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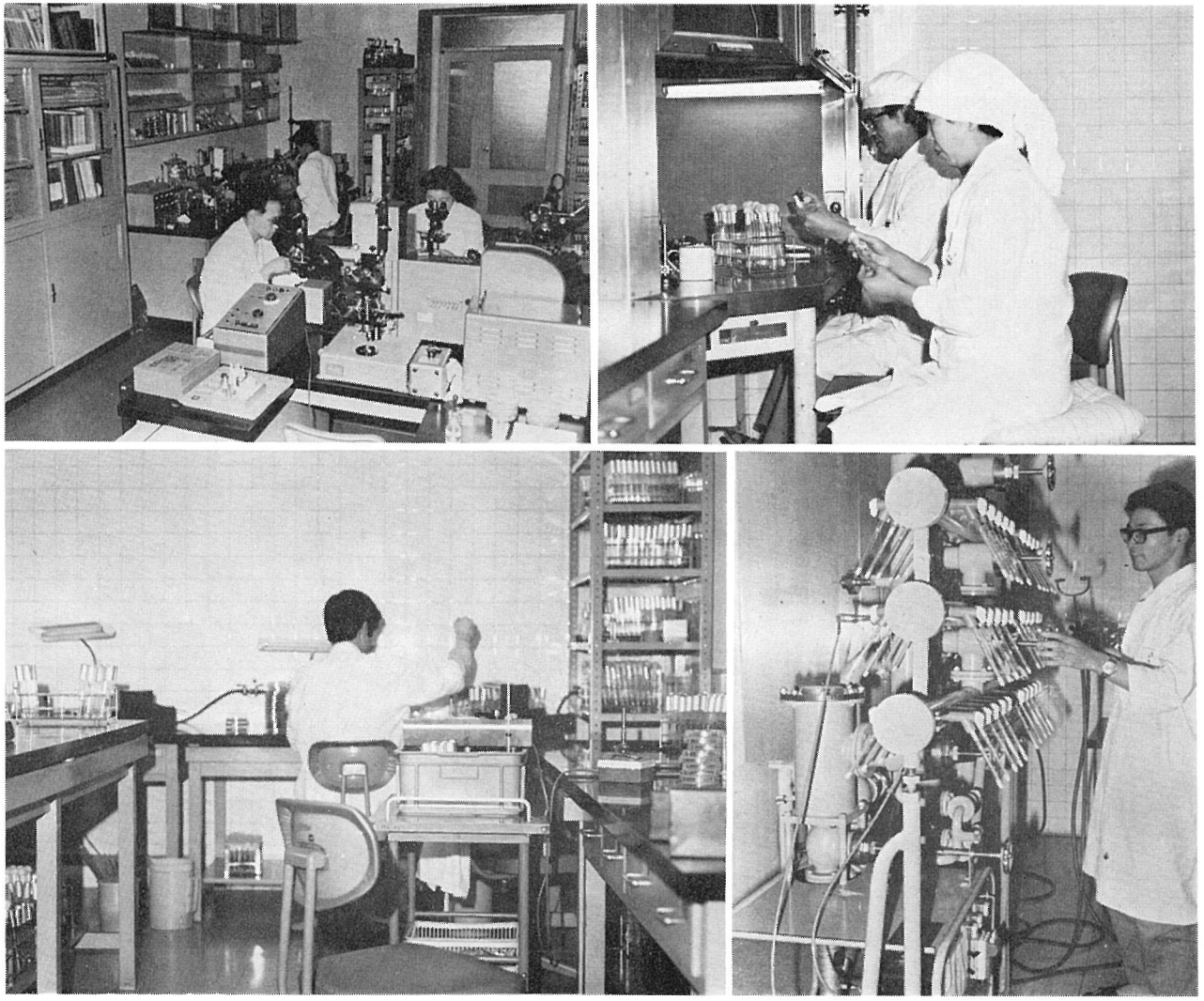

Laboratories

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ANNUAL REPORT, 1973-1974

REPORT OF THE DIRECTOR

The International Post-Graduate University Course in Microbiology developed from Group Training Course in Fermentation Technology at the Suita Campus of Osaka University in 1972 was started from 1973. Drs. Hasegawa, Tubaki and Banno were invited as lecturers on the microbial classification together with Dr. Kazuo Komagata, the University of Tokyo, in 1973 and 1974.

Dr. Banno attended the Fourth International Symposium on Yeasts that took place in Vienna during the period of July 8–12, 1974.

Drs. Hasegawa and Tubaki served as members of the organizing committee of the First Intersectional Congress of the International Association of Microbiological Societies held in Tokyo during the first week of September, 1974, to which congress Dr. Yokoyama presented an ecological paper. Drs. Banno, Tubaki and Yokoyama also joined the Special Mycology Symposium in Atami on August 29–31, and Post-Congress Mycological Meeting in Tottori on September 9–10, 1974. In addition, Drs. Hasegawa and Nakazawa attended the Post-Congress Symposium on Actinomycetes in Kyoto on September 8–9, and Dr. Yokoyama the Post-Congress Osaka Symposium of Phytopathology in Suita on September 9–11, 1974. These international scientific meetings were held by Japanese societies in cooperation with participants of the Tokyo Congress. In those days, the Institute often received visits of courtesy from many foreign microbiologists including the chief staff of the International Streptomyces Project.

Dr. Iijima and Mr. Sakamoto were present at the International Symposium on

Bacterial Resistance held by the Commission on Chemoresistance, International Society of Chemotherapy, on October 24–26, 1974 in Tokyo.

A supplementary edition to the IFO List of Cultures, 1972 was prepared. It is to be issued at the beginning of 1975, including 905 names of strains. During the two years of 1973 and 1974, the Institute added 545 cultures of fungi and bacteria to its culture collection, and the number of cultures reached 8759 in the IFO Collection. As to the distribution service, 8779 subcultures were sent to research organizations in Japan and abroad during these two years.

(T. Hasegawa)

IFO Res. Comm. 7,
3-12, 1975 (*March*)

AT THE THIRTIETH ANNIVERSARY OF THE IFO

Takezi HASEGAWA

As everybody has a birthday to be celebrated, so every organization usually has a day to commemorate its founding. The first of November, 1974 was the thirtieth anniversary of the Institute for Fermentation, Osaka. It was celebrated by the people working at the Institute, who have cultivated rich fruits on the tree of knowledge in microbiology through collection, preservation, and distribution of cultures.

The Institute was established in 1944 jointly by the government and Takeda Chemical Industries, Ltd. as one of the research institutes for microbiology according to the state policy then being pursued. The first director was Ryodi Nakazawa from the Government Research Institute of Formosa, who had been interested in such contributions to learned circles as culture collections and bibliographical works. He retired in March of 1946 leaving a recommendation that the Institute should bring up a culture collection of microorganisms as one of its main objectives. Thereafter he was devoted himself to the making of a bibliography of fermentation and biological chemistry until he died in December, 1974 at the great age of ninety-five.

Chobei Takeda, Chairman of the Board of Trustees, appointed Kikiti Sato to the post of director in April, 1946. Sato formerly worked at Nakazawa's laboratory in Formosa for about fifteen years after graduating from the University of Tokyo in 1921, and was engaged as the dupty director of the Institute in 1944. Yielding to the policy of the Board, he directed his efforts to establishing a public collection (service collection) of reference strains with the assistance of Kin'ichiro Sakaguchi, Professor of Faculty of Agriculture, University of Tokyo.

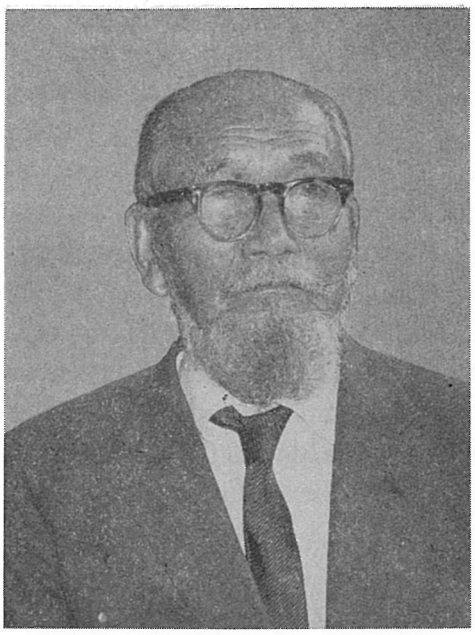
Since the beginning of the twentieth century, microbiological studies have made great progress in Japan. Many reference strains were collected by talented microbiologists in the course of their studies, and their cultures formed a number of collections at research organizations in Japan. Among them, four collections made at the Faculty of Agriculture, University of Tokyo (ATU), Faculty of Agriculture, Hokkaido University (AHU), Government Research Institute of Formosa (GRIF, now defunct) and at the Central Laboratory of the South Manchuria Railway Co. (CLMR, now defunct) are still now historically famous in the agricultural and industrial fields. In 1944 when the Institute was founded, the IFO Collection started with descendant cultures derived from the GRIF and ATU Collections. The cultures from the CLMR and AHU Collections were added afterwards to them in 1946 and 1952 respectively, through the courtesy of Hirosuke Naganishi, Professor of Hiroshima Technical College (Faculty of Technology, Hiroshima University, after 1949), and Yuji Sasaki, Professor of the Faculty of Agriculture, Hokkaido University. During a period previous to the

C. Takeda
Chairman, Board of Trustees

establishment of the Institute, strains of the CLMR Collection was transferred to some other collections with its curators' removal. And these cultures formed the main parts of the collections at the Hiroshima Technical College (HUT) in 1929, the Osaka University (Faculty of Technology, OUT) in 1933, and at the Nagao Institute (NI) in 1940. The Institute also obtained these overlapped cultures from them as materials for a comparative investigation by us on reference strains in Japanese culture collections mentioned afterwards in this paper.

Following the termination of World War II the financial support from the Government was cut off on account of radical reforms in the Government at that time. Since then the functions of the Institute have been exercised exclusively under the sponsorship of the company. There were tight circumstances all over Japan of those days, and the financial condition of the Institute was anything but satisfactory. It was hardly avoidable that members of the Institute conducted researches chiefly into the applied microbiology for the purpose of obtaining funds from companies. Up to the year of 1960, the activities had covered various fields including the production of antibiotics, ergot alkaloids, nucleotides, and of enzymes as well as the microbial transformation of organic substances. Under such a course of events, the Institute had been expanding rapidly to great proportions, and was getting to require a large budget for its administration.

World War II caused extensive damage to many local collections of microorganisms in the world, and it resulted in the establishment of the International Federation for Culture Collections of Microorganisms in 1949. It was the same situation then in Japan. Takezi Hasegawa, taking charge of the maintenance of cultures in the IFO Collection after 1946, published the IFO List of Cultures, 1st edition (1953), which included 1,867 names of cultures as a result of reidentification until then. He stated in the preface to the book that all reference strains preserved in the Japanese research organizations should be confirmed by identification tests because of the insufficient care taken in maintaining these cultures on account of the prevailing lack of man power and materials for keeping cultures for several years until the end of the War. The Higher Education and Science Bureau, Japan Ministry of Education inquired widely about cultures of microorganisms preserved in public and private research organiza-



R. Nakazawa

K. Sato

tions in Japan, and published the names of 22,000 cultures reported from 144 laboratories under the title of "A General Catalogue of the Cultures of Microorganisms Maintained in the Japanese Culture Collections" in 1953. This catalogue provided very useful material for inquiry into the strains preserved. Sakaguchi, as a board member of the Japanese Federation of Culture Collections of Microorganisms established in 1951, launched the formation of an official research team to confirm the whole of the cultures in the General Catalogue. The joint research was continued for six years from 1954, and the publication of the JFCC Catalogue of Cultures in 1962 resulted therefrom.

The second edition of IFO LIST OF CULTURES was brought out in 1956, and included 2,391 names of cultures. The Board of Trustees of the Institute decided to reorganize the research projects and laboratories to carry on basic studies in the fields of mycology, bacteriology, and microbial genetics. The research in such fields was considered to be very important for growth and development of the culture collection. During the half year from the summer of 1960, the Institute cut off the greater part of its research members who had been working in applied fields. Sato resigned his post as Director to Hasegawa in March, 1961. Sato was famous for his work on scientific administration of the process for manufacturing anchu, a native cereal wine of South China fermented with *Monascus anka*. He devoted the remainder of his life to guidance in the technique of brewing sake, and passed away in 1971 at the age of seventy-four.

The activities of the Institute after reorganization in 1961 are as stated below.

THE CULTURE COLLECTION

Among a great number of microbial cultures received by the Institute from Japanese

organizations before and after 1944, there were many groups of cultures each including several descendants from one reference strain. Each of these descendant cultures had been maintained by different organizations, hence Hasegawa's inquiry into collected strains began with the comparison of taxonomic properties among them. It was a long-term task, but the work was not monotonous. This study gave him and his coworkers precious knowledge of biological phenomena, for example, variation and mutation during long-term preservation, phenotypic changes among colonies grown on different media, and hereditary tendencies to variation in each strain. The inquiry into the reference strains maintained in the IFO Culture Collection is still continuing.

The third edition of the IFO LIST OF CULTURES was issued with 2,981 names of cultures in 1962, the fourth edition with 4,527 in 1968, and the fifth edition with 5,475 in 1972. Since 1969, the Institute has been one of the international subcenters responsible for the International Cooperative Project for Description and Deposition of Type Cultures of *Streptomyces* (International Streptomyces Project, ISP) in compliance with the request from the Society for Actinomycetes, Japan. The number of ISP strains deposited in the IFO Collection has reached about five hundred.

The distribution of cultures began with the first publishing of the IFO LIST OF CULTURES in 1953. Since then the request for strains from public and private research organizations in Japan and abroad showed a yearly increase, and the number of cultures distributed to them reached over five thousand per year in 1964. As such an increase was causing difficulties to disturb the curators' constant efforts in keeping cultures in the collection from symptoms of degeneration or variation, the Institute had to limit the number of cultures in each order for several years after 1966. The results of distribution of cultures in these ten years were recorded as shown in the table.

	To Japanese organizations		To foreign organizations		Totals	
	Number of strains	orders	Number of strains	orders	strains	orders
1964	5,241	463	231	52	5,472	515
1965	5,098	533	321	67	5,419	600
1966	3,305	504	102	34	3,407	538
1967	2,780	484	244	67	3,024	551
1968	2,631	566	163	45	2,794	611
1969	2,925	641	196	53	3,121	694
1970	3,297	753	365	77	3,662	830
1971	3,741	827	356	74	4,097	901
1972	2,880	760	221	80	3,101	840
1973	2,794	780	273	79	3,067	859

RESEARCH ACTIVITIES

I. Mycology

1) Taxonomic studies on fungi

Tubaki presented in the IFO ANNUAL REPORT No. 1 (1963) a generalized scheme for classification of Hyphomycetes, in which he divided the Hyphomycetes into six major groups named after the type of conidia developed.[28] Four new genera; *Gonytrichella* Emoto et Tubaki 1970,[4] *Stachybotryna* Tubaki et Yokoyama 1971,[34] *Subulispora* Tubaki et Yokoyama 1971,[34] and *Hyalohelicomina* Yokoyama 1974[37] were proposed in Hyphomycetes, in addition to seventeen newly published species of Hyphomycetes and of Ascomycetes. Taxonomic studies by Tatsuo Yokoyama also covered some genera in the plant pathogenic fungi, i. e., *Actinopelte*,[35] *Graphiola*,[31] and *Cristulariella*.[41] Species in Tremellaceae and Graphiolaceae were studied under culture. It appeared that daughter cells sprouted out from basidiospores of *Tremella* and its allied genera, and that sporidia of *Graphiola* reproduced by budding. The yeast-like colonies resulted as the imperfect states of them. Such relationships between Heterobasidiomycetes and Cryptococcaceae were discussed in 1965[17] and 1971.[32] Expeditions abroad to collect fungi were made to Australia and New Zealand (1965),[30] Antarctic (1965),[29] Alaskan Arctic (1967),[15] Spitzbergen (1968, 1969), Greenland (1971) and New Guinea (1973)[38] under the leadership of Yosio Kobayasi, one of the councillors of the Institute. Tubaki also joined a mycological expedition invited by the Korean Society of Mycology in the summer of 1973.

2) Studies on marine fungi

Studies on Japanese marine fungi were started by Tubaki in 1964 and dealt with the presence of lignicolous and algicolous ascomycetes and hyphomycetes in Japan. Twenty-four species were isolated from marine materials and described in 1966 and 1968. A review of his own papers was presented by Tubaki including taxonomic and physiologic data[31] Since 1969, brackish water fungi have been studied by Tubaki and his coworkers. Such fungi are an intermediate ecological group between marine and limnic fungi. Twenty-four species were described in 1973.[36]

3) Studies on the succession of leaf-litter fungi

Dealing with the microfungal flora associated with the decomposition of plant materials, attempts were made by Tubaki and his co-workers to obtain a new interpretation of the successive colonization by fungi of the naturally developing litter of the forest in five stations in Japan. The autoecology, seasonal differences in the flora, and the differences of successive colonization by fungi between central and southern Japan were discussed, and four stages of the succession of fungi in leaf-litter were concluded to be distinguishable.[33,35,39]

Yeast Taxonomy

1) Studies on the genus *Rhodotorula*

A comparative study by Hasegawa and his coworkers into each of taxonomic properties among descendant cultures from each reference strain of *Rhodotorula* gave rise to questions about the systematics given to the genus in monographs, and the taxonomic studies on the genus were carried out for about ten years. These studies resulted in the proposal of a new system of classification in 1960[5], and in the discovery of the

perfect state in some strains belonging to *Rh. glutinis* Harrison, on which a new genus, *Rhodosporidium* Banno 1964, was established in Ustilaginales.[1,2] The presentation of *Rhodosporidium* by Isao Banno, one of our colleagues, was the first proof of phylogenetic relationships between yeasts and basidiomycetes.

2) Other studies

The property of acid formation in yeasts was studied by Banno and his colleagues.[3] It was found that strains of fifty-five species in ten genera formed a remarkable amount of acids from glucose. According to the patterns of acid production these yeasts were divided into three types, each of which based on a character common among a group of genera. The groups also differed from each other in the method of cell reproduction. The phenetic relationships among the *Saccharomyces* species have been investigated by the method of numerical analysis. The study is still continuing. One report is published in this issue of IFO RESEARCH COMMUNICATIONS.

Bacteriology

1) Studies on lactic acid bacteria

Many strains of lactic acid bacteria were isolated from various materials in the process of sake-brewing, and an investigation was made into their taxonomical properties and nutritional requirements. The result was reported by Reijiro Kodama and his colleagues in 1961.[18] Three strains of lactic acid bacteria isolated from flowers failed to grow in a modified Thompson's synthetic medium. These strains were demonstrated to require at least two substances for their growth, both of which were contained in tomato juice. They were identified as fructose and nicotinamide by Kodama. Microbiological methods for quantitative analysis of these two substances with one of the strains, named *Lactobacillus fructosus* Kodama 1956, were described in IFO ANNUAL REPORT No. 1 (1963).[19] Using nineteen strains of heterofermentative lactic acid bacteria, leuconostocs and lactobacilli, Kodama and his colleagues carried out a comparative study upon microbiological activities of thiamine and its related compounds.[20]

2) Bacteriological studies on silkworm diseases

A method of rearing the domesticated silkworm, *Bombyx mori*, on an artificial diet was first reported by a Japanese research organization in 1960. Since then, studies on aseptic techniques applied to rearing the insect have been carried on in Japan.

Kodama and his assistants made unique and valuable contributions to the progress in this method.[21-25] They isolated many strains of bacteria from epizootics in populations of silkworms and dead bodies of the insect, and exhaustive investigations were conducted into their taxonomic situations and pathogenicity. *Proteus, Pseudomonas, Serratia,* and *Streptococcus* strains brought about disease by feeding and/or by injection in healthy silkworm larvae reared aseptically on an artificial diet. Two strains among these pathogens, each of which belonged to *Streptococcus faecalis-Streptococcus faecium* intermediate and *Serratia piscatorum* respectively, were used by the authors in advanced studies to elucidate the pathogenic mechanism of flacherie, one of the com-

mon microbial diseases of silkworms. Some synergistic effects of the two pathogens were reported on the disease of gnotobiotic silkworm larvae. Further studies elicited the synergism between the virus and the bacteria in the development of flacherie of silkworms.

