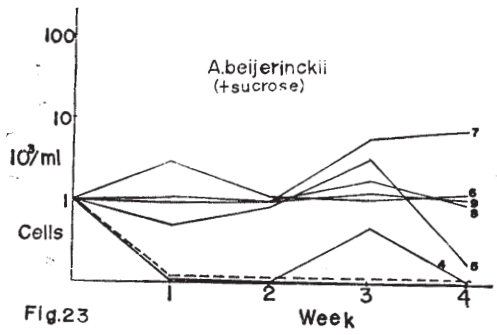


Fig.23

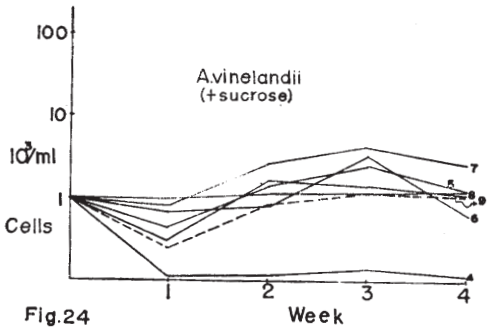


Fig.24

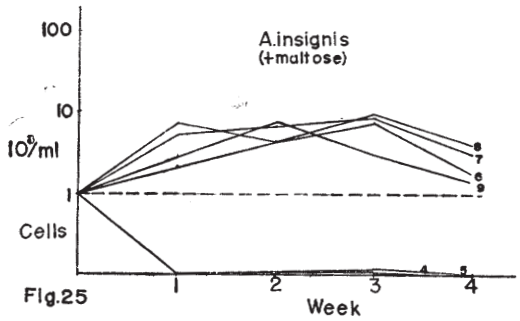


Fig.25

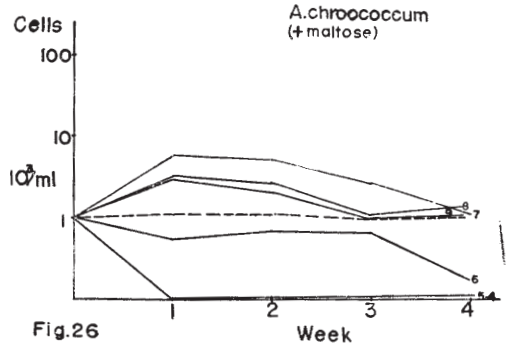


Fig.26

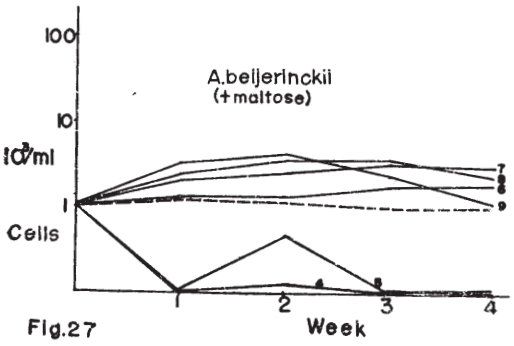


Fig.27

Fig. 25—27. The figure 25 shows the population changes of *Azotobacter insignis* in culture medium treated with maltose as carbon source. And the figure 26 and 27 represent the results of *Azotobacter chroococcum* and *Azotobacter beijerinckii*, respectively. The condition of culture were same as in glucose medium.

Fig. 25—27 represent the changes of population, when the isolates and control species are incubated in the Norris medium added with maltose as carbon source.

Population size was similar to that of the fructose, and larger than that of the sucrose. However, it was a particular case that population size in control wsa constant through 4 weeks.

(2) Nitrogen fixation in accordance with different carbon sources: After 4 weeks incubation(Myers *et al*, 1970) in 4 kinds of Norris medium with various carbon sources as examined above, the amounts of nitrogen fixation were measured. The results are described in Table 5—8.

And by the incubation in different condition of initial *p*H the amounts of nitrogen fixation were measured according to *p*H conditions.

Based upon Table 5—8, histograms were drawn up as shown in Fig. 28—31. The amounts of nitrogen fixation measured at the 4th week after inoculation of 10^6 cells of *A. insignis* to 500m*l* of liquid medium represent in Fig. 28.

Table 5. Nitrogen Fixation in Lab. Cultures(Glucose as carbon sources)

Species	*A. beijerinckii*	*A. insignis*	*A. chroococcum*	*A. vinelandii*
Initial *p*H				
4.0	0.76	0.24	0.01	0.43
5.0	1.46	2.45	0.42	0.68
6.0	2.31	12.22	15.27	10.25
7.0	6.36	16.69	58.43	42.24
8.0	7.50	5.08	8.77	9.47
9.0	4.85	6.12	4.11	17.8
Soil.	0.10	1.22	0.21	0.81

Unit: N mg/10^6 cells

Table 6. Nitrogen Fixation in Lab. Cultures(Fructose as carbon source)

Species	*A. beijerinckii*	*A. insignis*	*A. chroococcum*	*A. vinelandii*
Initial *p*H				
4.0	0.19	0.14	0.86	0.29
5.0	0.15	0.12	0.99	0.17
6.0	1.75	1.57	10.65	1.98
7.0	7.44	1.68	12.31	2.19
8.0	3.06	1.88	6.66	1.40
9.0	4.60	0.74	7.99	1.31
Soil	0.10	1.22	0.21	0.81

Unit: N mg/10^6 cell

Ref ering to Fig. 28, glucose is believed the most effective carbon source for nitrogen fixation. Fructose and maltose had similar effects each other, and sucrose was the least. On the other side, in the initial *p*H condition, the amounts

Table 7. Nitrogen Fixation in Lab. Cultures(Sucrose as carbon source)

Species Initial *p*H	*A. beijerinckii*	*A. insignis*	*A. chroococcum*	*A. vinelandii*
4.0	0.04	0.03	0.04	0.01
5.0	0.09	0.01	0.01	0.02
6.0	0.13	0.11	0.22	0.27
7.0	0.14	0.19	0.22	0.27
8.0	0.12	0.11	0.21	0.20
9.0	0.18	0.40	0.21	0.21
Soil	0.10	1.22	0.21	0.81

Unit: N mg/10^6 cell

Table 8. Nitrogen Fixation in Lab. Cultures(Maltose as carbon source)

Species Initial *p*H	*A. beijerinckii*	*A. insignis*	*A. chroococcum*	*A. vinelandii*
4.0	0.14	0.03	0.02	0.10
5.0	0.06	0.17	0.08	0.09
6.0	0.26	0.19	0.09	0.18
7.0	2.91	2.27	1.43	2.11
8.0	1.55	3.40	1.31	0.70
9.0	2.03	0.70	5.66	0.79
Soil	0.10	1.22	0.21	0.81

Unit: N mg/10^6 cells

of fixation were maximum at the range of *p*H 6.0—7.0 in initial conditions and above 7.0 not increased continuously.

Fig. 29 represents the amounts of fixation of the isolates, *A. beijerinckii*. The effects of carbon sources for the nitrogen fixation reduces in order of glucose, fructose, maltose, and sucrose, were also profitable and from 6.0 to 7.0 of *p*H range shown high peak. Fig. 31 represents the amount of nitrogen fixation of the control species, *A. vinelandii* IAM 1195, and then its level was similar to those of Fig. 28, 29, and 30. The amounts of nitrogen fixation in control cultures were measured 0.10698 mg in *A. beijerinckii*, 0.81581 mg in *A. vinelandii*, 1.22694 mg in *A. insignis* and 0.20912 mg in *A. chroococcum*, respectively, when 10^6 cells are inoculated.