3) Bacteriological studies on Streptomycetes

A crystal formation was found by Kôiti Nakazawa in a culture broth of *Streptomyces sindenensis* strain No. 5866, and a causal relation was observed between cell growth and crystals. It was ascertained thereafter that crystal formation occurred widely in culture broths of various species belonging to Streptomycetes.[27] A blue-colored mutant was found as one of the isolates from a reference strain of *Streptomyces lavendulae*.[26]

4) Studies on utilization of citric acid by bacteria

The utilization of citric acid has been used as a criterion in the systematics of bacteria, especially in that of Enterobacteriaceae. To define the physiologic and genetic backgrounds of this activity, the transport systems for tricarboxylic acids were examined by Ko Imai in one strain of *Salmonella typhimurium* whose genetic map had been well studied.[14,15] It was found in 1973 that *S. typhimurium* had four inducible transport systems for the acids examined. The first system was induced by citrate, isocitrate or *cis*-aconitate, and carried citric acid and isocitric acid. The second system, induced by the same acids, transported *cis*-aconitic acid but not citric and isocitric acids. The third system, induced by tricarballylate, transported citric, *cis*-aconitic and tricarballylic acids. The fourth system, induced by citrate, carried only citric acid. A genetic analysis of the systems is still continuing and one report is published in this issue of IFO RESEARCH COMMUNICATIONS. Another investigation into the citric acid utilization was made by Imai in reference strains of *Arthrobacter* since 1968.[12,13] It was found that the growth of *A. pascens* and *A. simplex* was inhibited by citrate and that the mechanisms of growth inhibition by citrate in these two bacteria were remarkably different. The inhibition in *A. pascens* was due to a defficiency of metal ions on account of their chelation by citrate, whereas that in *A. simplex* was caused by the binding of citrate to the cell surface.

Microbial Genetics

1) Studies on a temperate bacteriophage ϕ170

The laboratory started in 1962 and its first work was the isolation of temperate phages from natural sources by Teiji Iijima.[6] Among the phages obtained, Iijima chose a temperate phage, named ϕ170 by himself, that could transduce the *gal* markers among the mutant strains of *Escherichia coli* K12, and attach to the chromosome of K12 at the same locus as that of λ phage (att λ) though these phages had different immunities from each other. The phage ϕ170 has been used for a series of studies on genetics by Iijima.[7-9] It was found that the double lysogen for λ and ϕ170 was so unstable that it segregated single lysogens for each phage accompanying with the curing of the prophages at a constant rate. The application of this curing is very useful for the elimination of the λ phage from genetically marked mutants of K12 without

causing any marker change.[10] The mechanism of transduction by ϕ170 and the structure of the transductants were revealed by comparison with those of the λ phage.[7]

2) Studies on the preservation of cultures by drying

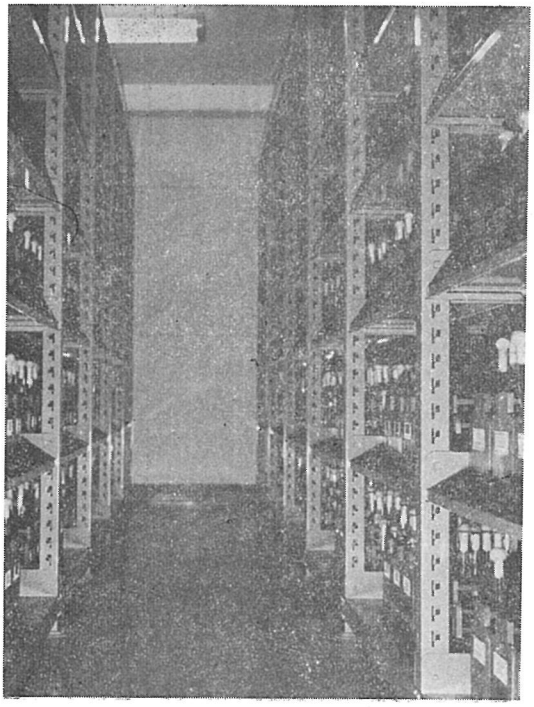
It became necessary to find a simplified method of preserving microorganisms with the increase of cultures in the collection. Iijima and his coworkers began the study on the method with mutant strains of *E. coli*, and reached the conclusion that drying *in vacuo* without freezing in advance was an excellent method for this purpose. The method was applied to other bacteria and bacteriophages.[11]

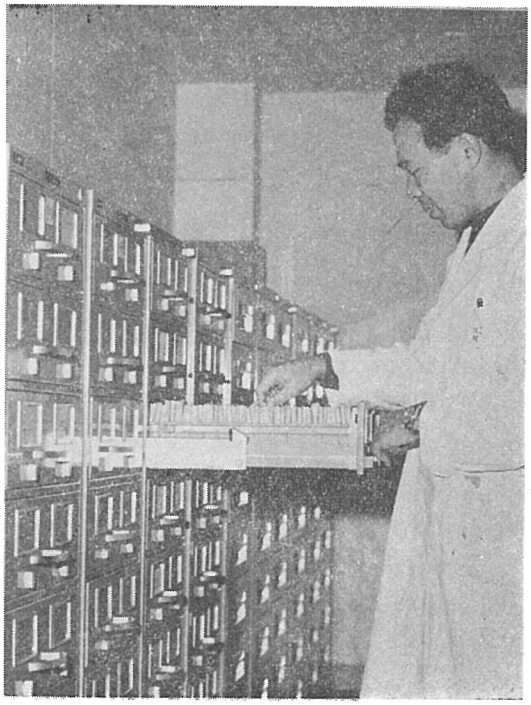
3) Other studies

As a genetic study on *Pseudomonas*, the phage typing of strains of *Pseudomonas aeruginosa*, isolated from natural sources, has been carried on by Yutaka Sakamoto since 1972. Four transport systems for tricarboxylic acids in *Salmonella typhimurium* have been analysed genetically by Teiji Iijima with the cooperation of the bacteriological laboratory of the Institute. Reports on these investigations are presented in this issue of IFO Research Communications.

The author did not describe here all other works carried out by many colleagues resigned before 1960 *who made a great contribution towards the development of the Institute, because it would require a volume to deal with them. He wishes to illustrate them under another title in the near future.*

Dr. Kodama retired from the Institute in October, 1972. *His valuable contributions should be appreciated.*





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RYODI NAKAZAWA
1878–1974

Takezi HASEGAWA

Ryodi Nakazawa, ex-director of the Institute for Fermentation, Osaka, died suddenly on December 2, 1974, about two weeks prior to his ninety-sixth anniversary.

Ryodi Nakazawa was born on December 18, 1878 in Tokyo. He graduated from the Institute of Agricultural Chemistry, Agricultural College, Tokyo Imperial University in 1905. He entered on his career of office at the Brewing Experimental Station, Tax Administration Agency of the Government next year. He studied in Germany for three years from June, 1907, on mycology at Technische Hochschule (Prof. Dr. C. Wehmer) in Hannover; zymology and brewing technique at Wissenschaftliche Station für Brauerei (Prof. Dr. H. Will) in Munich; wine making at Königliche Lehranstalt für Wein-, Obst-, und Gartenbau (Prof. Dr. J. Wortmann) at Geisenheim and so on.

In 1911, he moved to the Government Research Institute of Formosa (GRIF) in Taipei (Taihoku), and worked on microbiological researches promoting the development of the fermentation industry in Formosa. His most outstanding contributions concerned the scientific control of native fermentation technique there, especially the improvement in alcoholic fermentation of molasses. Among many industrial species of fungi he published, *Aspergillus awamori* Nakazawa 1915 and *Saccharomyces formosensis* Nakazawa 1933 are the most famous. He obtained his doctor's degree from Tokyo Imperial University in April, 1921. He was designated to the Head of Fermentation Department of the GRIF in August of the same year. Besides, he held a professorship at the Faculty of Agriculture, Taihoku Imperial University concurrently from 1930 to 1939.

He retired from the GRIF and the University under the age clause in August, 1939. Complying with the Government's request, he established the Institute for Fermentation in Osaka, and took office of the director in December, 1944. He resigned in April, 1946.

Since 1939, he had acted as an adviser on scientific administration to Takeda Chemical Industries, Ltd., and at the same time, devoted himself to the making of the Bibliography of Fermentation and Biologoical Chemistry that was completed in 11 vols. in 1964, and of the Bibliography of Fermentation Microorganisms that was left unpublished on account of his death except three volumes from beginning.

Nakazawa cultivated many excellent specialists especially during about thirty years of his stay in Formosa. He was a zealous advocate of the Romanization of the Japanese Alphabet, and his contributions to the Society for the *Romadi* continued throughout his life. Japan lost a person of world-wide fame in the field of microbiology.

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15–23, 1975 (*March*)

SACCHAROMYCES YEASTS ISOLATED IN JAPAN: (I) A NUMERICAL ANALYSIS OF *S. CEREVISIAE* AND ITS ALLIED SPECIES*

Isao BANNO

Summary

Over one hundred strains of *Saccharomyces* were isolated from natural sources and identified. These new isolates and strains of *Saccharomyces* spp. selected from the IFO collection were subjected to a numerical analysis and arranged in a form of dendrogram. Most of the isolates of *S. cerevisiae* and its allied species formed a major and a minor clusters. On the basis of distribution of positive characters in the isolates, a strain (K–40) was selected as one of the typical yeasts of *S. cerevisiae* isolated in Japan.

As to the classification of the genus *Saccharomyces*, many investigations have been made from various points of view such as morphological, physiological, serological and genetical points.

Numerical analyses of the yeasts of this genus have been made by Kocková-Kratochvirová and others (1,2) and by Campbell (3–5). They reported that the yeasts of the genus formed phenetic clusters generally corresponding to species formerly recognized by Lodder in 1952 (6). In the mean time, Windisch and Neumann (7,8) suggested from their breeding experiment that most of yeast types of *Saccharomyces sensu stricto* might not be different species but specialized ecotypes of the same species.

The concept of species of the genus *Saccharomyces* is not clearly defined as yet. The present investigations were conducted in order to examine the variation of characteristics in the natural population of *Saccharomyces* species and to obtain a numerical concept of the biological species.

Materials and Methods

Saccharomyces yeasts to be used were isolated from natural materials found in various places of Japan archipelago. The materials were tree slime flux, tree bark, leaves, litter, flowers, mashrooms and soil. Orange juice containing 5% glucose (pH 3.7) and kôji-extract supplemented by 100 ppm chloramphenicol were used as enrichement culture media. A small piece of the collected materials was inoculated into the two enrichment culture media in test tubes. The inoculated media were incubated at 25C without beeing stirred and then spread on the YM agar plate. After a 2– to 5–days incubation, the plate was observed under a dissecting microscope. Each type

* This paper was read in the 4th International Symposium on Yeasts held in Vienna, 1974.

of various yeast-colonies on it was picked and then reisolated on another plate of the same kind.

These new isolates were identified according to the procedures described in *THE YEASTS* 2nd edition (9). A total of 105 new isolates were of the genus *Saccaromyces* Meyen emend. Reess. Among them 79 isolates were identified as *S. cerevisiae* Hansen, 6 as *S. uvarum* Beijerink, 1 as *S. bayanus* Saccardo, 1 as *S. capensis* v. d. Walt et Tscheuschner, 4 as *S. florentinus* (Castelli) Lodder et Kreger-van Rij, 3 as *S. kluyveri* Phaff, Miller et Shifrine, 2 as *S. globosus* Osterwalder, 2 as *S. exiguus* Hansen and 1 as *S. unisporus* Jörgensen, and the remaining 6 were not identified as a known species.

In addition to these 105 isolates, 31 brewery yeasts and 61 reference strains of 30 species of the genus maintained in the IFO culture collection were examined for morphological, physiological and biochemical characteristics. In addition to ordinary tests, tests for assimilation of 72 miscellaneous compounds other than carbohydrates and for the resistance to blasticidin S, pyronine Y and crystal violet were carried out.

In the numerical analysis, the characteristics showing negative expressions in all the strains were omitted. As a result, 90 unit characters given in Table 1 were used

Table 1. Unit characters used in numerical analysis.

	Morphology (10 characters)		Fermentation (10 characters)
1	cell, size more than 4 μm in diameter	23	glucose
2	oval to ellipsoid	24	galactose
3	much long	25	sucrose
4	pseudomycelium, present	26	maltose
5	well developed	27	trehalose
6	pellicle, present	28	lactose
7	spore, number per ascus	29	raffinose
8	spherical	30	inulin
9	other shape	31	starch
10	conjugation proceed ascus formation	32	α-methylglucoside
	Physiology (12 characters)		Assimilation of C-compounds (53 characters)
11	growth at 37C	33–53	21 kinds of saccharide
12	growth in vitamin-free medium	54	1 kind of glycoside
13	pellicle on NaCl-medium	55–58	4 kinds of alcohol
14	acid formation in chalk agar	59–66	8 kinds of polyol
15	tolerance to 12% EtOH medium	67–78	12 kinds of organic acid
16	tolerance to 7% NaCl medium	79–82	4 kinds of amino acid
17	resistance to actidion (100 μg/ml)	83–85	3 kinds of amine
18	resistance to blasticidin (40 μg/ml)		Assimilation of N-compounds (5 characters)
19	resistance to pyronine Y (100 μg/ml)	86	sodium nitrate
20	resistance to crystal violet (20 μg/ml)	87	lysine
21	decomposition of arbutin	88	histidine
22	decomposition of gelatin	89	tryptophan
		90	ethylamine

in computation.

The numerical analysis was made according to the Sneath's method (10). Matching coefficient of Sokal and Michener in which positive and negative matches were assessed as similarity was calculated as a similarity value between strains. A dendrogram was prepared by arranging the strains in clusters at the highest mutual similarity value.

Result and Discussion

This paper presents mainly the analytical result on the strains of *Saccharomyces cerevisiae* and its allied species.

Figure 1 shows a dendrogram resulting from the analysis of the new isolates. The dendrogram is illustrated in a summarized form. Seventy-seven isolates formed a large cluster at 94% level of similarity value. They consist of 72 isolates of *S. cerevisiae* and 5 isolates of *S. uvarum*. This main cluster will be called Cluster A. Other 7 isolates of *S. cerevisiae* formed another minor cluster at 94% level also. This cluster will be called Cluster B. To the clusters A and B, 1 isolate identified as *S. uvarum* was joined at 92% level, and then 1 isolate of *S. bayanus* and 1 isolate of *S. capensis* were linked to these two clusters at 90% level. In addition to the above-mentioned clusters, 2 isolates of *S. globosus* and 1 isolate of *S. unisporus* also formed a cluster at 92% level. Two isolates of *S. exiguus* were joined at 98% level. Four isolates of *S. florentinus* formed a cluster at 92% level and 3 isolates of *S. kluyveri* formed a cluster at 88% level. Finally these clusters were joined forming a single cluster at as low as 84% level.

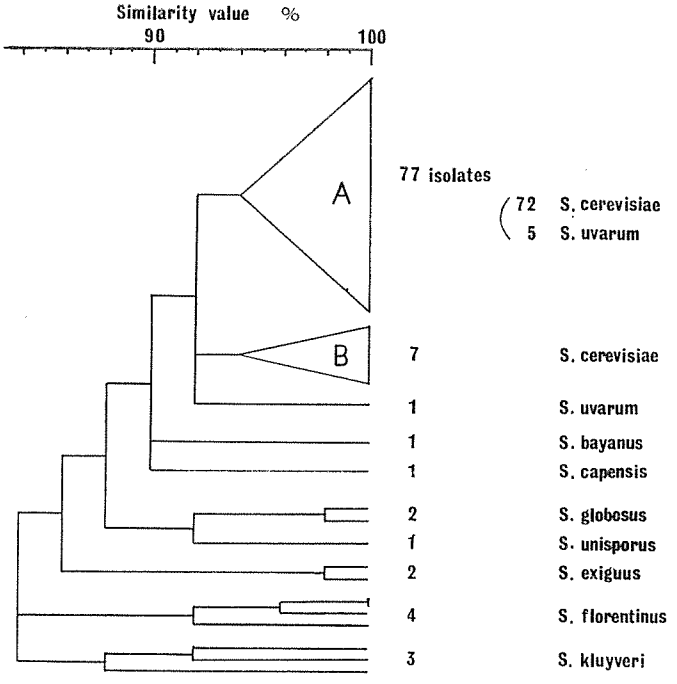

Fig. 1. Dendrogram of 99 new isolates identified.

Most of the isolates identified as *S. cerevisiae* and *S. uvarum* were included in Cluster A at such a high level as 94%. Therefore, the organisms of *S. cerevisiae* and *S. uvarum* isolated in Japan are very closely related to one another.

Next, the new isolates and a selection of brewery yeasts were analyzed at the same time and compared. Eleven sake yeasts, 7 alcohol yeasts for distillery, 5 wine yeasts, 4 beer yeasts and 4 bakery yeasts given in Table 2 were arbitrarily selected from the IFO culture collection and used in this analysis.

Figure 2 presents a dendrogram obtained from such a mixed analysis of the isolates and brewery yeasts. All of the 11 sake yeasts, 3 of bakery yeasts and 1 of alcohol yeast

Table 2. Brewery yeasts for comparison.

	Strains (IFO No.)
Sake brewery yeasts	0304, 0305, 0306, 0308, 0309, 2141, 2342, 2343, 2345, 2346, 2347
Alcohol-industrial yeasts	0233, 0234, 2065, 2112, 2114, 2115, 2373
Wine brewery yeasts	2220, 2231, 2260, 2317, 2359
Beer brewery yeasts	2005, 2023, 2031, 2033
Bakery yeasts	2039, 2043, 2044, 2045

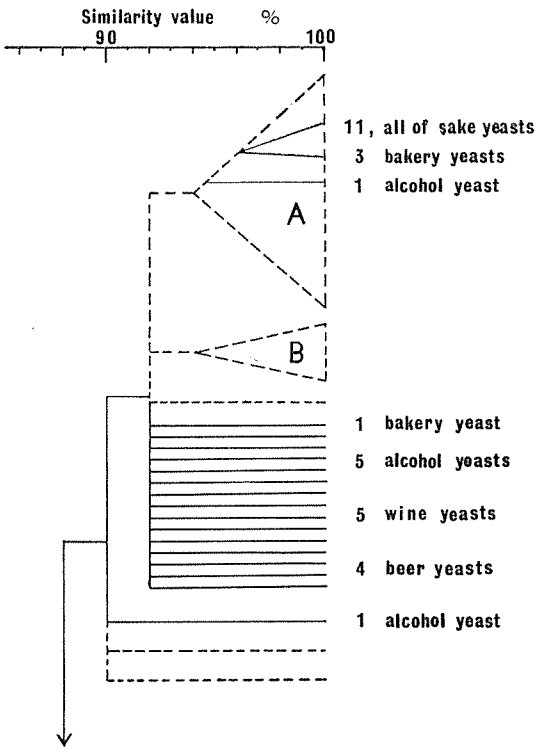

Fig. 2. Dendrogram of the new isolates and 31 brewery yeasts.
Solid lines indicate brewery yeasts and broken lines indicate the same isolates as in Figure 1.

were included in the main Cluster A. Other 16 of alcohol, wine, beer and bakery yeasts independently linked to the main cluster at 92% and 90% level.

The finding that all sake yeasts joined to Cluster A at a level higher than 94% is significant as a demonstration of a possible origin of the sake yeast.

On the other hand, 61 reference strains of the genus were analyzed together with the new isolates at the same time. They are of 30 different species shown in Table 3.