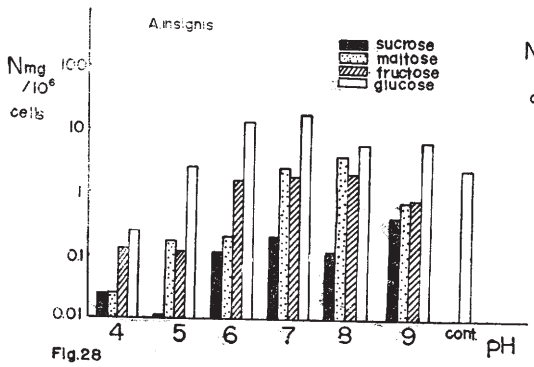


Fig.28

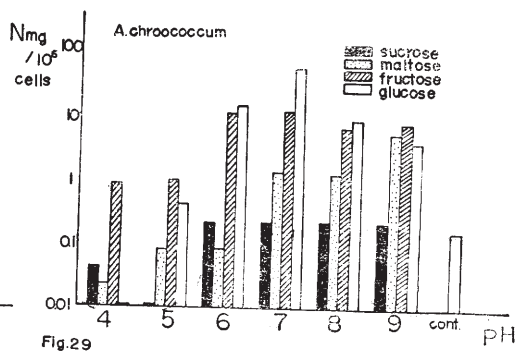


Fig.29

Fig. 28—31. The histogram represent the amount of fixed nitrogen by *Azotobacter insignis* in accordance with 4 kinds of carbon sources (glucose, fructose, sucrose, and maltose).

The media were prepared with 4 kinds of sugars, adjusted initial *p*H 4.0—9.0 and incubated with *Azotobacter* cells of 10^6/500m*l* for 4 weeks at 25°C.

After incubation the amount of fixed nitrogen were measured by micro-Kjeldahl apparatus.

The figure 29 and 30 mean the results of *Azotobacter chroococcum* and *Azotobacter beijerinckii*, respectively.

The figure 31 means the gains of nitrogen by control species, *Azotobacter vinelandii* IAM 1195.

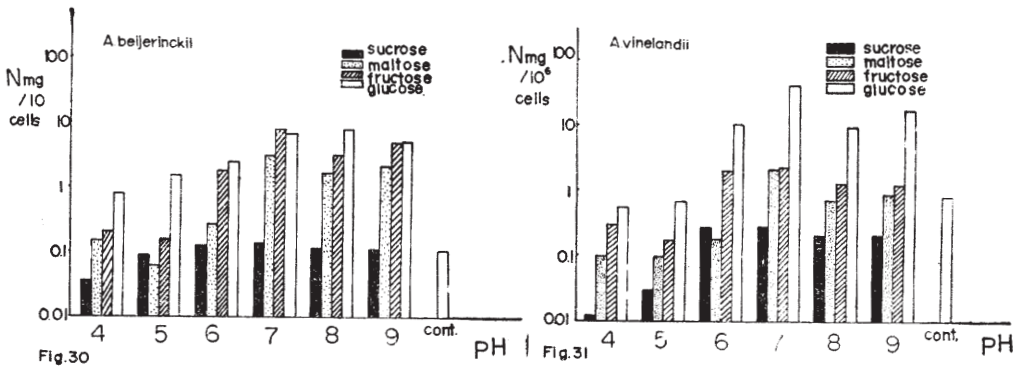
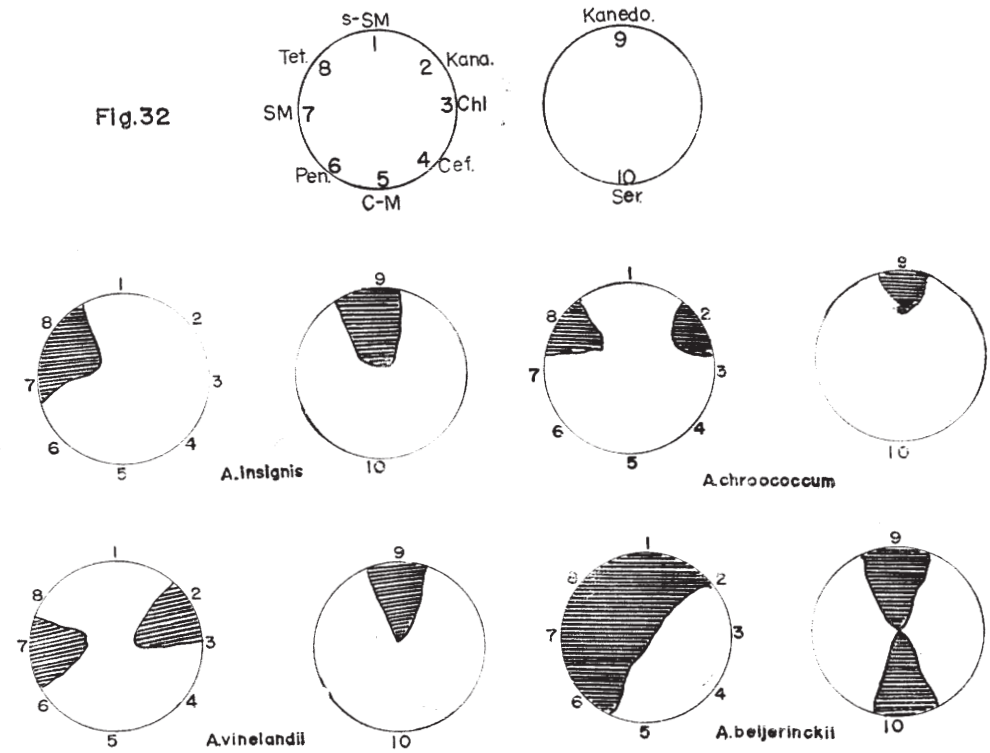


Fig. 32. Bamboo forest soil were treated with 10 kinds of antibiotics and inoculated 4 species of *Azotobacters* to elucidate the antagonistic reaction to soil microbes. The pictures in figure 32 represent the result of test obtained by soil replica plate technique.



Abbreviations: 1. S—SM(Streptomycin sulfate), 2. Kana(Kanamycin acid sulfate), 3. Chl(Cholramphenicol), 4. Cef(Cefamezin), 5. C—M(Colistin—M), 6. Pen(Penicilline), 7. SM(Streptomycin), 8. Tet(Tetracycline), 9. Kanedo(Kanedomycin), 10. Ser(Cycloserine).

(3) Nitrogen Fixation in Soil Incubation: Cells of 3 isolates were inoculated the number of 15.13×10^4 to 50g of bamboo forest soil and incubated 4 weeks at 4°C, 15°C, 20°C and 25°C, respectively. The amount of fixed nitrogen were 0.0014, 0.0021, and 0.0025 mg/4 weeks/10g-soil at 4°C, 0.026, 0.025, and 0.078 mg/4 weeks/10g-soil at 15°C, 0.074, 0.0896, and 0.09 mg/4 weeks/10g-soil at 20°C, and 0.154, 0.186, and 0.123mg/4 weeks/10g-soil at 25°C in order of *A. insignis*, *A. beijerinckii*, and *A. chroococcum*, respectively.

The gains were gradually increased with the increase of temperatures.

(4) Colonization in Soil: Thirty gram of bamboo forest soil were added with 6m*l* of Norris medium, the isolates were inoculated, and thereafter antagonistic