Figure 3 shows a dendrogram resulting from this analysis. Two reference strains of *S. cerevisiae* and 1 of *S. oleaginosus* were included in the main Cluster A, and 3 more strains of *S. cerevisiae,* 1 strain of *S. uvarum* and 2 strains of *S. chevalieri* were joined at 92% level together with Cluster A and B. Furthermore, 2 strains of *S. cerevisiae,* 3 strains of *S. uvarum,* 4 strains of *S. bayanus* and 1 each strain of *S. diastaticus, S. heterogenicus* and *S. italicus* were individually linked to the main cluster at 90% level.

The reference strains of other *Saccharomyces* species formed separate clusters as shown in Figure 3. This result is similar to that obtained by Campbell in 1972.

The fact that most of the new isolates of *S. cerevisiae* and *S. uvarum* formed a definite Cluster A at a higher similarity level than did the reference strains of *S. cerevisiae* and brewery yeasts supports the idea that the natural population of *S. cerevisiae* and *S. uvarum* in Japanese islands is an topological form. Because these isolates and strains of *S. cerevisiae* and of its 7 allied species did not form separate clusters but made up a large mixed cluster at 90% level which seemes to be a basic taxonomic unit of these yeasts, this 90% level cluster probably corresponds to the phenetic species.

Table 3. Reference strains of the genus *Saccharomyces.*

Species	Strains IFO No.	Species	Strains IFO No.
S. bailii	1098	*S. inconspicuus*	1621
S. bayanus	0539, 0853, 1127	*S. inusitatus*	1343
	1612	*S. italicus*	0253
S. bisporus	1131, 1055	*S. kloeckerianus*	1160
S. cerevisiae	0205, 0282, 0313	*S. microellipsodes*	1255
	0317, 0849, 1009	*S. montanus*	0021
	1234	*S. oleaginosus*	1014
S. chevalieri	0217, 0490	*S. rosei*	1129
S. coreanus	0573	*S. rouxii*	1130
S. dairensis	0211, 1168	*S. saitoanus*	1624
S. delbrueckii	0955	*S. telluris*	1017
S. diastaticus	1015	*S. transvaalensis*	1625
S. exiguus	1128	*S. unisporus*	1173
S. fermentati	1618	*S. uvarum*	0218, 0293, 0615
S. florentinus	1088		1225
S. globosus	0254	*S. vafer*	1626
S. heterogenicus	1620	*S. veronae*	1050

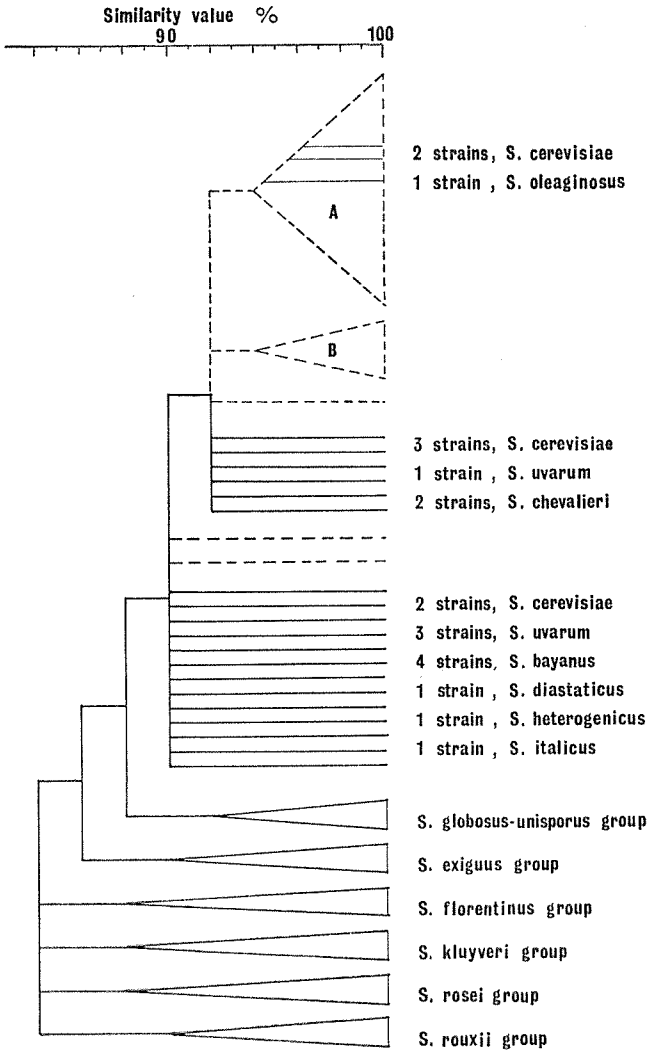

Fig. 3. Dendrogram of the isolates and reference strains of *Saccharomyces*.
Solid lines indicate reference strains and broken lines indicate the same new isolates as in Figure 1.

Another analysis was made concerning the diversity of characteristics of the new isolates included in Cluster A and concerning that of all the members (the new isolates, brewery yeasts and reference strains) of *S. cerevisiae* and its allied species included in the 90% level cluster. The frequency of positive expressions in each of the unit characters among the members and the mean number of positive characters of the members were calculated. On the calculation of these items, a hypothetical mean organism (HMO) of the members was determined as follows. The HMO is an imaginary organism introduced by Tsukamura, having a mean characteristics of the members (11). First a character appearing at the highest frequency and then a character at a successively lower frequency were selected. The selection was continued until the number of selected characters became equal to the mean number of positive characters of the

Table 4. Deviation of similarity values.

	Mutual similarity values			Mean similarity value to HMO*	Mean number of positive characters
	mean	max.	min.		
Cluster A	87.88±4.28	100	78	92.15±3.19	30.82
The 90% level cluster	85.10±5.17	100	71	87.66±4.18	29.58

* HMO: Hypothetical mean organism.

members. It is HMO that would show positive expression in all the selected characters but negative expression in the remaining characters.

In addition, the minimum, maximum and mean of mutual similarity values were obtained from the similarity matrix of the members. Subsequently the mean similarity value of the members to the HMO was calculated.

Table 4 shows the calculated result. As for the isolates in Claster A, the mean of mutual similarity values is approximately 88% and the mean similarity value to their HMO is 92%. These two figures are relatively high and their standard deviations are small. Therefore the members of this Cluster A seem likely to be homogeneous. However, as for all the members of the 90% level cluster of *S. cerevisiae* and its allied species, the mean of mutual similarity values is approximately 85% and the mean similarity value to their HMO is 88%. These figures are lower than those of Cluster A and their standard deviations are somewhat greater. If this 90% level cluster truly corresponds to the biological species, the result implies that this species contains a great variety of characteristics.

Table 5 shows the HMOs of the isolates of the Cluster A and of the members of the 90% level cluster. The HMO of Cluster A is the organism that has positive expression in the 31 characters indicated by + signs in the second column. The HMO of the 90% level cluster is the organism that expresses positive reactions in the 30 characters indicated by + in the last column. Figures in parentheses indicate the actual frequencies on which positive expressions appeared.

The HMOs of the isolates in Cluster A and of the members in the 90% level cluster are different from each other in several characters, that is, in tolerance to 12% ethanol and to 7% NaCl, and assimilation of mannitol, propyl alcohol and glycerate. This means that main group of the isolates of *S. cerevisiae* and its allied species (Cluster A) has a somewhat deviated distribution of characters from that has the whole population of the phenetic species consisting of these species, indicating that a population of this phenetic species in Japan may be a topological group. The yeast closest to the HMO of Cluster A is an isolate called K–40, which is a typical strain of such a population.

The author wishes to express the deepest appreciation to Dr. K. Kodama for sending him many yeasts newly isolated.

Table 5. Hypothetical mean organism.

Characters	Cluster A	The 90% level cluster
Cell, oval more than 4 μm in width	+ (1.00)	+ (0.93)
Pseudomycelium	+ (0.93)	+ (0.72)
4 spores per ascus	+ (1.00)	+ (0.89)
Spherical spore	+ (1.00)	+ (1.00)
Growth at 37C	+ (0.98)	+ (0.86)
Tolerance to 12% EtOH		+ (0.53)
Tolerance to 7% NaCl		+ (0.48)
Resistance to pyronine Y	+ (1.00)	+ (0.99)
Resistance to crystal violet	+ (0.74)	+ (0.48)
Acid formation in chalk agar	+ (0.90)	+ (0.79)
Fermentation		
Glucose	+ (1.00)	+ (1.00)
Galactose	+ (0.98)	+ (0.90)
Sucrose	+ (1.00)	+ (0.98)
Maltose	+ (0.93)	+ (0.89)
Raffinose	+ (0.52)	+ (0.61)
α-Methylglucoside	+ (0.96)	+ (0.77)
Assimilation as sole C-sources		
Galactose	+ (1.00)	+ (0.95)
Sucrose	+ (1.00)	+ (1.00)
Maltose	+ (1.00)	+ (0.97)
Terhalose	+ (0.96)	+ (0.88)
Raffinose	+ (1.00)	+ (0.97)
Melezitose	+ (0.92)	+ (0.53)
Ethanol	+ (1.00)	+ (0.98)
Glycerol	+ (0.78)	+ (0.57)
Mannitol	+ (0.70)	
α-Methylglucoside	+ (1.00)	+ (0.98)
Lactate	+ (0.98)	+ (0.98)
Acetate	+ (0.56)	+ (0.63)
Propyl alcohol	+ (0.67)	
Pyruvate	+ (0.89)	+ (0.82)
Glycerate	+ (0.52)	
Assimilation as sole N-sources		
Tryptophan	+ (0.92)	+ (0.94)

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TYPING OF *PSEUDOMONAS AERUGINOSA* BY PHAGE RESISTANCE AND LYSOGENY*

Yutaka SAKAMOTO, Teiji IIJIMA, Shizuko IYOBE,**
and Susumu MITSUHASHI**

Summary

A phage typing method for *Pseudomonas aeruginosa* was improved by using the phage groups instead of the individual phages and by employing the prophage typing as the subsidiary method. Therefore, the results were more clear and reproducible, and nontypable strains decreased to 3.7% (total 707 strains) by the combining typing system. An application of this typing system to the identification of the strains from patients and of the reference strains of independent serological typing systems was performed. This system is specific for *P. aeruginosa* and its closely related species.

One of the gram-negative bacteria, *Pseudomonas aeruginosa*, has been isolated from various parts of human body in addition to other animals, plants, soil and water. It is recognized as the etiological agent of a variety of human infections. If we had a suitable typing method for differentiation of *P. aeruginosa*, it would be easier to chase the sources of nosocomial infections and to take proper treatments for the patients.

There were several typing methods for differentiation of bacteria, and those idea were applied to *P. aeruginosa* (Table 1). But there was no useful method for differentiation of *P. aeruginosa* except the serological typing (Bergan 1972 e, 1973 a, b,

Table 1. Typing methods for *Pseudomonas aeruginosa*.

Typing	Type	Method	References
Serological typing	Serotype	Agglutination with a set of type sera	5, 6, 7, 8, 15, 18, 22, 23, 24, 25, 27, 35, 36
Phage typing	Phage type	Sensitivity to a set of phages	3, 4, 5, 7, 8, 9, 12, 13, 15, 17, 19, 26, 28, 31, 33, 34
Prophage typing	Lysotype	Production of phages on a set of indicators	12, 13, 20, 30
Pyocin typing I	Pyocin type	Production of pyocins on a set of indicators	6, 7, 8, 11, 12, 14, 16, 20, 27, 30, 36
II	Pyocin type	Sensitivity to a set of pyocins	12, 29, 32
Chemical typing	Chemotype	Chemical analysis of lipopolysaccharides	10

* A part of this paper was presented to the International Symposium on Bacterial Resistance (Oct. 24–26, 1974. Tokyo).

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c; Gould & McLeod, 1960; Habs, 1957; Homma et al., 1951, 1970, 1974; Lányi, 1966–1967; Matsumoto et al., 1968; Verder & Evans, 1961; Wahba, 1965). The serological typing, however, requires skillful technique for the preparation of antisera and also requires careful observation on agglutination reaction. Phage typing method (Bergan, 1972 a, b, c, d, e, 1973 a, b; Bergan & Lystad, 1972; Farmer & Herman, 1969; Feary et al., 1963; Gould & McLeod, 1960; Graber et al., 1962; Hoff & Drake, 1961; Lindberg et al., 1964; Meitert, 1965; Postic & Finland, 1961; Sjöberg & Lindberg, 1968; Sutter et al., 1965) is easy to apply and does not require special techniques and special materials, but there were some faults in a usual phage typing method.

If we have a typing method which is accurate and reproducible as well as easy and simple, this would be used for a routine work. So, we intended to improve the phage typing method used by Sjöberg (Sjöberg and Lindberg, 1968) to be more accurate and reproducible.

Table 2. Bacterial strains used.

No. of Strains	Source	References
33	IFO Culture Collection	
22	Phage-propagating strains	33
7	Genetical study	
564	Clinical sources*	
81	Reference strains of serotyping**	24
Total 707		

* Strains collected and identified in the Mitsuhashi laboratory in Gunma University.
** Strains obtained from Prof. J.Y. Homma in the University of Tokyo.

Table 3. Phages used.

Group	Phage	Group	Phage
I	2 7 352	V	24 M4
II	44 109 1214	VI	21 F7
III	16 F8 E79*	VII	C11
IV	31 C188	VIII	119X
		Others	F116* M6 F10 73

All phages except both E79 and F116 were obtained from Dr. L. Sjöberg (26, 33) by courtesy of Prof. J.Y. Homma in the University of Tokyo.
* These two phages were isolated by Prof. B.W. Holloway (21) and supplied by Dr. M. Kageyama in the Mitsubishi-Kasei Institute of Life Sciences (E79), and by Prof. T. Iino in the University of Tokyo (F116).

Material and Methods

Bacterial strains and bacteriophages. Strains of *P. aeruginosa* and typing bacteriophages used are listed in Table 2 and Table 3, respectively. Single plaque isolation was made for each phage before stock propagation in order to exclude contamination, but was not made before mass-production to avoid selection of variants. All phages were isolated independently, but they were divided some groups by their nature as described later in Results and Discussion.

Media. For the cultivation, dilution or plating, the following three media were employed.

Nutrient broth (NB): Polypeptone(Daigo) 10 g, yeast extract(Daigo) 3 g, NaCl 2 g, and distilled water 1000 ml (pH was adjusted to 7.2 by 1N of NaOH solution).

Nutrient agar(NA): agar medium containing 1.5% of agar in NB.

Soft agar: agar medium containing 0.8% of agar in NB.

For the cultivation of bacteria, 0.4% of KNO_3 was added to NB in order to prevent the dissociation.

Phage propagation. Phage-propagating strains used were the set of bacteria obtained from Dr. Sjöberg together with phages except E79 and F116. Each phage except E79 and F116 has the individual propagating bacterium corresponding to each phage. In this study, Phages E79 and F116 propagated in the bacteria M88 and M10, respectively. All phages propagated on the lawns of the bacteria by the soft agar layer method. Phage lysates were sterilized by addition of chloroform except for the two phage lysates (E79 and F116) which were sterilized by the Millipore-filtration. The plaque forming units (PFU) were examined on the propagating bacteria. All lysates and diluted suspensions were stored at 5 C.

Phage typing. After shaking cultivation for 18 hr at 37 C, bacterial cultures to be tested were plated on NA plates with 4.5 ml of Soft agar and stored in refrigerator before testing. A drop (0.01 ml) of each of the 21 phage suspensions containing 1 to 3×10^5 PFU/ml was spotted on the test plate with an apparatus devised in our laboratory (IFO). After drying of the drops at room temperature, these plates were incubated at 37 C for 10 to 18 hr and checked the formation of plaques or lysis zones. The apparatus is provided with 20 syringes for the tuberculin test. Twenty phage suspensions are spotted onto a plate at the same time by the apparatus.

Prophage typing. A lysates was prepared from each strain after cultivation for 18 hr without inducer. In order to kill and lyse the cells, chloroform was added to the culture. Those lysates were spotted on 9 indicators without dilution. Indicators used in this typing were selected from about 200 strains. In Table 9, 6 indicators (I to VI) were selected for their sensitivities to many of the lysates and the remaining 3 strains (VII to IX) were selected for their sensitivities to the limited number of the lysates. In some cases the plates were replica-plated to the same indicator plates for

the differentiation of the plaques of phages (transferable) from the killing zone of pyocins (not transferable).

Results and Discussion

Phage typing

Table 4 shows the numbers of phage-sensitive strains among 707 strains tested. In the third column, the numbers of strains which indicated the weak or vague results are shown. Data for 4 phages, F116, M6, F10 and 73, were not used for the final classification procedure, because these 4 phages were only active to minor strains and did not show clear results. The remaining 17 phages were divided into 8 groups according to their high correlation of lytic spectra (Table 5) and similar sensitivity-patterns (Table 6). Most of the strains tested gave clear and reproducible results for the upper three phage groups, I, II and III (Table 4). Therefore, typing was performed primarily with these three phage groups.

Table 6 shows sensitivity-patterns of some strains to each phage. The sensitivity to each phage in one phage group is similar except minor differences. Therefore, in our typing method, phage-sensitivity to the group of phages was checked, instead of

Table 4. Numbers of strains sensitive to each phage.

Phage		C	NC	Total
I	2	153	19	172
	7	274	11	285
	352	265	10	275
II	44	238	39	277
	109	242	44	286
	1214	236	30	266
III	16	87	32	119
	F8	210	23	233
	E79	207	34	241
IV	31	52	115	167
	C188	58	89	147
V	24	101	13	114
	M4	87	37	124
VI	21	54	112	166
	F7	36	12	48
VII	C11	126	56	182
VIII	119X	144	35	179
Others	F116	20	10	30
	M6	25	12	37
	F10	7	18	25
	73	12	25	37

C: Clear results. NC: Not clear results.
Total strains examined are 707.

Table 5. Correlation between phages.

Phage	352	7	2	1214	44	109	E79	F8	16	C188	31	M4	24	F7	21	C11	119X	M6	F10	73	F116
352	100	96	55	25	28	34	34	33	21	10	11	19	22	6	11	25	24	3	1	3	3
7		100	55	26	28	34	34	34	21	10	11	19	22	6	11	25	26	3	1	4	4
2			100	28	30	36	31	30	26	9	9	30	25	8	10	27	25	3	9	6	4
1214				100	79	60	51	51	30	12	9	18	20	9	11	20	30	6	1	2	5
44					100	66	56	56	30	13	11	18	23	9	13	22	27	6	1	3	4
109						100	64	64	38	14	11	22	32	9	13	30	30	6	2	3	5
E79							100	99	36*	14	10	21	31	7	15	29	28	4	1	3	6
F8								100	37*	14	10	21	31	7	15	28	28	4	1	3	6
16									100	15	13	25	25	15	13	17	20	7	5	5	11
C188										100	53*	8	8	2	4	12	11	4	2	2	3
31											100	4	4	1	3	7	11	4	2	0	3
M4												100	56*	17	12	24	25	1	2	8	4
24													100	15	16	30	24	2	0	8	5
F7														100	24*	4	18	15	0	0	5
21															100	10	21	9	0	6	10
C11																100	22	4	3	7	5
119X																	100	8	2	3	10
M6																		100	0	0	14
F10																			100	0	4
73																				100	11
F116																					100

In this table, similarity index(%) (Bergan, 1972a, b) of the lytic spectra of phages on 707 strains of *P. aeruginosa* are shown.

similarity index $(S_{JK})=\dfrac{n_{JK}}{n_{JK}+n_{jK}+n_{Jk}}$

n_{JK}: total number of bacteria which are sensitive to both phage J and phage K.

n_{jK}: total number of bacteria which are sensitive to phage K and resistant to phage J.

n_{Jk} : total number of bacteria which are sensitive to phage J and resistant to phage K.

In this calculation, "sensitive" means positive reaction (+) and "resistant" contains negative (−) and weak-positive (±) reactions.

* In these combinations there were many weak-positive reactions. If the numbers of weak-positive reactions were added to numbers of positive reactions, higher values of S_{JK} as follows were obtained.

E79–16: (47%), F8–16: (52%), C188–31: (77%), M4–24: (62%), and F7–21: (61%).

Table 6. Sensitivity-patterns to phages.

Strain tested	Phage type	Phage group																
		I			II			III			IV		V		VI		VII	VIII
		2	7	352	109	44	1214	16	F8	E79	31	C188	24	M4	21	F7	C11	119X
GN3981	A6	+	+	+	+	+	+	+	+	+	−	−	+	+	+	−	+	+
GN4195	A23	−	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	+
M10	A25	−	+	+	+	+	+	+	+	+	−	−	−	−	+	+	−	+
GN4565	B20	+	+	+	+	+	+	±	−	−	+	+	−	−	−	−	+	−
GN4026	B30	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−	+	−
GN3328	C28	+	+	+	+	−	−	+	+	+	−	−	+	+	−	−	−	−
GN4585	D15	−	−	−	+	+	+	+	+	+	−	−	+	+	−	−	+	+
C11	D30	−	−	−	+	+	+	−	+	+	−	−	−	−	−	−	+	−
GN4561	E21	+	+	+	−	−	−	−	−	−	−	−	+	+	−	−	+	−
GN3955	F32	−	−	−	+	+	+	−	−	−	−	−	−	−	−	−	−	−
GN4578	G31	−	−	−	−	−	−	−	+	+	−	−	−	−	−	−	−	+
GN5051	H16	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+

+: Sensitive −: Resistant ±: Weak

phage-sensitivity to the individual phages. Consequently, we could exclude misjudgement of results and identify more simply. For example, when one member of a phage group showed vague or negative while two other members showed positive, the sensitivity to this group was defined as positive. We have some mutants of the bacterium which lost or newly possessed the sensitivity to all phages in the same phage group simultaneously. This is a reason why we used the phage groups instead of the individual phages. Numerical analysis by Bergan (1972 a, b) showed the correlation of typing phages. Some of his clusters of typing phages resemble to our phage groups. In his papers he did not use phage groups for typing but used a new set of phages in which each phage was selected as a representative from each cluster.

Primarily the bacterial strains to be tested were classified into main types A to H according to the patterns of sensitivity to the upper 3 phage groups in Table 7. Then these 8 types were divided into 1 to 32 subtypes systematically, according to the patterns of sensitivity to the remaining 5 phage groups (Table 7). A phage type is indicated as type A3 or type G12 and so on.

Table 8 shows the distribution of strains in each phage type. The type H32 is not sensitive to any phages of the 8 groups and is nontypable. So the H32 was separated from type H.

By this phage typing, 27.2% of strains tested were nontypable. Then we attempted to identify these nontypable strains by another typing method. By using other typing phage sets for their strains (Bergan, 1972 c, d, 1973 b), there were few nontypable strains, but reproducibilities were not good (Bergan & Lystad, 1972).

Table 7. Phage type.

Phage group	Main type A	B	C	D	E	F	G	H
I 2, 7, 352	+	+	+	−	+	−	−	−
II 44, 109, 1214	+	+	−	+	−	+	−	−
III 16, F8, E79	+	−	+	+	−	−	+	−

Phage group	Subtype 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
IV 31, C188	+	+	+	+	+	−	+	+	+	−	+	+	−	+	−	−
V 24, M4	+	+	+	+	−	+	+	+	−	+	+	−	+	−	+	−
VI 21, F7	+	+	+	−	+	+	+	−	+	+	−	+	+	−	−	+
VII C11	+	+	−	+	+	+	−	+	+	+	−	−	−	+	+	+
VIII 119X	+	−	+	+	+	+	−	−	−	−	+	+	+	+	+	+

Phage group	Subtype 17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32
IV 31, C188	+	+	−	+	−	−	+	−	−	−	+	−	−	−	−	−
V 24, M4	+	−	+	−	+	−	−	+	−	−	−	+	−	−	−	−
VI 21, F7	−	+	+	−	−	+	−	−	+	−	−	−	+	−	−	−
VII C11	−	−	−	+	+	+	−	−	−	+	−	−	−	+	−	−
VIII 119X	−	−	−	−	−	−	+	+	+	+	−	−	−	−	+	−

+: Sensitive −: Resistant

Table 8. Numbers of bacterial strains belong to each phage type.

Phage type	Phage group I	II	III	Sources* IFO	Clinical materials	(R)	Serotyping	Phage-propagating & genetical study	Total	(%)
A	+	+	+	12	68	(4)	19	8	106	15.0
B	+	+	−	3	16	(5)	0	1	22	3.1
C	+	−	+	1	20	(1)	0	1	22	3.1
D	−	+	+	7	55	(3)	12	6	80	11.3
E	+	−	−	4	110	(15)	14	1	129	18.2
F	−	+	−	3	51	(8)	8	5	70	9.9
G	−	−	+	1	16	(1)	0	0	17	2.4
H	−	−	−	2	52	(6)	13	3	69	9.8
(H32)	−	−	−	0	176	(24)	15	4	192	27.2
Total				33	564	(67)	81	29	707	100.0

* Sources are cited in Table 2.
(R): Numbers of strains harbouring R factors.

Prophage typing

Many strains of *P. aeruginosa* have one or more prophages and the detection of phages is easy (Farmer & Herman, 1969; Feary et al., 1963; Holloway, 1960; Paterson, 1965). In our laboratory (IFO) there are some series of *P. aeruginosa* mutants which were changed their phage types by treatment with a mutagen, nitrosoguanidine but those mutants belong to the original prophage groups (lysotype) as their parents. (In this paper, the term 'lysotype' means the lysogenicity type classified by a prophage typing.) So we intended to employ this prophage typing method for the subsidiary

Table 9. Lysotype.

Indicator bacteria		Main type 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
I	109	+	+	+	+	+	+	−	+	+	+	+	−	+	+	+	−	+	+	−	+	−	−
II	M6	+	+	+	+	+	−	+	+	+	+	−	+	+	+	−	+	+	−	+	−	+	−
III	Pd16	+	+	+	+	−	+	+	+	+	−	+	+	+	−	+	+	−	+	+	−	−	+
IV	M4	+	+	+	−	+	+	+	+	−	+	+	+	−	+	+	+	−	−	−	+	+	+
V	GN3310	+	+	−	+	+	+	+	−	+	+	+	+	−	−	−	−	+	+	+	+	+	+
VI	Pd18	+	−	+	+	+	+	+	−	−	−	−	−	+	+	+	+	+	+	+	+	+	+

Indicator bacteria		Main type 23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44
I	109	+	+	+	−	+	+	−	+	−	−	+	+	−	+	−	−	+	−	−	−	+	+
II	M6	+	+	−	+	+	−	+	−	+	−	+	−	+	−	+	−	−	+	−	−	+	−
III	Pd16	+	−	+	+	−	+	+	−	−	+	−	+	+	−	−	+	−	−	+	−	−	+
IV	M4	−	+	+	+	−	−	−	+	+	+	−	−	−	+	+	+	−	−	−	+	−	−
V	GN3310	−	−	−	−	+	+	+	+	+	+	−	−	−	−	−	−	+	+	+	+	−	−
VI	Pd18	−	−	−	−	−	−	−	−	−	−	+	+	+	+	+	+	+	+	+	+	−	−

Indicator bacteria		Main type 45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64
I	109	−	+	−	−	+	−	−	−	+	−	−	−	−	+	−	−	−	−	−	−
II	M6	+	−	+	−	−	+	−	−	−	+	−	−	−	−	+	−	−	−	−	−
III	Pd16	+	−	−	+	−	−	+	−	−	−	+	−	−	−	−	+	−	−	−	−
IV	M4	−	+	+	+	−	−	−	+	−	−	−	+	−	−	−	−	+	−	−	−
V	GN3310	−	−	−	−	+	+	+	+	−	−	−	−	+	−	−	−	−	+	−	−
VI	Pd18	−	−	−	−	−	−	−	−	+	+	+	+	+	−	−	−	−	−	+	−

Indicator bacteria		Subtype 1	2	3	4	5	6	7	8
VII	31	+	+	+	−	+	−	−	−
VIII	F7	+	+	−	+	−	+	−	−
IX	Pd173	+	−	+	+	−	−	+	−

+ : Positive
− : Negative

method of phage typing. Lysotypes were classified into 64 main types and then into 8 subtypes (Table 9). By combining use of these two typing methods the percentage of nontypable strains decreased to 3.7% from 27.2% which was obtained by using the phage typing only.

Application of Phage typing

In Table 10 there are 14 strains isolated from the urine of six patients. These strains are traceable to their origins. A few drug resistance markers occasionally changed but both phage type and lysotype were rather stable. In the last case in Table 10, the difference in both phage type and lysotype of strains are apparent, even though they were isolated from the same patient. It is considered that these two strains are not the same because of their remarkable difference in their drug resistance along with their phage type and lysotype.

Table 10. Phage type of *P. aeruginosa* from the urine of patients.

Patient	Date	Phage type	Lyso-type	Drug resistance SA	TC	CM	SM	KM	NM	GM	LVM	CPC
4–T.M.	14 Aug. '72	H–32	55–8				r	r			r	
	23 Aug. '72	H–32	55–8	r			r	r				
4–M.U.	3 Oct. '72	H–32	6–7				r	r		r	r	
	16 Oct. '72	H–32	6–7		r	r	r	r		r	r	
	6 Nov. '72	H–32	6–7		r		r	r		r	r	
1–H.T.	9 Jan. '73	H–31	1–8							r		
	12 Jan. '73	H–31	1–8							r		
14–K.H.	4 May '73	F–28	37–2	r			r	r			r	
	8 May '73	F–28	37–2	r			r	r			r	
14–T.M.	3 Jan. '73	D–15	6–7		r							r
	5 Mar. '73	D–15	6–7		r					r		r
	6 Apr. '73	D–15	6–7	r								r
1–Y.H.	8 Jan. '73	B–20	59–8				r		r	r	r	r
	3 Apr. '73	H–5	29–6	r		r		r		r		

SA: Sulfanilamide TC: Tetracycline CM: Chloramphenicol SM: Streptomycin
KM: Kanamycin NM: Neomycin GM: Gentamicin LVM: Lividomycin
CPC: Carbenicillin r: Resistant

The relation between phage type and serotype are shown in Table 11. The correspondences of serotypes of Habs' strains, of Lanyi's strains, and of strains of Verder and Evans to the Homma's serotypes (from 1 to 15) were examined and arranged by Homma (1974). In Table 11, the strains which were arranged by the above data were identified by the phage typing. For example, the serotype of Habs' reference strain 13264 which corresponds to the Homma's serotype 1 was determined by the phage

Table 11. Phage typing of the reference strains of serotyping.

Serotype (Homma)	Homma		Habs		Lányi		Verder & Evans	
	Strain No.	Phage type	Strain No.	Phage type	Strain No.	Phage type	Strain No.	Phage type
1	IID1001	A27	13264(3)	A30	170001(1) 170017(8)	A20 D32	58F(6)	D32
2	IID1002	D32	A486(5)	E31	170007(3)	D32	G2312(10)	H32
3	IID1003	H27	5940(8) 14811(9)	E32 E32	170011(5) 170012(5) 170013(5)	E32 E32 E32	T488(8)	E32
4	IID1004	A4	——		170019(10) 170020(10)	A6 H3	T6370(9)	A30
5	IID1005	H27	121(11)	H32	170015(7) 170016(7)	H32 F16	Lawson(1) 2108(3) 1174(7)	H28 H31 H32
6	IID1006	H26	5936(4)	H26	170021(11)	H26	——	
7	IID1007	F32	4821(2)	F32	170006(3)	H32	2243(1)	F31
8	IID1008	D2	5939(6)	D32	170008(4) 170009(4) 170010(4)	A30 A30 A26	1369(2)	D32
9	IID1009	F27	U3433(10)	F32	170002(2)	F12	——	
10	IID1010	A3	5933(1)	A6	170014(6)	A29	Mills(4)	A1
11	IID1011	H32	——		170022(12)	H32	——	
12	IID1012	E32	——		——		1M(5)	H32
13	IID1013	H32	——		170003(3) 170005(3)	D32 A31	359(1)	D12
14	IID1014	H32	5945(12)	H32	170023(13)	H32	2915(7)	H32
15	IID5018	H32	——		170018(9)	H32	——	

Number in a parenthesis indicates the serogroup of Habs, of Lányi, or of Verder & Evans. Relation of these serogroups was examined and arranged by Homma (1974).

typing as type A30. The letters such as A27, D32 or F31 indicate the phage type determined by these procedures. The similarity of phage types in a same serogroup can be read in this Table, and it may reflect the existence of the similar site on the surface of the bacteria reacting to the both phage and serum. And there are a few differences of phage types in the same serogroup, or there are the same phage types among different serogroups in Table 11. Perhaps, it means the differences between serological typing and phage typing.

Because phage typing is affected not only by the difference of the surface material but also by the immunity or the restriction mechanism. In addition, the prophage typing method did not give a clear result for classification of serotypes.

Species specificity of the phage typing

In the Culture Collection of IFO, there are 43 species of Pseudomonadaceae other than *P. aeruginosa*, and their phage types were examined (Table 12). Some strains of *P. maltophila, P. nitroreducens, P. polycolor, P. solanacearum* and *P. vendrelli* were sensitive to the typing phages while other strains did not react. So, it is noticed that our typing system is specific for *P. aeruginosa* and its closely related species.

It is considered that our improved typing system will be an easy and convenient tool for the identification of strains belonging to *P. aeruginosa.*

Table 12. Test of sensitivity to typing phages of strains belong to Pseudomonadaceae.

Species	No.		Species	No.	
Pseudomonas aeruginosa	707*	+	*Pseudomonas mildenbergii*	1	−
P. alkanolytica	2	−	*P. nitroreducens*	2	+1, −1
P. aptata	1	−	*P. oleovorans*	1	−
P. aureofaciens	5	−	*P. ovalis*	8	−
P. auricularis	2	−	*P. oxalaticus*	1	−
P. caryophylli	1	−	*P. pavonacea*	2	−
P. chlororaphis	2	−	*P. phaseolicola*	1	−
P. convexa	3	−	*P. polycolor*	1	+
P. coronafaciens	1	−	*P. putida*	5	−
P. cruciviae	2	−	*P. putrefaciens*	5	−
P. dacunhae	3	−	*P. reptilivora*	1	−
P. denitrificans	2	−	*P. riboflavina*	2	−
P. desmolytica	1	−	*P. schuylkilliensis*	2	−
P. diminuta	1	−	*P. solanacearum*	1	+
P. fluorescens	11	−	*P. striafaciens*	1	−
P. fragi	2	−	*P. striata*	1	−
P. indoloxidans	2	−	*P. synxantha*	3	−
P. iodinum	1	−	*P. stutzeri*	2	−
P. maltophilia	1	+	*P. tabaci*	1	−
P. malvacearum	1	−	*P. taetrolens*	1	−
P. marginalis	2	−	*P. trifolii*	1	−
P. melanogenum	2	−	*P. vendrelli*	1	+

* These contain 192 of nontypable strains.

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FUNGUS FLORA OF LAKE SEDIMENT

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Summary

Fungus flora of the sediment of Lake Sengari was investigated throughout the years. The sediments were collected from six stations of the lake once a month and the isolation of fungi was carried out. As a result, eighty six genera of fungi have been obtained, which mostly consist of Ascomycetes and Fungi Imperfecti. Common terrestrial fungi such as *Alternaria* and *Cladosporium* are rather rare in the sediment, while those of the ascomycetes such as *Eupenicillium, Gymnoascus, Neosartorya, Talaromyces* and *Westerdykella* are characteristically dominant in the deepest place.

Introduction

Fungi are known to exist in aquatic sediments, marine and limnic, and the sedimentary deposits in lakes have been extensively studied in several countries. However, there have not been much information on the fungi in lakes except for special fungus groups such as those found among the Mastigomycotina and the aquatic Hyphomycetes. Only so-called "water molds" or "aquatic fungi" have been investigated extensively both qualitatively and quantitatively. In other cases, only fragmental informations on those fungi in the lake sediments are available in the literature published for recording the substrate. Many kinds of fungi have been carried to the sediments because they are washed into lakes from surrounding land and may remain dormant in the sedimentary muds. Their sediments have been accumulated since the lake was formed, containing a variable amount of organic matters derived from the land surface and organisms inhabiting in the water of the lake. Naturally, many of these fungi from the land must have died because of unfavourable conditions of the sediment, but some survive for a long period of time even under the low temperature and anaerobic conditions prevailing. From the previous studies (3), many individual species have been encountered in the sediments of seawater and brackish water. Then we have tried to accumulate the data of the fungi from the fresh water of the lake. The purpose of the present paper is to elucidate the fungus flora of the lake sediments throughout the year and to give the data of the isolation of such fungi in relation to the physical and biological features of the lake.

Materials and Methods

Lake Sengari is located in Hyogo Prefecture, a western part of Japan, and is an artificial reservior for tap water for the residents of Kobe City. The foundations of

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the dam were laid in 1919. As shown in Plate 1 and Fig. 1, the lake is narrow, some three kilometers long, and meandering. Two rivers flow into the lake at the northern ends and the whole margin is surrounded by the natural forest mainly with pines and shrubs. Sediment samples were obtained from the following strations as indicated in Pl. 1 and Fig. 1: Hazu (St. 1), Hatsuka (St. 2), Goryu (St. 3), Gunkai (St. 4), Zendana (St. 5) and Shusui (dam) (St. 6). The samples were collected by the staff of the Water Quality Laboratory of the Kobe Water Works nearly once a month during the period of September, 1972, through December, 1973, from the bottom of the six stations with an average depth of 3, 5, 8, 15, 27 and 31 meters respectively as already indicated (4). The sediments were returned to the laboratory of the Institute for Fermentation as

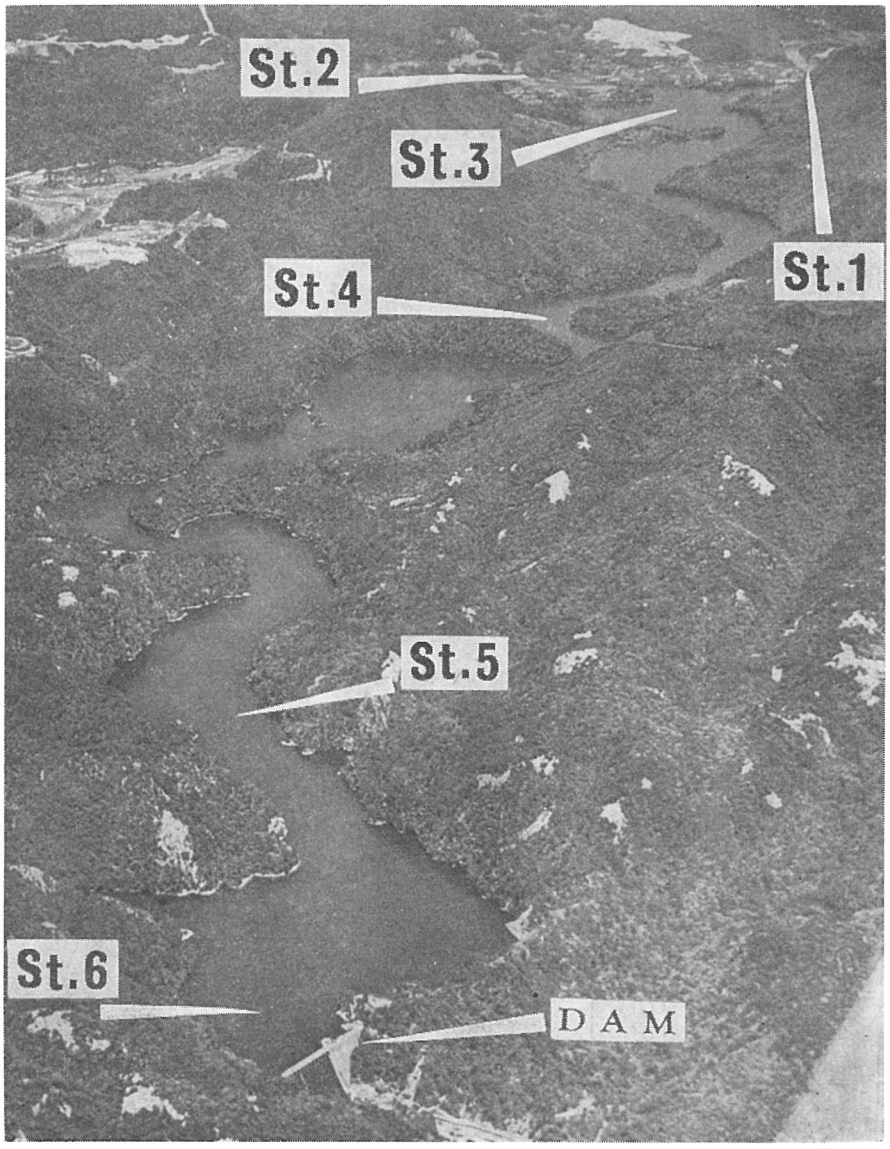


Plate 1. A bird's view of Lake Sengari.
Collecting stations are indicateN by white arrows.