Fig.33

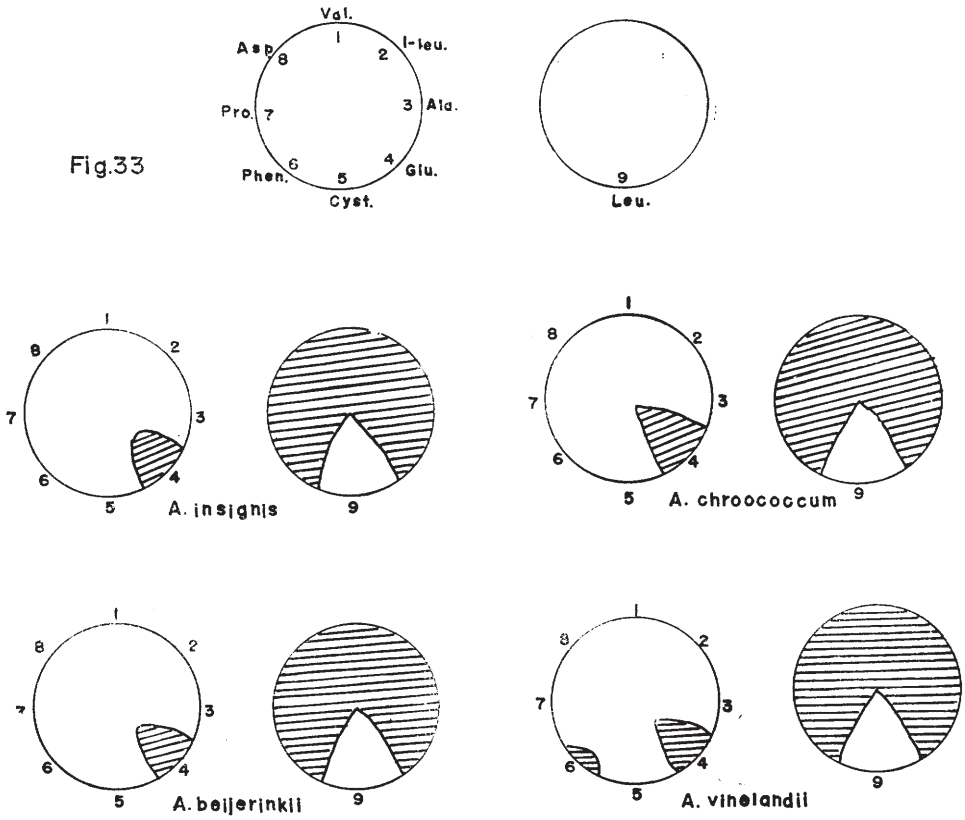


Fig. 33. Nine amino acids which are relatively high contents in bamboo forest soil were tested in soil replica methods to elucidate the effects of them on colonization of *Azotobacter* in soil.
Abbreviations:
1. Val(Valine), 2. I-leu(iso-leucine), 3. Ala(Alanine), 4. Glu(Glutamic acid), 5. Cyst(Cysteine), 6. Phen(Phenylalanine), 7. Pro(Proline), 8. Asp(Aspartic acid), 9. Leu(Leucine).

phenomena on 10 kinds of antibiotics have been observed by means of the soil replica plate technique. The results showed slight differences according to the species of *Azotobacter*.

It was found out that the colonization of *Azotobacter* was substantially inhibited by Kanamycin, streptomycin, Kanedomycin, and tetracycline originated from Streptomyces, Colistin—M from bacillus and Penicilline from penicillium. Fig. 33 represents the effects of 9 amino acids on the colonization. It was confirmed that glutamic acid do no affect the colonization of *Azotobacter* in soil.

DISCUSSION

1. Environmental Factors affecting to the Distribution of *Azotobacter* in Bamboo forest soil.

With regard to the biological environment, Acinomycetes were dominant group in bamboo forest soil and its population size was also the largest one(Rovira 1956b, Mishustin 1956, Kuster 1967). The population size of general bacteria and general fungi occupied in next order. Compared with the fact that population of general bacteria is generally larger than that of general fungi in the soil of Korea(Hong and Choi, 1974), such relationship had reciprocal values in bamboo forest soil. Since that probably the soil samples were collected only within 10 cm from the surface, such results might be obtained.

The population of *Azotobacter* represents the level of $3.0—8.6\times10^4$ cells/g-dried soil through the site A, B, and C. It was actually well known facts that population size of *Azotobacter* is high in the plants as the phylosphere of bamboo (Ruinen, 1970), rhizosphere of oak, maiz, tomato, and wheat(Rovira 1956abc), and onion and radish(Strzelczyk 1961).

It is believed that Actinomycetes may regulate the population size of *Azotobacter*. It was reported that streptomyces and Nocardia of Actinomycetes(Schreven 1963, Pinck 1960ab, 1962) excrete antibiotics and carry out antagonistic action to bacteria, to control the population size of them(Patel 1969). Furthermore, it was reported that fungi and sporogeneous bacteria were the main *Azotobacter* antagonists in some soil (Yugina, 1958). Therefore the population size of *Azotobacter* may be slightly large in the site A, where that of Actinomycetes are relatively small in size and it is indicated that the less sensitive general fungi to antibiotics might be larger than general bacteria in population size. Antagonistic reaction between *Azotobacter* and Actinomycetes were proved indirectly by the soil replica plate techniques in this experiment.