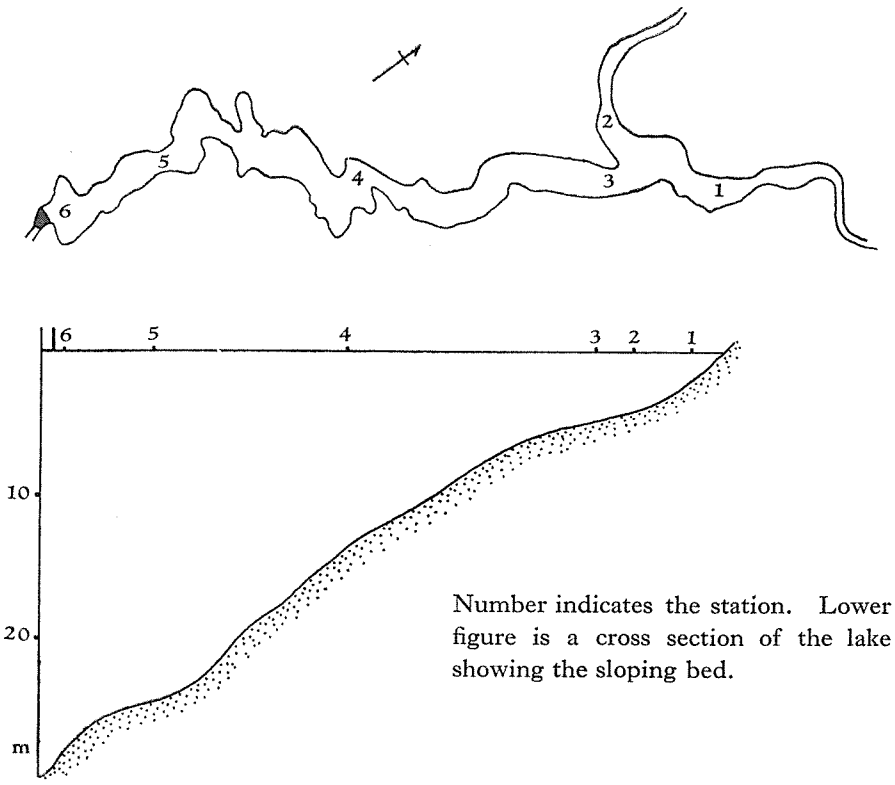


Fig. 1. Map of Lake Sengari surveyed.

quickly as possible and the isolations were carried out by spreading each 0.1 ml of the heavy aqueous sediment-suspension (approximately 3 g of each wet sediment was aseptically transferred to 5 ml of sterilized water) onto an agar plate for the isolation*. The plates were incubated at 25 C for three days. Then the plates were scanned carefully on the whole surface under the dissecting microscope (×30–50) and all colonies developed were transferred to agar slants. Even very small or very slow growing colonies, hardly recognizable with the naked eye, can be thus found through this procedure. In addition to above method, heat- and alcohol-treatment procedures were also applied. The heat-treatment was carried out by heating the above-mentioned heavy suspension at 80 C for 15 minutes, then 0.2 ml of each was spread onto the same agar plate. The alcohol-treatment was carried out by adding 5 ml of absolute ethyl-alcohol to each 5 ml of the sediment suspensions and mixing thoroughly for 15 minutes, then 0.2 ml of each suspension was spread on the plate. These two procedures are effective for the isolation of the ascomycetous fungi.

The depth of water, temperature, pH and the dissolved oxygen were recorded just above the bottom at each station by Yano. The methods and techniques employed for the determination of each element were as follows: Temperature: Thermometer,

* glucose 1%, malt extract 0.3%, yeast extract 0.3%, peptone 0.5% and agar 1.5%; 50 μg/ml of tetracycline was added to the medium.

standard; pH: Glass electrode pH meter (Hitachi); Dissolved oxygen: Winkler's method.

The estimation of the number of fungi per gram of the sediment was made from January through December of 1973 by Matsuda by diluting each 5 g of the sediment samples with 45 ml of sterilized water and incubating each 1 ml of the suspension onto the plates of potato-sucrose, yeast-malt extract, glucose peptone, Czapek's, and Waksman's agar media respectively. All plates were incubated at 25 C for one to three weeks before fungus colonies are counted, and then the average number of the colonies per gram of the sediment was obtained.

Results

Taxonomy

As might be expected, the sediments of the lake provide a large variety of fungi. In addition to the thermophilic fungi, reported previously (4), there are many terrestrial fungi. Eventually over 170 species of fungi representing 86 genera of the Zygomycotina, Ascomycotina and Deuteromycotina were recorded from the sediments of Lake Sengari in which six stations were set up. Many isolates were also obtained which provide with clamp-connections on the mycelium and must be the mycelial states of the Basidiomycotina. Species of the fungi isolated are listed in Table 1 and the frequency of the occurrence in the sediments is indicated by ○, ◎ and ● in the table. Those indicated with ○ have been isolated less than three occasions from each station during the period of isolation; ◎, four or five occasions; ●, over six occasions. Those species of the thermophilic fungi were also included in this table and are indicated by *. Those species which were found for the first time in Japan are underlined and are described elsewhere.

Zygomycotina

Many isolates of *Absidia* belong to *A. corymbifera* and are less common in its occurrence in the sediment.

Circinella-species are fairly rare and two isolates are *C. simplex* and another one is *C. mucoroides.*

Among the species of the genus *Mortierella* isolated, those of the Section Isabellina are very common and have been found at all the stations. Among them, *M. ramanniana* is predominant. One distinct species, listed as *Mortierella* sp. No. 1, is significant in its morphological properties. It is isolated very often from the sediments, and the growth is flat, tough and leathery, and pale pinkish brown in its surface color. Sporangiophores are numerous, and very short, less than 60 μm, and the sporangia are predominantly three-spored. Sporangiospores are very peculiar in that they are always of the *Camellia*-seed shape even after liberated, consisting of two flat and one roundly curved planes. This species seems to be close to *Mortierella*- or *Haplosporangium*-species. However, it does not fit to any known species of both genera in many respects.

Table 1. List of fungi isolated from the sediments of Lake Sengari.

species \ station	1	2	3	4	5	6
Zygomycotina						
Absidia spp.		○	○	○	○	○
Circinella simplex v. Tieghem	○				○	
Circinella umbellata v. Tieghem & Le Monnier				○		
Gongronella butleri (Lendner) Peyr. & D. Vesco		○				
Mortierella sp. No. 1	○		○	○	○	◎
Mortierella spp.	○	○	○	○	○	○
Mortierella ramanniana (M.) Linn.	○	○	◎	○	○	○
Mucor circinelloides v. Tieghem		○			○	
M. genevensis Lendner	○					
M. hiemalis Wehmer	○	○				○
M. pusillus Lindt*	○		○	○	○	
M. racemosus Fres.				○		○
Rhizopus spp.			○	○	○	○
Rhizopus sp.*(thermophilic)	○	○	○	○	●	◎
Zygorhynchus moelleri Vuill.					◎	
Basidiomycotina						
Basidiomycetes	○	○	○	○	○	○
Basidiomycetes No. 1.					○	○
Ascomycotina						
Chaetomium funicola Cooke					○	○
C. fusiforme Chivers	◎	◎	○	○	○	
C. globosum Kunze & Fr.	○	○				○
C. indicum Corda					○	
C. subspirale Chivers						○
C. torulosum Bainier						◎
C. turgidopilosum Ames						○
Coniochaeta sp.	○			○	○	
Emericella nidulans (Eidam) Vuill.*						○
Emericellopsis microspora Backus & Orpurt	◎	○	○	◎	○	○
E. minima Stolk				○	○	
E. salmosynnemata Groskl. & Sw.	○					
Eupenicillium brefeldianum (D.) Stolk & Scott				○		○
E. javanicum (B.) Stolk & Scott	○	○			○	●
E. ornatum Udagawa	○				○	
E. pinetorum Stolk	○					
E. shearii Stolk & Scott						○
E. spp.	○			○	○	
Eurotium rubrum Konig et al	○			○	○	
E. tonophilum Ohtsuki						○

Table 1. (continued)

species \ station	1	2	3	4	5	6
E. umbrosum (B. & S.) Malloch & Cain		○				
Gymnoascus roseus (Raillo) Apinis			○	◎	○	◎
Hamigera avellanea (T. &. T.) Stolk & Samson*				○		
Myxotrichum cancellatum Phillips					○	
Neosartorya fischeri (W.) Malloch & Cain			○			○
N. fischeri var. *glabra* (F. & R.) M. & C.*	○	○	◎	●	◎	◎
N. quadricincta (Y.) Malloch & Cain*				○		
Pseudeurotium ovalis Stolk	◎		◎	◎	◎	
P. zonatum v. Beyma	◎	◎	◎	◎	◎	◎
Sordaria spp.				○	○	○
Talaromyces emersonii Stolk*		○				○
T. flavus var. *flavus* Stolk & Samson	◎	◎	●	●	●	●
T. flavus var. *macrosporus* Stolk & Samson				○		
T. trachyspermus (S.) Stolk & Samson*	○	○			○	○
T. wortmannii C. R. Benjamin		○	○	○		○
Thermoascus aurantiacus Miehe*				○	○	
Thielavia minor (R. & B.) Malloch & Cain		○				
T. terricola (G. & A.) Emmons			○	○	○	○
Westerdykella dispersa (C.) Cejp & Milko			○	○		
W. multispora (S. & M.) Cejp & Milko	○	○	◎	●	●	●
Zopfiella leucotricha (Sp.) Malloch & Cain			○			
Deuteromycotina						
Acremonium butyri (v. Beyma) W. Gams				○		
Acrophialophora fusispora (Saksena) Samson			○			
Alternaria alternata (Fr.) Keissler	○					
Arthrinium phaeospermum (Corda) Ellis				○		
A. sphaerospermum Fuckel		○				
A. spp.	○	○	○			
Aspergillus cervinus (Massee) Neill						○
A. clavatus Desm.		○	○	○	○	○
A. flavus-oryzae group			○			
A. fumigatus Fres.*	◎	◎	●	●	●	●
A. niger v. Tieghem				○		
A. parvulus Smith			○	○		
A. sydowi (B. & S.) Thom & Church				○		
A. tamarii Kita						○
A. terreus Thom			○	○		
A. versicolor (V.) Tirab.	○					
Aureobasidium pullulans (d.B.) Arnaud		○	○	○		○
Beauveria sp.					○	

Table 1. (continued)

species \ station	1	2	3	4	5	6
Cephalosporium spp.	○	○	○	○	○	○
Chaetophoma spp.			○	○		
Chloridium chlamydosporis (v.Beyma) Hughes	○	○				○
C. *viride* Link ex Link			○			
C. sp.		○				
Chrysosporim merdarium (Link) Carm.			○			
C. *pannorum* (Link) Hughes	○	○	○	○	○	●
Cladosporium cladosporioides (Fres.) de Vries	○					
Clonostachys cylindrospora Arnaud	○	○				○
Coniothyrium spp.	○	○	○	○	○	○
Curvularia clavata Jain	○					
Cylindrocladium sp.		○				
Dactylaria sp.					○	
Diplodina sp.	○			○		
Doratomyces microsporus (Sacc.) Mort. & Smith	○					○
Fusarium oxysporum Schl.	○					
F. spp.	○	○	○	○	○	○
Fusicoccum sp.				○		
Geotrichum candidum Link	○	○	○	○	○	○
Gilmaniella humicola Barron		◎			◎	○
Gliocladium catenulatum Gilm. & Abb.	○		○	○		○
G. *roseum* Bainier		○	○			○
G. *vermoeseni* (Biourge) Thom		○				
G. *virens* Miller et al	○					
G. spp.	○	○	○		○	○
Gliomastix murorum var. *felina* (M.) Hughes					○	○
Gloeosporium sp.		○				
Gonytrichum macrocladum (Sacc.) Hughes	○	◎				○
Graphium sp.						○
Humicola spp.	○	○	○	○		○
Hyalodendron sp.				○		
Hyalopycnis sp.	○					
Isaria spp.		○	○			○
Leptodiscella africana (P.) Papend.		○				
Leptographium spp.				○	○	
Macrophoma sp.	○					
Malbranchea pulchella var. *sulfurea* (M.) C. & E.*			○	○	○	○
Mammaria echinobotryoides Ces.			○		◎	
Melanconium sp.			○			
Metarrhizium anisopliae (M.) Sorokin		○				○

Table 1. (continued)

species \ station	1	2	3	4	5	6
Myrothecium roridum Tode ex Fr.	◎	○	◎		○	◎
Oidiodendron griseum Robak					○	○
O. *truncatum* Barron				○		
O. sp.		○				
Paecilomyces elegans (Cda.) Mason & Hughes	○					
P. *marquandii* (Mass.) Hughes				○		
P. *varioti* Bainier		○				
P. sp.	○	○	○	○		
Penicillium charlesii Smith						○
P. *citrinum* Thom	○	○			○	
P. *claviforme* Bainier					○	
P. *clavigerum* Dem.					○	
P. *commune* Thom		○				
P. *corylophiloides* Abe		○				
P. *corylophilum* Dierckx	○		○	○		○
P. *crustosum* Thom					○	
P. *cyaneum* (B. & S.) Biourge					○	
P. *cyclopium* Westling			○			○
P. *decumbens* Thom		○				
P. *digitatum* Saccardo				○		
P. *expansum* Link	○					
P. *frequentans* Westling						○
P. *funiculosum* Thom	◎	◎	○	○	◎	◎
P. *herquei* Bain. & Sart.			○			
P. *implicatum* Biourge					○	
P. *janthinellum* Biourge	○	○	○	○	○	○
P. *jenseni* Zaleski		○				
P. *lanoso-viride* Thom			○			
P. *lanosum* Westling						○
P. *lilacinum* Thom		○		○		
P. *lividum* Westling	○					
P. *multicolor* G. M. & P.	○	○	◎	○	○	○
P. *nigricans* (Bain.) Thom		○			○	◎
P. *oxalicum* Currie & Thom	○	◎	◎	◎	◎	◎
P. *paraherquei* Abe ex Smith						○
P. *purpurogenum* Stoll					○	
P. *purpurogenum* var. *rubri-sclerotium* T.						○
P. *rubrum* Stoll	◎		○	○	○	
P. *rugulosum* Thom		○	○			○
P. *stoloniferum* Thom					○	

Table 1. (continued)

species \ station	1	2	3	4	5	6
Penicillium tardum Thom	○	○	○		○	○
P. thomii Maire	○				○	○
P. trzebinskianum Abe						○
P. velutinum v. Beyma						○
P. verruculosum Peyronel	○	○	○	◎	○	○
P. spp.	○	○	○	○	○	○
Phialocephala humicola Jong & Davis	◎	◎				
Phialophora alba v. Beyma	○		○			
P. jeanselmei (L.) Emmons		○	○	○	○	●
P. spp.	○	○	○	○	○	○
Phoma violacea (Bertel) Eveleigh				○		
P. spp.	○	○	○	○	○	○
Phomopsis sp.	○					
Pyrenochaeta spp.	○	○				○
Rhinocladiella sp.	◎	◎				
Scolecobasidium constrictum Abbott	○					
S. humicola Barron	○		○			
S. terreum Abbott	◎		○	○	○	○
Scopulariopsis brevicaulis (Sacc.)Bain.		○				
Scopulariopsis sp.	○					
Sporothrix sp.	○	○	○	○	○	○
Sporotrichum thermophile Apinis*				○		○
Staphylotrichum coccosporum Meyer & Nicot		○				
Stilbum sp.	○	○	○		○	○
Thermomyces lanuginosus Tsiklinsky*	○		○	○	○	○
Trichoderma viride complex	◎	○	◎	◎	◎	○
Torula caligans (Batista et al) Ellis				○		
Torulomyces lagena Delitsch						○
Verticllium luteo-album (Link ex Fr.) Subr.					○	○
V. sp.		○				
Volutina concentrica Penzig & Sacc.	○					
Mycelia Sterilia	○	○	○	○	○	○

Description of the species will be given elsewhere.

Not frequent, but *Mucor* has been found in all the stations. A thermophilic species, *M. pusillus*, has been isolated in many occasions.

Rhizopus sp., a thermophilic species, is predominant, isolated mostly from deeper samples. These thermophilic isolates are close to *R. nigricans*, but are strongly thermophilic as described previously(4). Generally the mucoraceous fungi are rather uncommon in the sediments so far as investigated.

Ascomycotina

Seventeen genera have been encountered during the present investigation. Many of them were obtained also through the isolation procedures of the heat- and alcohol-treatments of the samples.

Chaetomium torulosum is exceptional among the seven species of the genus isolated in that it has been isolated over three occasions only from the deepest place, St. 6. *C. fusiforme* also has been not infrequent species from rather shallower places.

Emericellopsis microsporus has been predominant among the species of the genus isolated. This genus is known as a common fungus in the aquatic sediment, marine or limnic (3). *E. salmosynnemata* was rarely found. Generally *Emericellopsis*-species seem to be distributed in rather deep places in the lake.

In *Gymnoascus*, only one species, *G. roseus*, was encountered and this species seems to be distributed in rather deeper places.

Eupenicillium is not infrequent during the study. *E. javanicum* is predominant, and is peculiar because of its occurrence in the deepest place of the lake.

Eurotium rubrum is predominant among the species of the genus isolated, but the *Eurotium*-species is generally not so commonly found from the sediment although the members of this genus are so common on land.

In *Neosartorya, N. fischeri* var. *glabra* is peculiar in its distributional pattern. This species has been found in all the stations, especially over three occasions in Sts. 3, 5 and 6, and over six occasions in St. 4.

Pseudeurotium is significant in the occurrence because both species, *P. ovalis* and *P. zonatum*, have been found in all the stations in more than three occasions from most of the sediments collected. These species must be dominant fungi in the sediment of the lake throughout the year.

Sordaria-species, commonly known as coprophilous fungi, and *Thielavia terricola* have been found also from only deeper places, Sts. 4, 5 and 6.

Talaromyces flavus var. *flavus* is also significant in the distributional pattern. It has been found in over three occasions in Sts. 1 and 2, and over six occasions in Sts. 3, 4, 5 and 6. Only this species seems to distribute commonly in rather deep places.

Westerdykella multispora has been found in the relatively deep places only. It has been isolated in over three occasions in the sediments of St. 3 and over six occasions in Sts. 4, 5 and 6.

Deuteromycotina

Nearly sixty two genera of the Deuteromycotina have been encountered. Among them, coelomycetous fungi, such as *Coniothyrium* and *Phoma*, are not uncommon in all the stations.

Unexpectedly *Aspergillus* is not so common in the sediments and only ten species have been encountered. Among them, *A. clavatus* is quite significant in its distributional pattern and has been found nearly in all the stations except St. 1. *A. fumigatus* is extremely common as in the terrestrial case, and has been isolated in over three occasions in Sts. 1 and 2, and over six occasions in Sts. 3, 4, 5 and 6. This species has been also isolated as the thermophilic fungi very often (4). Other very common terrestrial members such as *A. flavus-oryzae* group, *A. niger, A. sydowi, A. terreus,* and *A. versicolor* have been rather rare in the sediments so far as investigated.

Cephalosporium is also common throughout the study as was expected. Many of the isolates might be the conidial states of *Emericellopsis* (3).

Chrysosporium pannorum has been found in all the stations, especially in the deepest place, St. 6, in over six occasions. This species is known as a psychrophilic and provided with thicker walled conidia, and therefore is probably able to tolerate the lower temperature and the water pressure in the deepest bottom. The occurrence of this species must have a connection with that of *Gymnoascus* which has been found in the deeper places very often.

Geotrichum candidum has been well known as the most common sewage and polluted water fungus. However, in the present study, the occurrence of this species is apparently not so frequent.

Gilmaniella humicola has been frequently isolated in Sts. 2 and 5. The thick walled conidia must be able to tolerate those conditions.

Myrothecium roridum seems to be a common species in the lake, and has been isolated very often from the sediments, over three occasions in Sts. 1, 3 and 6.

Paecilomyces-species has been rarely isolated although it is very common in the land like *Aspergillus*.

Penicillium is very commonly found throughout the study in contrast with *Aspergillus*. Thirty seven species have been isolated. Among them, following species were predominant: *P. janthinellum, P. tardum, P. verruculosum, P. rubrum, P. funiculosum, P. oxalicum,* and *P. multicolor*. *P. nigricans* is peculiar in that it has been siolated only from the deepest place, St. 6, over three occasions.

Trichoderma is very common as was expected and is treated here as the *Trichoderma viride*-complex. This group, distributed widely in the lake as on land, probably has been introduced from the marginal habitats of the lake.

All isolates of *Phialocephala* belong to *P. humicola* which was originally isolated by Jong & Davis (2) from the soil of Cape May, New Jersey.

Phialophora is generally known as not uncommon in the aquatic habitat, and, in the present study, it was also found very often. Among the species isolated, *P. jeanselmei*

has been found in nearly all the stations, especially in St. 6 in over six occasions.

Basidiomycotina

Basidiomycetous fungi have been isolated frequently. The fact that the basidiomycetous fungi like other groups are so common in the sediment is reasonable because many of the fungi treated here are largely terrestrial washed away by the rain action. In the case of the basidiomycetous fungi, they are considered to be merely transients in nature. Unfortunately all isolates of such do not develop fruitbodies under culture and no generic name can be applied to them. So far as investigated, these isolates have a tendency to develop the conidia from hyphae provided with clamp-connections. Conversely those which develop the conidia might have grown on the isolation medium. Among them, some isolates are significant because of the conidia-bearing fructifications. Such conidia develop within the spherical, globose mycelial mass which looks like pycnidia in appearance lined by mycelium. However the structure of the fructification is not ostiolated and not pseudoparenchymatous but is only surrounded by the loose mycelial network. Conidiogenous cells are similar to those of microconidia of *Riessia* (1). Additional isolations of this species have been also made from the aquatic sediments of other lakes and a description will be given elswhere.

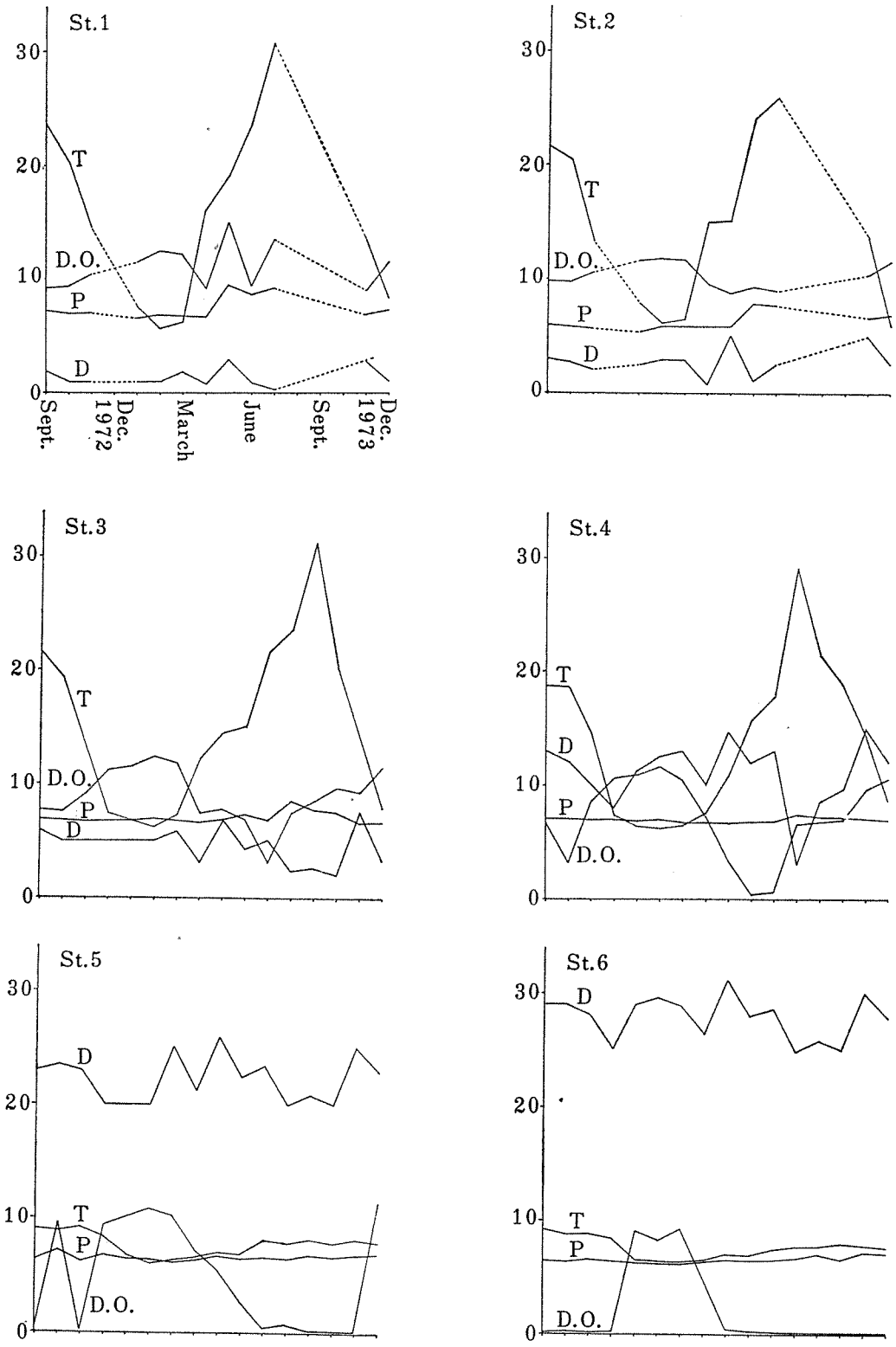
Physical data

The vertical change of those elements of water temperature, pH, dissolved oxygen and depth of water are summarized in Fig. 2. The number of fungi in the sediment of each station, examined by Matsuda, one of the authors, are summarized in Fig. 3.

Discussion

A shallow lake is generally known as fertile and supports many living organisms while a deep lake is rather barren and has been considered to produce only a few organisms. Lake Sengari is long and narrow with a gentle current of water from north to south, shallower (1–3 m) at the northern and deeper (28–30 m) at the southern end where a dam is settled. Because of the nature of this lake, general knowledges on living organisms is also applicable. What is obvious for the reader in comparing the data shown in Fig. 2 and 3 is the generally similar pattern of the occurrence of fungi among the stations. Sts. 1 and 2, 3 and 4, and, 5 and 6 are somewhat similar to each other from the view-point of physical data. The number of fungi obtained by the plate method is shown in Fig. 3, and this estimate refers to the number of viable cells and mycelial fragments in the sediments samples capable of growing rapidly on the agar medium employed for the isolation. Therefore, the number of fungi shown in Fig. 3 is different in the contents of species from that demonstrated in Table 1. Fungi shown in Table 1 include those found by the plate method and also those recognized on the agar medium under the dissecting microscope as described previously.

The present Lake Sengari is somewhat different from other lakes. Lake Sengari



T...water temperature (C) D.O....dissolved oxygen (mg/l)
P...pH D...depth of water (m) dotted line...no sample was obtained

Fig. 2. Physical data of the water in each station.

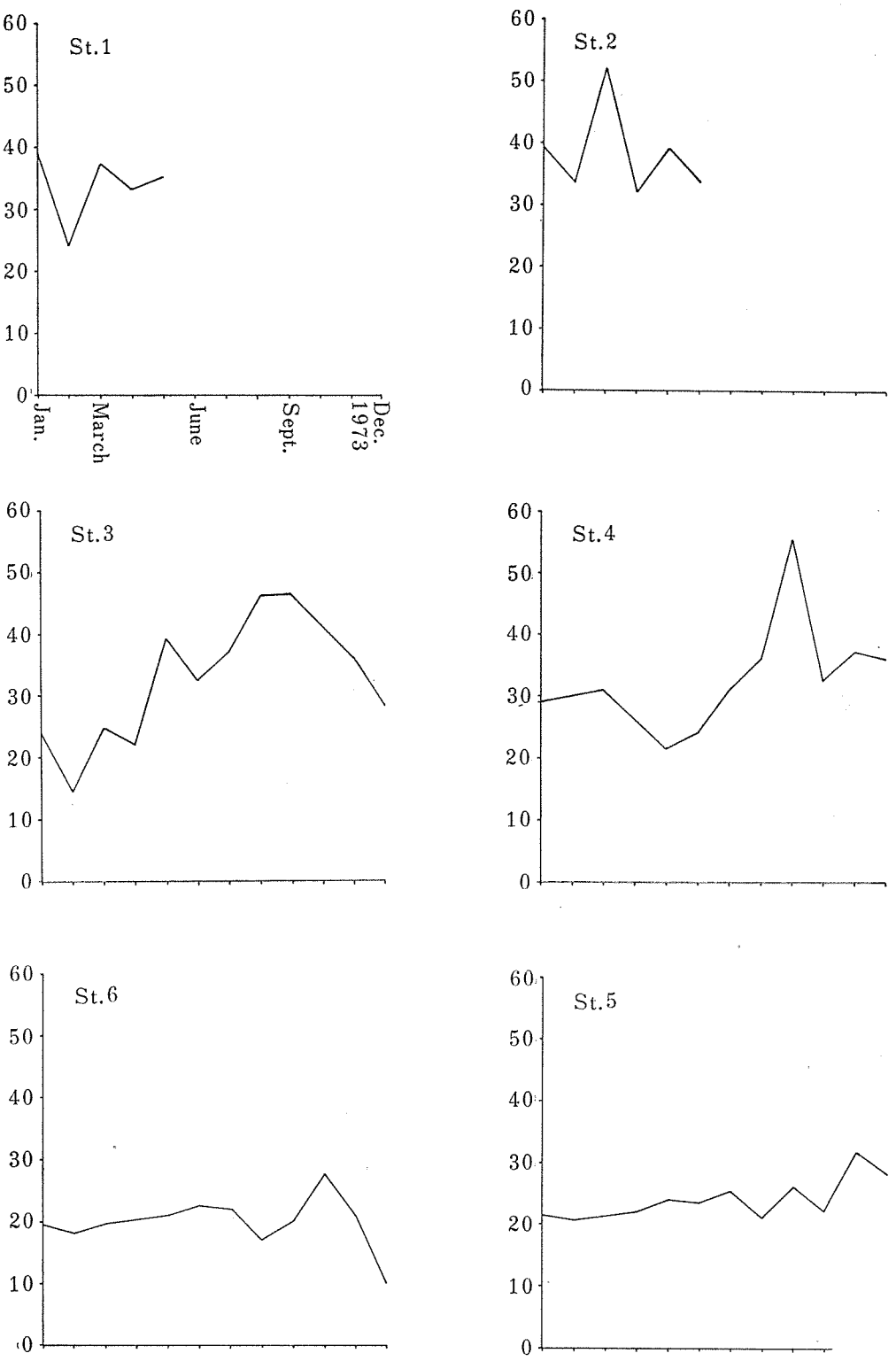


Fig. 3. Seasonal change of the number of fungi per gram of the sediment in each station.

is a dammed-up artificial reservior and was originally an abysmal ravine. So, as shown in Fig. 1, the bottom of the lake is sloping gradually and has been always washed by a gentle current of the water. These conditions of the lake may make the variability of the fungus flora of the sediment. Some fungi can remain for a long time because of their dormant structures, some can not survive under the water, and some may be transients. Therefore, from the results of the present investigation, it is difficult at moment to say definitely about the general tendency of the occurrence of fungi in the sediment comparing with the distribution of common terrestrial fungi.

At the present, however, the following points may be mentioned from the results of the present investigations.

1. The most significant is that common terrestrial fungi such as *Alternaria, Cladosporium, Curvularia* are extremly rare as compared with the flora of the surrounding habitats of the lake. Among *Alternaria,* only one species, *A. alternata,* has been isolated only from St. 1. *Cladosporium cladosporioides* and *Curvularia clavata* are of the same kind and were found only in St. 1. Station 1 is located in Hazu (Pl. 1) from where the river flows into the lake, and the station is close to several small farms, therefore, it is reasonable to find these terrestrial fungi in the sediment. These fungi must have been washed into the river from surrounding plant materials, but could not remain dormant in the sedimentary muds during the process of flowing down to the deeper places.

2. With the exception of above three genera, what is seen in the Table 1 is the presence of dominant distributional pattern of the dematiaceous fungi imperfecti in the sediment. The species indicated by ◎ are mostly of dematiaceous, viz. *Gilmaniella, Gonytrichum, Mammaria, Phialophora, Rhinocladiella* and *Scolecobasidium.* Conversely, the tendency of this occurrence may be due to the dormancy of these fungi showing inactive survival in the unfavourable circumstances.

3. Ascomycetous fungi are very common, mostly of the Plectomycetes and Pyrenomycetes. Especially those of *Eupenicillium, Gymnoascus, Neosartorya, Talaromyces* and *Westerdykella* have been found fairly frequently. It is interesting to note that *Eup. javanicum, Neos. fischeri* var. *glabra, Tal. flavus* var. *flavus* and *West. multispora* are more frequently found in the deeper places where the temperature is low and the dissolved oxygen is generally very low. Depth, temperature and dissolved oxygen content will most surely circumscribe both the number and variety of the species. Above mentioned ascomycetous species must have survived because of their resting structures such as ascospores in the sediment under certain unfavourable circumstances for a long time. However, it is uncertain why they have been isolated only from the deeper places. Two running rivers provide a variety of fungi, and the areas of Sts. 1 and 2 have become loaded with propagules of the terrestrial fungi. Then a number of fungi of extraneous origin have been introduced to the center of the lake as transients in nature. Many of the propagules may survive for some time and then decline in the frequency with which they can be isolated from the sediment because of the unfavourable

circumstances. Many other fungi may remain more or less constantly throughout an extended period and then become inactive and produce resting structures. These resting structures must be accumulated into the sediment near the deepest areas, St. 6, by the gentle current of water. Such resting structures must be ascospores, chlamydospores or often thick-walled conidia.

4. The successive microflora associated with the decomposition process of the organic materials are generally well known. Even in the case of both aquatic and marine saprobic fungi, seasonal difference in the flora has been investigated. However, in the present investigation, there is no definite seasonal difference in the occurrence of fungi probably because of less activity of decomposition of the sediment materials by these terrestrial fungi. Of course, there is a seasonal variation in the number of fungi themselves as shown in Fig. 3, but no clear conclusion can be drawn with regard to successive occurrence of fungi in the sediment of the lake. Problem on successive change of the microflora in the sediment is the subject for a future study and involves many points to be solved.

The authors are gratefully indebted to Dr. K. Harimaya, director of the Water Quality Laboratory of the Kobe Water Works, for his many helps during this study.

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TRANSPORT SYSTEM FOR THE C_4-DICARBOXYLIC ACIDS IN *SALMONELLA TYPHIMURIUM*

Ko IMAI

Summary

The transport system for the C_4-dicarboxylic acids in *Salmonella typhimurium* was studied. It was found that *S. typhimurium* possesses a constitutive system which transports succinic, fumaric, L-malic, and L-aspartic acids into the cell.

Previous reports (2, 3) from this laboratory provided evidence that *S. typhimurium* possesses four inducible transport systems for the tricarboxylic acids (citric, isocitric, *cis*-aconitic, and tricarballylic). During those studies it was found that *S. typhimurium* cells constitutively oxidized succinic, fumaric, and L-malic acids. It had been published that *Escherichia coli* (4) and *S. typhimurium* (6) possessed an inducible transport system for these C_4-dicarboxylic acids.

The present work here shows the evidence that *S. typhimurium* possesses a constitutive transport system which carries succinic, fumaric, L-malic, and L-aspartic acids.

Materials and Methods

Chemicals. Monopotassium isocitrate and benzylpenicillin were obtained from the Microbiological Research Laboratories of the Central Research Division, Takeda Chemical Industries, Ltd., Osaka. Succinic acid-2,3-^{14}C was purchased from the Radiochemical Centre, Amersham, England. Other chemicals used here were purchased from Wako Pure Chemical Industries, Ltd., Osaka.

Bacterial strain. Salmonella typhimurium SU453 was obtained from Dr. K.E. Sanderson. This strain possesses genetic markers as follows: *hisF*, *trpB*, *metA*, *xyl*, and *strA* (7).

Media. The constituents of the media used in this study were described previously (2).

Uptake of succinic acid. A 100-μl amount of a reaction mixture containing cells (ca. 10^8 cells), succinic acid-2,3-^{14}C (ca. 2,000 dpm/nmole), and other supplements, was incubated on a reciprocal shaker at 37 C. The reaction mixture was added to 5 ml of ice-cold 0.1 M potassium phosphate buffer (pH 7.2), and the cells were collected by filtration through a Millipore membrane (0.45 μm pore size). The filter membrane was washed twice with 5 ml of ice-cold buffer and rapidly transferred into a vial containing 10 ml of a scintillation fluid. The constituents of the scintillation fluid were described previously (2). The radioactivities were measured with the use of an Aloka liquid scintillation counter.

Preparation of intact cell suspension. The cells grown on the basal medium containing 0.5% carbon source (glucose, acetate, citrate, L-glutamate, or succinate) were collected, washed twice with 0.1 M phosphate buffer, and resuspended in the buffer. These suspensions contained about 4×10^9 cells per ml and were used in the oxygen consumption experiments.

Oxygen consumption. Oxygen consumption in the presence of organic acid was measured at room temperature by a Beckman 777 oxygen analyzer. The intact cell suspension (5 ml) was aerated in a Voltex mixer after incubation for 1 min at 25 C and poured into a glass vessel with a Clark oxygen electrode. The organic acid to be tested was added, at a final concentration of 1 mM, to the suspension that was agitated continuously by a magnetic stirrer throughout the measurement. Oxygen content in the cell suspension was expressed in percent saturation in the vessel open to the air.

Assay for malic dehydrogenase. Malic dehydrogenase activities were measured by the method of Hsu and Lardy (1).

Results

Succinic acid uptake by S. typhimurium

Salmonella typhimurium SU453 cells grown in nutrient broth linearly took up labeled succinic acid during initial 20 min without lag (Fig. 1).

To obtain a Michaelis-Menten constant (*Km*) for succinic acid transport by *S. typhimurium,* the influence of concentration on the rate of this uptake was investigated over the range 2×10^{-5} M to 4×10^{-4} M. A plot of the results yielded a typical saturation curve, from which the *Km* value was estimated to be about 3×10^{-5} M (Fig. 2).

Influence of the carboxylic acids on succinic acid uptake

To determine the substrate specificity of the succinic acid trasnport system of *S. typhimurium* SU453, the influence of the carboxylic acids on the succinic acid uptake was examined in 100 μl of a reaction mixture containing about 10^8 cells, 0.5 mM labeled succinic acid (100,000 dpm), and 50 mM carboxylate to be tested. Succinic acid uptake was inhibited by fumarate, L-malate, and L-aspartate, but not by methylsuccinate, 2-oxoglutarate, and L-glutamate. Tricarboxylates such as citrate, *cis*-aconitate, isocitrate, and tricarballylate, did not inhibit this uptake (Table 1).