Except for the biological factors, neutral condition (*p*H 6.7—7.0), moisture contents(17.06—34%) and proper temperatures were confirmed to favourable for the distribution of *Azotobacter*(Anderson 1958ab). The fact that the content of potassium in rhizosphere soil is higher than the average level of the soil in Korea(Park 1971) it becomes a factor to maintain alkaline condition of soil. Total organic matter had occupied relatively high content of 16.18—18.7%(Chung *et al* 1971). It is pointed out for this fact that the origin of soluble sugar could be 3—8 times higher, compared with the amount of soluble sugar measured in loam soil (Eacon 1968). Soluble sugar and 15 kinds

of amino acids may be originated by decomposition of the organic matter in the rhizosphere or excreted from the root hair of bamboo(Gams 1967, Rovira 1956 abc). Four kinds of sugar(glucose, fructose, sucrose and maltose) and 15 kinds of amino acids with respect to their contents may be directly a reason why *Azotobacter* and other soil microbes have high population size in bamboo forest soil(Katznelson 1961, Rovira 1956abc, and Clark 1970).

A specific phenomena is the fact that glutamic acid had no effect on the colonization of *Azotobacter*. However, it may be explained on the basis of that glutamic acid, a first amino acid transformed from ammonia, may be synthesized within *Azotobacter* cell (Virtanen, 1953).

2. Growth phases of *Azotobacters* and Carbon source for Nitrogen fixation.

The effects of carbon source for population size of *Azotobacter* and nitrogen fixation were determined in high values as the order of glucose, fructose, maltose and sucrose. Such results are consistent with Sasson's consequence(1967) that the rate of oxygen absorption shows same orders in the cultures treated with various sugars. Katznelson(1940) had reported that the rate of consumption of glucose is faster than that of maltose in soil. Anderson(1958b) also reported that *Azotobacter*, 12 month incubated in the soil with K_2HPO_4 and manitol or K_2HPO_4 and sucrose, had large population size, compared with that of *Azotobacter* incubated in soil with sucrose alone. The consequence, that potassium contents of the bamboo forest are larger than that of results of some reports(Park *et al* 1971), indicated that potassium can exist in K_2HPO_4 and KH_2PO_4 as 9 to 1(Katznelson 1940). In this experiment, the fact that final *p*H stays on the range of 6.0 —7.0 may be explained as the role of potassium in nature.

Referring to the population size and fixed nitrogen by *Azotobacter*, when 4 kinds of carbon sources are added to bamboo forest soil, carbon sources consumed by *Azotobacter* is principally glucose and next in order of fructose, maltose, and sucrose. The advantage of maltose, compared with sucrose, may be the fact that maltose are decomposed to monomer, glucose only and sucrose to glucose and fructose.

3. Asymbiotic nitrogen fixation in the Rhizosphere of bamboo forest by *Azotobacter* species.

Increase in the nitrogen contents of soil with cover of non-leguminous plant, due to non-symbiotic nitrogen fixation have been recorded both in the field and in the labolatory(Stevenson 1959, Moore 1963, and Parker 1957). In the field, the gains reported 22 to 65 kg per hectare annually. In 1963, Moore reported the possibility of nitrogen increase in the plants-soil system of 100 to 130 kg per hectare annually under grass as against 100 to 200 kg per hectare annually under legumes. Allison(1955) expressed gains of nitrogen ranging up to 100 kg/ha per year.

Based on the amount of fixed nitrogen obtained from 4-weeks incubation of isolated *Azotobacter* in bamboo forest soil, the seasonal productivity of nitrogen can be calculated with following formula;

$$\frac{\text{Population in field count}}{\text{Population in lab. cultures}} \times \text{Nmg(fixed}$$

nitrogen in lab. cultures at a certain

temperature)=N mg/10g soil/Season(3 months)

Spring(15°C)

site A: 6.73÷15.13×0.1304=0.0552 Nmg/10g soil/3mo

site B: 3.0÷15.13×0.1304=0.0258 〃

site C: 4.1÷15.13×0.1304=0.0351 〃

Summer(25°C)

site A: 8.6÷15.13×0.463=0.2631 〃

site B: 6.0÷15.13×0.463=0.1836 〃

site C: 6.8÷15.13×0.463=0.2080 〃

Autumn(20°C)

site A: 8.1÷15.13×0.2336=0.1250 〃

site B: 5.9÷15.13×0.2336=0.0910 〃

site C: 5.9÷15.13×0.2336=0.0910 〃

Winter(4°C)

site A: 3.6÷15.13×0.006=0.0014 〃

site B: 4.1÷15.13×0.006=0.0016 〃

site C: 7.2÷15.13×0.006=0.0028 〃

Therefore, annual total amounts are as follows;

site A: 0.04447(mg/g. soil/yr)×Weight of soil(within 10cm from surface of hectare)