Isolation of the dicarboxylic acid transport-negative mutants

To isolate the mutant strains lacking the ability to transport the C_4-dicarboxylic acids, *S. typhimurium* SU453 cells grown in 2 ml of nutrient broth were added into 8 ml of nutrient broth containing 3 mg of N-methyl-N′-nitro-N-nitrosoguanidine (nitrosoguanidine) and were incubated for 15 min at 37 C. The cells were harvested by centrifugation and were starved in 10 ml of the basal medium for 16 hr at 37 C. The cells were transferred to 20 ml of the basal medium containing 100 mg of succinate and 4 mg of benzylpenicillin and were incubated for 8 to 10 hr at 37 C. After peni-

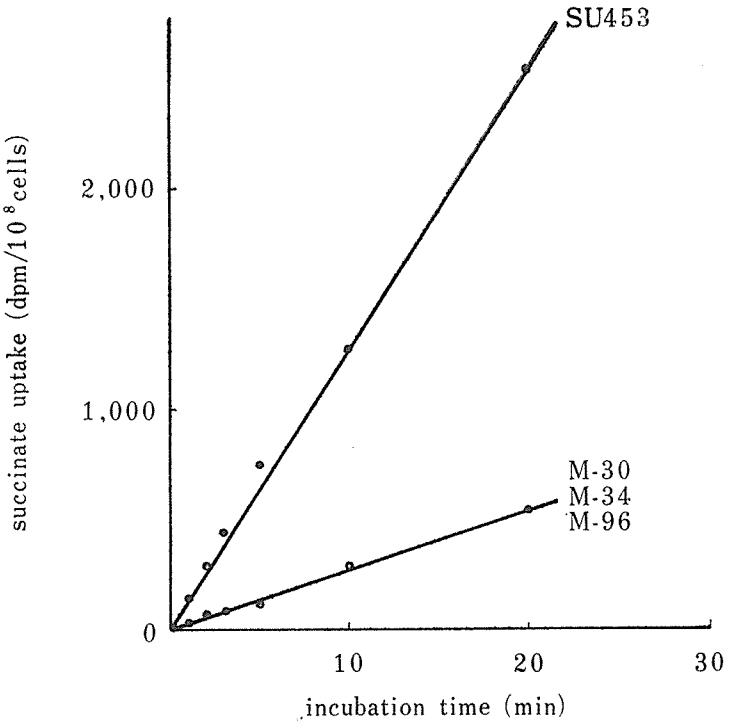

Fig. 1. Uptake of succinic acid by *S. typhimurium*.

The cells of *S. typhimurium* SU453, M-30, M-34, or M-96 grown in nutrient broth were collected by centrifugation and were resuspended in 0.1 M phosphate buffer (pH 7.2). A 50-μl amount of this suspension was added to 50 μl of a medium containing 0.2 mM succinic acid-2, 3-^{14}C (20,000 dpm) and 0.1 M phosphate buffer. The mixture was incubated on a reciprocal shaker at 37 C. Uptake of succinic acid was measured by the Millipore membrane method.

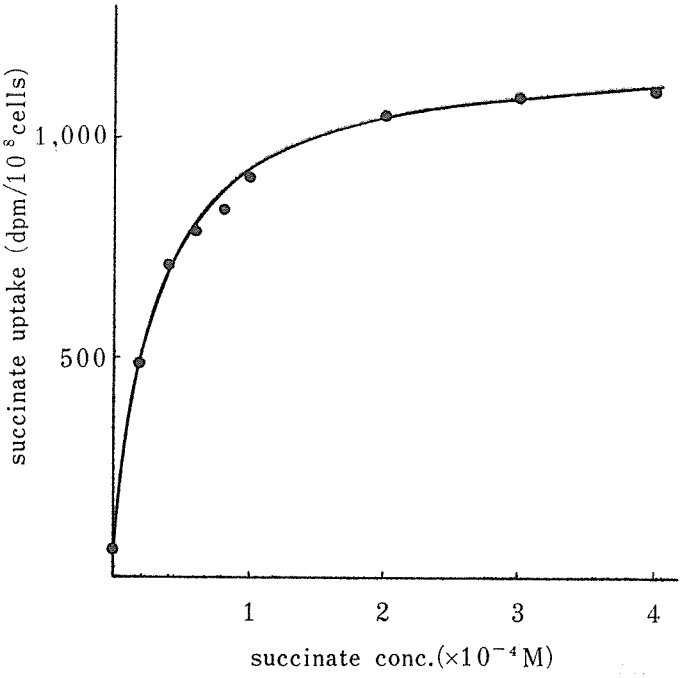

Fig. 2. Influence of concentration on the rate of succinic acid uptake.

Uptake of succinic acid was measured by the method described in Fig. 1.

Table 1. Influence of the carboxylic acids on succinic acid uptake.

Competitor	Succinic acid uptake during 5 min (dpm/10^8 cells)
None	2,350
Succinate	142
Fumarate	137
L-Malate	253
L-Aspartate	580
Methylsuccinate	2,188
2-oxo-Glutarate	2,786
L-Glutamate	2,193
Acetate	2,718
Citrate	2,108
cis-Aconitate	2,125
Isocitrate	2,395
Tricarballylate	2,411

A 50-μl amount of the intact cell suspension of *S. typhimurium* SU453 grown in nutrient both was added to 50 μl of a medium containing 1 mM succinic acid-2,3-^{14}C (100,000 dpm), 100 mM carboxylate to be tested, and 0.1 M phosphate buffer. Uptake of succinic acid was measured by the Millipore membrane method.

cillin enrichment the cells were washed twice with the basal medium, incubated in 50 ml of nutrient broth for 16 hr, and then were spread on nutrient agar plates. The resulted colonies were examined for the utilization of glucose, acetate, citrate, and succinate as sole carbon sources. Fourteen mutant strains, which did not utilize succinate but utilized glucose, acetate, and citrate, were isolated and were retested for the utilization of 9 kinds of organic acids shown in Table 2. It was found that these 14 mutants did not utilize fumarate, L-malate, and L-aspartate (Table 2), and spontaneous revertants of these mutants for the utilization of succinate or fumarate did utilize all of the C_4-dicarboxylic acids. The uptake of labeled succinic acid by these mutants was markedly lower than that by the parent strain (Fig. 1).

Table 2. Utilization of the organic acids.

Strain No.	Carbon source								
	ace	cit	aco	iso	tri	suc	fum	mal	asp
SU453	+	+	+	+	+	+	+	+	+
M-30, -34, -64, -96, -106, -145, -158, -167, -171, -172, -216, -223, -227, -282	+	+	+	+	+	−	−	−	−

Abbreviations: ace, acetate; cit, citrate; aco, *cis*-aconitate; iso, isocitrate; tri, tricarballylate; suc, succinate; fum, fumarate; mal, L-malate; and asp, L-aspartate.

+, utilized.

−, not utilized.

*Oxygen consumption by the intact cells of S. typhimurium SU*453

In previous paper (2), it was reported that the intact cells grown in nutrient broth did oxidize acetate, succinate, fumarate, and L-malate, but not citrate, isocitrate, *cis*-aconitate, and tricarballylate. These tricarboxylates were oxidized by the tricarboxylate-induced cells (the cells grown in nutrient broth containing tricarboxylate), so *S. typhimurium* possesses inducible transport systems for the tricarboxylic acids. On the other hand, acetate, succinate, fumarate, and L-malate were constitutively transported into the cell.

By reason of that nutrient broth contains aminodicarboxylic acids that may induce the dicarboxylic acid transport system, the oxidation of succinic acid was examined by the intact cells grown on the basal medium with glucose, acetate, citrate, L-glutamate, or succinate as a sole source of carbon (Fig. 3). The cells grown on acetate, citrate, L-glutamate, or succinate oxidized succinic acid, but the cells grown on glucose did not.

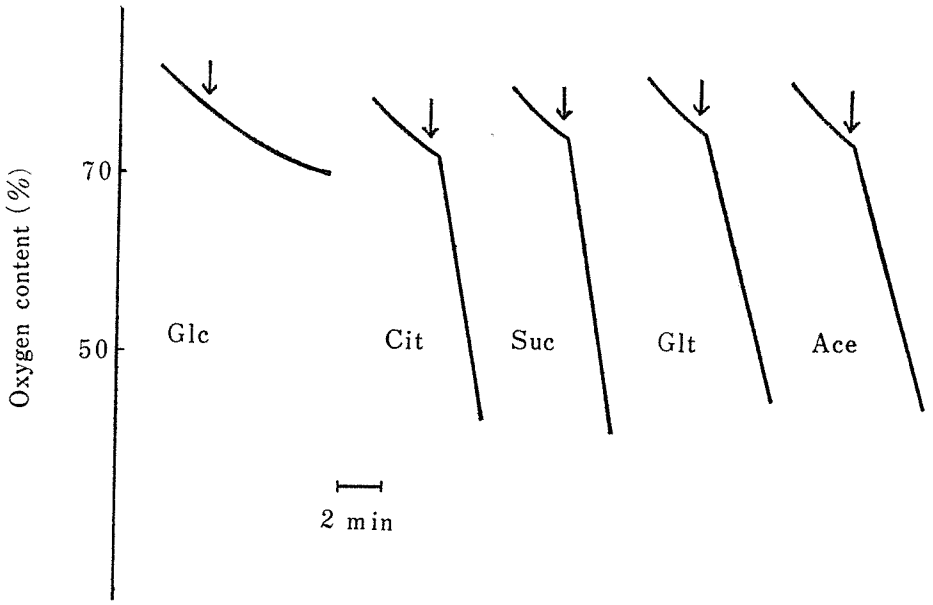

Fig. 3. Oxygen consumption by the intact cells in the presence of succinic acid. The arrows indicate addition of succinate.
Abbreviations: Glc, glucose grown cells; Cit, citrate grown cells; Suc, succinate grown cells; Glu, glutamate grown cells; and Ace, acetate grown cells.

*Malic dehydrogenase activities in the cells of S. typhimurium SU*453

Murai et al (5) reported that NADP-linked malic enzyme in *E. coli* is repressed by glucose and takes a role in the supply of acetyl-CoA from malate *via* pyruvate. To reveal why the cells of *S. typhimurium* SU453 grown on glucose did not oxidize succinic acid, NADP-linked enzyme activities in the cells grown on various compounds as carbon source were measured (Table 3). It was found that NADP-linked enzyme in *S. typhimurium* SU453 was repressed by glucose but not by acetate, citrate, and succinate.

Table 3. Malic dehydrogenase activities.

Enzyme source	Malic dehydrogenase activity (unit/mg protein)
Glucose grown cells	18
Acetate grown cells	237
Citrate grown cells	294
Succinate grown cells	303

The cells of *S. typhimurium* SU453 grown on glucose, acetate, citrate, or succinate were suspended in 0.1 M phosphate buffer (pH 7.4) and was disrupted by an ultra sonic oscillator at 20 kc for 5 min. The cell debris were removed by centrifugation at 12,000 $\times g$ for 20 min. The supernatants were used immediately for malic dehydrogenase assay.

One unit of enzyme is defined as that amount which causes an initial rate of increase in optical density (ΔE_{340}) of 0.001 per minute under the conditions described by Hsu and Lardy (1).

Succinic acid uptake by the cells grown on glucose

Succinic acid uptake by the cells of *S. typhimurium* SU453 grown on glucose was examined in the presence or absence of glucose (Fig. 4). The glucose grown cells did take up succinic acid in the presence of glucose but the cells were saturated with succinic acid during 5 min in the absence of glucose.

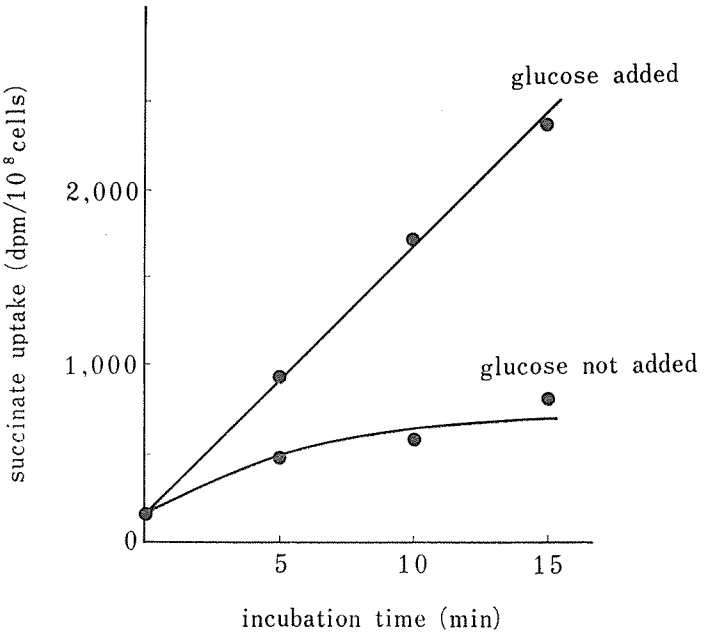

Fig. 4. Uptake of succinic acid by the glucose grown cells.
The cells grown on glucose were harvested by centrifugation and resuspended in 0.1 M phosphate buffer. A 50-μl amount of this suspension was added to 50 μl of the medium with or without 10 mM glucose. The medium contained 1 mM succinic acid-2,3-^{14}C and 0.1 M phosphate buffer.

Discussion

Previous papers (2, 3) defined that *S. typhimurium* possesses four inducible transport systems for the tricarboxylic acids. It is of particular interest to study the transport system for succinic, fumaric, and L-malic acids, because these acids are intermediates of the tricarboxylic acid cycle and resemble each other in their chemical structures. Present work here concludes that *S. typhimurium* possesses a constitutive transport system that carries succinic, fumaric, L-malic, and L-aspartic acids. The reasons will be described below.

The cells of *S. typhimurium* grown in nutrient broth linearly took up labeled succinic acid during the initial 20 min without lag (Fig. 1), and this uptake was inhibited by fumarate, L-malate, and L-aspartate (Table 1). Succinate utilization-negative strains with a single mutation did not utilize fumarate, L-malate, and L-aspartate (Table 2), and succinic acid uptake by these mutants was markedly reduced compared with the parent strain (Fig. 1).

These findings indicate that *S. typhimurium* possesses single transport system which carries succinic, fumaric, L-malic, and L-aspartic acids.

Parada et al (6) reported that the C_4-dicarboxylic acid transport system in *S. typhimurium* was inducible one, because the cells grown on glucose did not take up the dicarboxylic acids, but the cells grown on succinate, fumarate, or malate did take up these acids. In present work it was found that glucose grown cells of *S. typhimurium* SU453 did not oxidize succinic acid. A possible reason for it was considered below. Malic dehydrogenase activities in glucose grown cells of *S. typhimurium* SU453 were repressed (Table 3), so the cells could not convert succinate to acetyl-CoA *via* pyruvate and could not oxidize succinate through the tricarboxylic acid cycle. If glucose grown cells were supplied with glucose, the cells should convert glucose to acetyl-CoA by the glycolysis and should oxidize succinate through the tricarboxlic acid cycle, and uptake of labeled succinic acid by the cells should be enhanced. Present work showed that glucose grown cells of *S. typhimurium* SU453 did take up succinic acid when the cells were supplied with glucose (Fig. 4).

These results indicate that *S. typhimurium* possesses a constitutive transport system for the C_4-dicarboxylic acids.

Utilization of citric acid is an important characteristic for the identification of the species belonging to Enterobacteriaceae, but that of dicarboxylic acid is not so. *Salmonella typhimurium* possesses four inducible transport systems for the tricarboxylic acids (2, 3), but *E. coli* did not have any tricarboxylic acid transport system (unpublished data). Beside, both organisms possess a resemble transport system for the C_4-dicarboxylic acids except that *E. coli* has inducible system, whereas *S. typhimurium* has constitutive one (Table 4). It was demonstrated that the gene specified the C_4-dicarboxylic acid transport system located on the genome close to the *xyl* marker in *E. coli* (4) and *S. typhimurium* (6).

It may be concluded that the C_4-dicarboxylic acid transport system in *E. coli* and

Table 4. Transport systems for di- and tricarboxylic acids in *S. typhimurium* and *E. coli*.

	S. typhimvrium	*E. coli*
Dicarboxylic acids	constitutive system. substrate I. suc, fum, mal, asp	inducible system. substrate I. suc, fum, mal, asp
Tricarboxylic acids	inducible system. substrate. inducer I. cit, iso. cit, iso, aco II. aco. cit, iso, aco III. tri, cit, aco. tri IV. cit. cit	

Dicarboxylic acid transport system in *E. coli* is based on the data of Kay and Konberg (4). Abbreviations are the same as in Table 2.

S. typhimurium have been conserved without considerable changes in the process of evolution.

I am grateful to Dr. K.E. Sanderson for sending me the strains of *S. typhimurium*. I also wish to thank Dr. T. Hasegawa and Dr. T. Iijima for critical reading of the manuscript.

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GENETIC LOCUS OF *tct* (TRICARBOXYLIC ACID TRANSPORT) GENE IN *SALMONELLA TYPHIMURIUM*

Teiji IIJIMA and Ko IMAI

Summary

Salmonella typhimurium possesses four transport systems for tricarboxylic acids. Mutant strains which lacked the transport sustem(s) were isolated. One of these mutants, M72, was analysed to elucidate the genetic locus of the mutation on the chromosome of *S. typhimurium*. From interrupted conjugation experiments, it is suggested that the genetic locus of *tct* gene of M72 was located between *his F* and *thy A*.

An ability to utilize tricarboxylic acids for a sole carbon source has been an important characteristics for the identification of various species in the Enterobacteriaceae. *Salmonella typhimurium* is able to grow on a medium containing citrate as a sole carbon source. However, its taxonomically related species *Escherichia coli* is unable to utilize citrate as a sole carbon source, eventhough it has a enzyme which metabolize citrate in the cell extract. The inability of *E. coli* to utilize citrate is due to the absence of transport system(s) for tricarboxylic acids.

It was revealed that *S. typhimurium* possesses four different transport systems for tricarboxylic acids (Imai et al, 1973) and various mutant strains lacking the transport system(s) were isolated in this laboratory.

In this report a preliminary result of an analysis of genetic locus for this transport systems is present.

Materials and Methods

Basal media. Modified Davis' medium containing the following components, K_2HPO_4 7 g, KH_2PO_4 3 g, $(NH_4)_2SO_4$ 1 g, distilled water 1000 ml, was used as a basal medium. This was solidified by 1.6% agar. L-amino acids were supplemented at a concentration of 20 μg/ml when a nutritional mutant was used. In the case of thymine requiring mutants, the concentration of thymine in a medium was 20 μg/ml at a final concentration. Isocitrate-Davis medium contains isocitrate as a carbon source in place of glucose.

Nutrient medium. It contained peptone 1%, yeast extract 0.02% and NaCl 0.02%. This was also solidified by 1.6% agar.

Bacterial strains. Transport-negative mutants of *S. typhimurium* were isolated from SU453 (*hisF trpB metA xyl*-1 *strA* F^-) and used as recipient. One of the mutants, M72, lacked the transport system I and II simultaneously (Imai, 1975). Three kinds of Hfr strains were used in this experiment. These strains were obtained from

the Salmonella Genetic Stock Center in the University of Calgary, through courtesy of Dr. Sanderson.

Mating technique. Broth mating and interrupted conjugation were carried out according to the method of Sanderson (1972). One ml amount of a logarithmic phase broth culture of a donor strain was mixed with 2 ml of an overnight stationary phase culture of an F^- strain in 2 ml of broth. Aliquots are withdrawn from the mixture and diluted to tenfold with Davis' medium and plated on appropriate selective media. In the case of interrupted conjugation, the samples were diluted to tenfold and blended for 1 min in a high speed mixer (Nihon Koki Co.). A 0.1-ml of aliquot was plated on appropriate selective media after a serial dilution.