$0.04447\times2\times10^{9}\div1000$(g)
=88940g/ha/yr.
=88.94kg/ha/yr.

site B: $0.0302\times2\times10^{9}\div1000$
=60400g/ha/yr.
=60.4kg/ha/yr.

site C: $0.03369\times2\times10^{9}\div1000$
=67380g/ha/yr.
=67.38kg/ha/yr.

As the discussed above, annual product of nitrogen fixed by *Azotobacter* in the surface soil of bamboo forest are calculated to 88.94 kg/ha in site A, to 60.4 kg /ha in site B, and to 67.38kg/ha in site C. These values mean the possible amount of expected by *Azotobacters*. Therefore, if the amounts fixed by such anaerobes as *Clostridium* are added, total productivity may be far more increased (Rice *et al* 1967, Jurgensen 1971). In spite of the more contents of soluble sugar in site C than that in site A, the reason why the amounts of fixed nitrogen is smaller, may be based on the population size of *Azotobacter*.

And the reason why the population of *Azotobacter* is smaller than that of site A may be due to the antagonistic reaction with Actinomycetes(Schreven 1963).

CONCLUSION

This experiment can be concluded as follows; It is believed that high contents of soluble sugar, various amino acids and amount of organic matters in the rhizosphere of bamboo forest are resulted in larger population sizes of general bacteria, general fungi, and Actinomycetes as well as *Azotobacters*, compared with those of non-rhizosphere(Hong *et al* 1974b, Bacon 1968). Especially, the contents of potassium in bamboo forest soil are higher than those of other soil, so the actual condition of soil is essential for the growth of *Azotobacter* is maintained as neutral one.

On the other hand, it is considered that the population size of *Azotobacter*

in the bamboo forest soil is regulated by that of Actinomycetes.

In the rhizosphere of bamboo forest, the main carbon source for nitrogen fixation was determined as glucose and the minors were utilized in order of fructose, maltose and sucrose.

Annual gains of nitrogen by *Azotobacter* in soil of bamboo forest(within 10 cm from surface) were estimated as of 88.94 kg/ha at site A, 60.4 kg/ha at site B, and 67.38 kg/ha at site C, respectively.

적 요

죽림(*Phyllostachys reticulata* Koch)의 근권토양에서 *Azotobacter*의 분포 및 균체수에 미치는 환경요인과 *Azotobacter*에 의해서 기대될 수 있는 년간 총 질소고정량을 규명하고자 본 실험을 실시하였던 바 다음과 같은 결론을 얻었다.

1. 죽림의 근권토양은 일반 양토에 비하여 유리당류의 함량이 3—8배 많았으며 총 유기물량, 아미노산류의 함량도 비교적 높은 수준이었다.
2. Potassium의 함량이 높으므로 인하여 토양의 중성적 조건을 이루어 평균 *p*H는 7.0에 가까운 것이었다.
3. 위와 같은 근권토양의 환경조건으로 인하여 Actinomycetes, general fungi, general bacteria, *Azotobacter*는 비근권토양 및 다른 계통의 근권토양에 비하여 Population size가 컸으며 Actinomycetes는 우점종으로서 판단되며 그 다음으로 일반균류, 일반세균의 순서이다.
4. 죽림의 근권토양에서 *Azotobacter*의 Population size는 Actinomycetes population size에 의하여 제어를 받는 것으로 판단된다.
5. 죽림의 근권토양에서 *Azotobacter*에 의하여 질소고정이 행하여 질 때 탄소원으로 쓰이는 당류는 주로 glucose이며 fructose, maltose, sucrose의 순서이다.
6. 표층으로부터 10cm 깊이 이내의 죽림의 근권토양에서 *Azotobacter*에 의해서 기대될 수 있는 년간 질소 고정량은 1년생 죽림에서 88.94 kg/ha, 5년생 죽림에서 60.4 kg/ha, 50년생 죽림에서 67.38 kg/ha이다.

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