Results and Discussion

*Mating; SU*418×*M*72 *and SA*536×*M*72

Tricarboxylic acid transport-negative mutant M72 was crossed with two different Hfr strains SU418, and SA536. Recombinants which received *tct*$^+$ gene from the donor appeared only in the cross SU418×M72 (Table 1). This results means that *tct* gene would locate on the region which SU418 transfers at a high frequency. For further analysis, it is necessary to isolate a recipient strain which possesses a new mutant marker in the region between *hisF* and *metA* on the chromosome linkage map. The most suitable candidate for this purpose would be *thyA*, because *thyA* mutant is specifically selected by treatment with aminopterin (Okada et al 1960). Thymine requiring mutants were isolated from M72 by treatment with aminopterin according to the method of Okada (1962), and among the isolated thymine-requiring mutant, STF69 was isolated as a suitable recipient.

Table 1. Transfer of *tct* gene from two donor strains.

Selection	Cross	
	SU418×M72(P22)	SA536×M72
his$^+$	561	140
met$^+$	41	7
his$^+$ *tct*$^+$	35	0
met$^+$ *tct*$^+$	1	0

Donor and recipient cells were mixed in a tube and incubated at 37 C for 1 hr. Aliquots were plated on the following four kinds of plates: Davis+methionine+tryptophan (*his*$^+$ selection), Davis+histidine+tryptophan (*met*$^+$ selection), isocitrate-Davis+methionine+tryptophan (*his*$^+$ *tct*$^+$) and isocitrate-Davis+histidine+tryptophan (*met*$^+$ *tct*$^+$). Figures in the Table show the numbers of recombinants on these plates.

*Mating; SU*418×*STF*69

Both donor SU418 and recipient STF69 were cultured overnight and diluted with fresh broth in five to ten fold. The diluted cultures were incubated for 3 hrs at 37 C

Table 2. Transfer of various markers in the cross SU418×STF69.

Selection	Time after the mixing (min.)			
	0	30	60	140
his$^+$	0	5×10^2	3.2×10^3	5.7×10^3
thy$^+$	0	0	2.0×10^2	1.8×10^3
met$^+$	0	0	0	2.0×10^2
trp$^+$	0	0	0	0

Donor (SU418) and recipient (STRF69) cells were mixed in a tube and incubated at 37 C. Samples were withdrawn from the mixture at 0, 30, 60 and 140 min after the mixing, and plated on the following four kinds of plates: Davis+methionine+thymine+tryptophan, (*his*$^+$ selection), Davis+histidine+methionie+tryptophan (*thy*$^+$ selection), Davis+histidine+thymine+tryptophan (*met*$^+$ selection) and Davis+histidine+thymine+methionine (*trp*$^+$ selection). Figures in the Table show the numbers of recombinants on the selective plates.

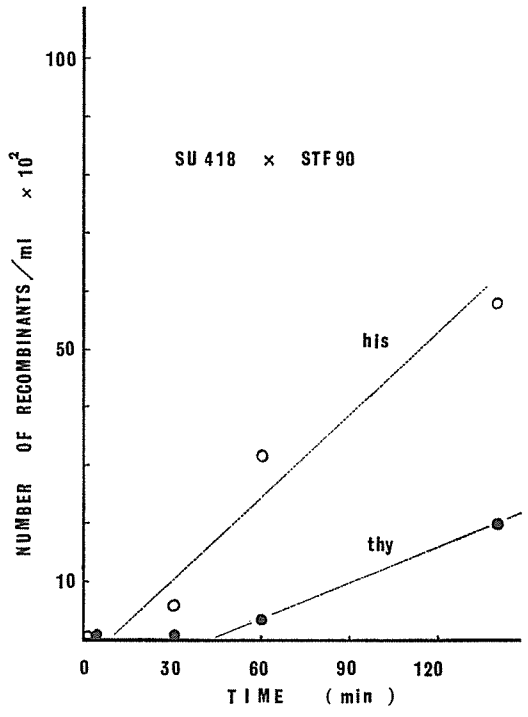

Fig. 1. Interrupted conjugation between SU418 and STF69.

Donor and recipient cells were mixed in a tube and incubated at 37 C. Samples were withdrawn from the mixture and diluted in tenfold with Davis' medium. The diluted samples were blended in high speed mixer for 1 min and aliquots were plated on appropriate selective plates.

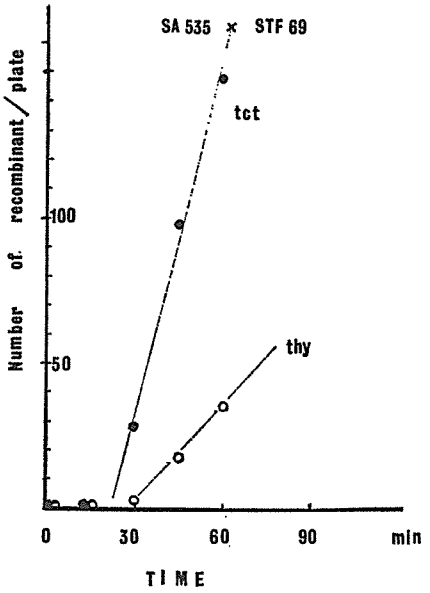

Fig. 2. Interrupted conjugation between SA535 and STF69.

Method was as described in the legend of Fig. 1.

without shaking. The donor and recipient cells were mixed in a tube and aliquots were withdrawn from the mixture at an interval and the recombinants were counted by plating on appropriate selective plates. The numbers of recombinants appeared on the

selective plates are shown in Table 2. The result shows that the new marker *thy* is located between *his* and *met* on the chromosome of *S. typhimurium*. A result of interrupted conjugation between SU418 and STF69 is shown in Fig. 1. The result shows that this donor transfer its chromosome as the following order; *his*···*tct*···*thy*···*met*···.

*Mating; SA*535×*STF*69

To elucidate the more exact location of *tct* marker, blending experiment was carried out at 15 min interval in mating between SA535 and STF69. The result is shown in Fig. 2. From the result in Fig. 2, *tct* gene is transfered about 8 min prior to the entrance of *thy* under the experimental condition. For the determination of the exact locus of *tct*, however it is necessary to carry out transduction experiment using recipients which have mutant markers located in this region, for example *cysC*, *pheA* or *cysA*. These tests are now in progress.

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ON THE CRYSTAL-PRODUCING STRAINS OF STREPTOMYCETES

Kôiti NAKAZAWA

In the course of study on streptomycetes, the author isolated a strain of *Streptomyces sindenensis* (No. 5866) from a soil sample collected in 1954 in Japan. The strain had a characteristic to form water-droplet like fruit-bodies on glucose asparagine agar.

As stated in the previous papers (1,2,3,4), it was found that crystals were formed in the culture media of *Streptomyces sindenensis* No. 5866. In addition, mycelial growth from the crystals and a phenomenon of conversion of species were observed. Namely when the crystal was incubated on bouillon agar, mycelia of *Streptomyces sindenensis* No. 5866, grew from the crystal and when the crystals were buried among the mycelia of various species of streptomycetes, the crystals were converted to another crystals, from which another species of streptomycetes grew. Moreover, the production of crystals by some species of streptomycetes has been observed usually in bouillon agar (5). These results suggested that the possibility of producing crystals by streptomycetes in the medium is considered as a general phenomenon.

I have streaked the strain of streptomycetes collected in our laboratory on bouillon agar and incubated at 28 C for 3 weeks and have observed that the following species of streptomycetes produced crystals in bouillon agar beneath the colony. About 17% of test strains produced crystals in the medium. It was considered that the ability of producing crystals in bouillon agar was widely distributed among the species of streptomycetes (Table 1). Whether the formation of these crystals has some relation with the life cycle of streptomycetes or not is not yet clear.

Crystals were observed by a poralizing microscope (ca. ×60) (Plate 1). And the surface features of the crystal formed by *Streptomyces sindenensis* (No. 5866) were observed by a scanning electron microscope (Plate 2, 3, 4, 5).

Neither conidium nor a piece of mycelium was observed by a scanning electron microscope.

Table 1. Crystal-producing strains

	IFO No.	ISP No.
Streptomyces afghaniensis	12831	5528
S. albofaciens	12833	5268
S. albogriseolus	12834	5003
*S. albohelvatus**	13012	5410
S. albus	3195	
S. antibioticus	3126	

Table 1. (continued)

	IFO No.	ISP No.
S. aureus	3175	
S. calvus	13200	5010
S. canus	12752	5017
S. capoamus	13411	5494
S. cavourensis	13026	5300
S. cellulosae	13027	5362
S. chryseus	13377	5420
*S. coeliatus**	13030	5422
S. coelicolor	12854	5233
*S. coeruleofuscus**	12757	5144
S. collinus	12759	5129
S. colombiensis	13454	5558
*S. cretaceus**	13457	5561
S. cyaneus	13346	5108
S. diastatochromogenes	3337	
S. filipinensis	12860	5112
S. flaveolus	3240	
*S. flavescens**	13038	5428
S. flaviscleroticus	13357	5270
S. flavochromogenes	13443	5541
*S. flavotritici**	12770	5152
S. flavus	3359	
S. fradiae	12177	
S. fungicidicus	12439	
*S. globisporus**	12208	
S. graminofaciens	13455	5559
S. griseoflavus	13044	5456
S. griseofuscus	12870	5191
S. griseolus	3300	
S. griseoluteus	13375	5392
S. griseorubens	12780	5160
S. griseoruber	12873	5281
S. griseosporeus	13458	5562
S. griseus	3237	
S. hawaiensis	12784	5042
S. indigocolor	13383	5432
S. kanamyceticus	13414	5500
*S. lavendocolor**	12881	5216
S. lavendulae	3361	
S. lavendulae	3145	
S. lavenduligriseus	13405	5487
*S. levoris**	21883	5202
S. lusitanus	13464	5568

Table 1. (continued)

	IFO No.	ISP No.
*S. malachiticus**	12795	5167
S. melanogenes	12890	5192
S. murinus	13799	5091
S. mutabilis	12800	5169
S. nitrosporeus	3362	
S. nogalater	13445	5546
S. olivaceus	3150	
S. parvus	3388	
S. perviensis	13481	5592
S. phaeochromogenes	12041	
S. pseudogriseolus	12092	5026
S. purpurogeniscleroticus	13358	5271
S. regensis	13448	5551
S. rochei	12908	5231
*S. roseofluvus**	13194	5172
S. scabies	12914	5078
S. showdoensis	13417	5504
S. sindenensis	12915	5255
S. steffisburgensis	13446	5547
S. termitum	13087	5329
S. venezuelae	13096	5230
S. verne	13097	5079
S. versipellis	13409	5491
*S. violaceochromogenes**	13100	5181
*S. violaceorectus**	13102	5279
S. violaceus	13103	5082
S. viridis	13373	5381
S. viridochromogenes	12339	
S. viridodiastaticus	13106	5249
S. yokosukaensis	13108	5224
S. zaomyceticus	3856	

Those indicated by * were first validly reported as Actinomyces.

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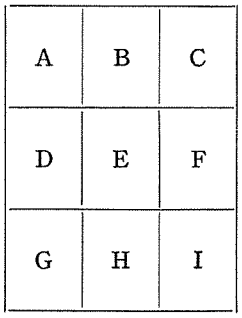
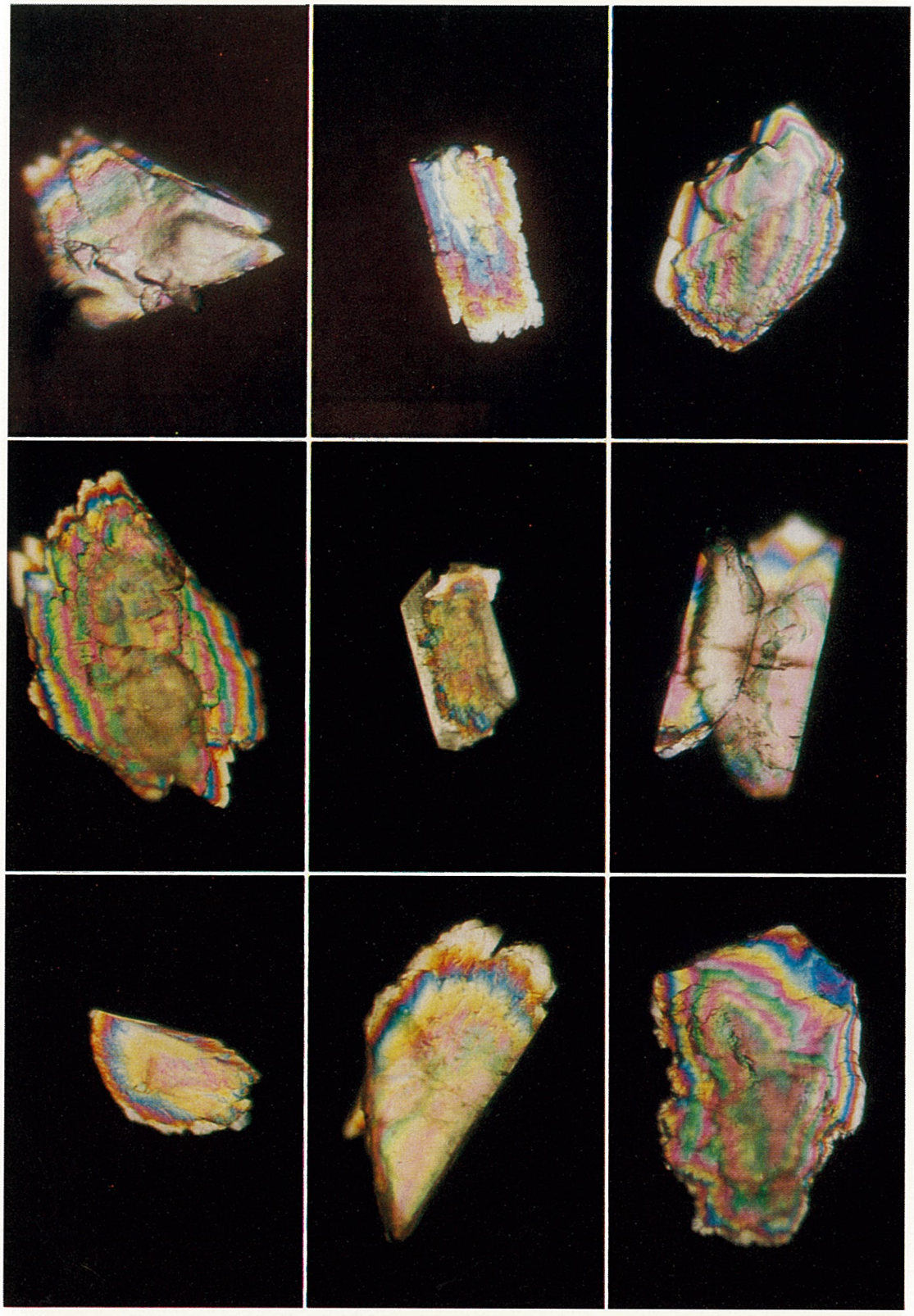
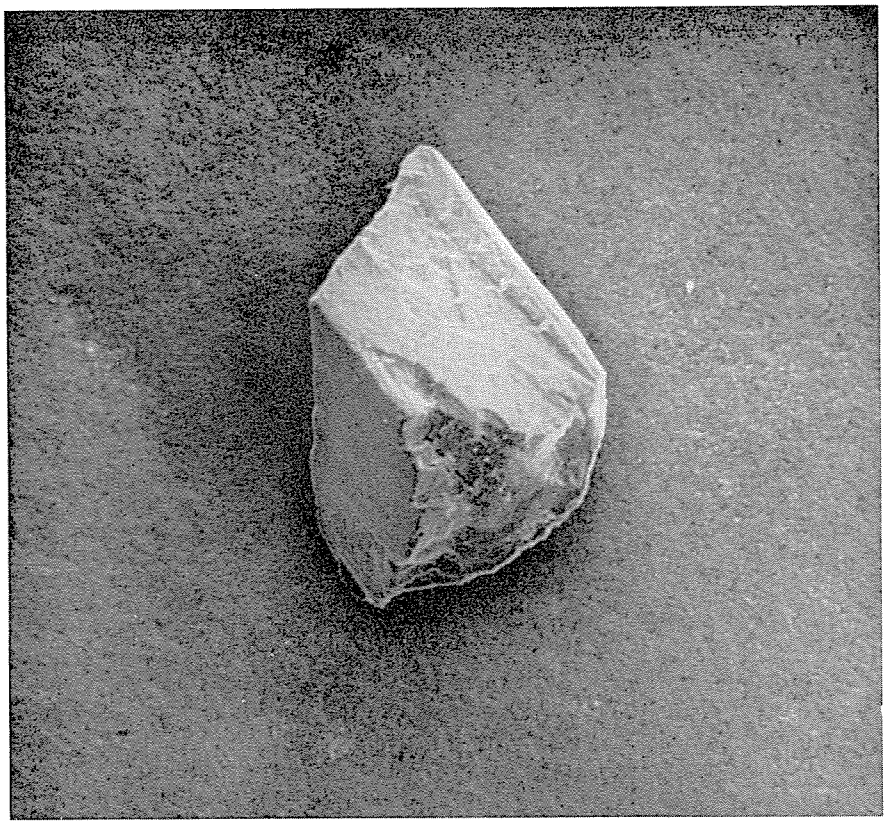


Plate 1. Crystals produced in the medium by Streptomycetes

A. *Streptomyces aureus* B. *Streptomyces chryseus* C. *Streptomyces flavochromogenes*
D. *Streptomyces fradiae* E. *Streptomyces graminofaciens* F. *Streptomyces kanamyceticus*
G. *Streptomyces mutabilis* H. *Streptomyces nitrosporeus* I. *Streptomyces parvus*





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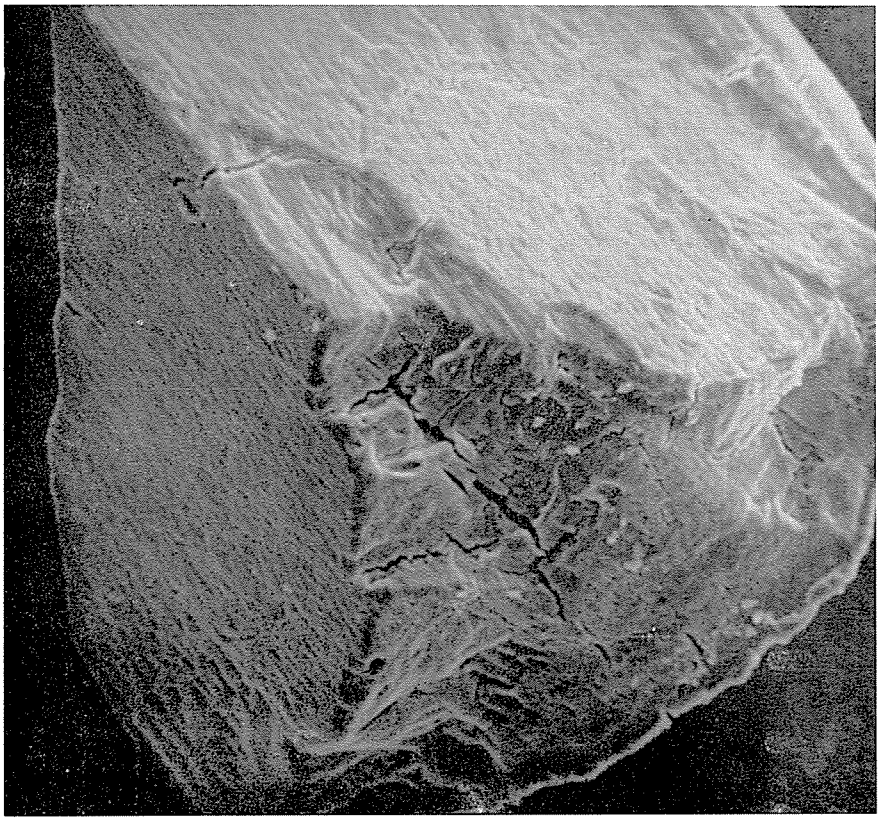
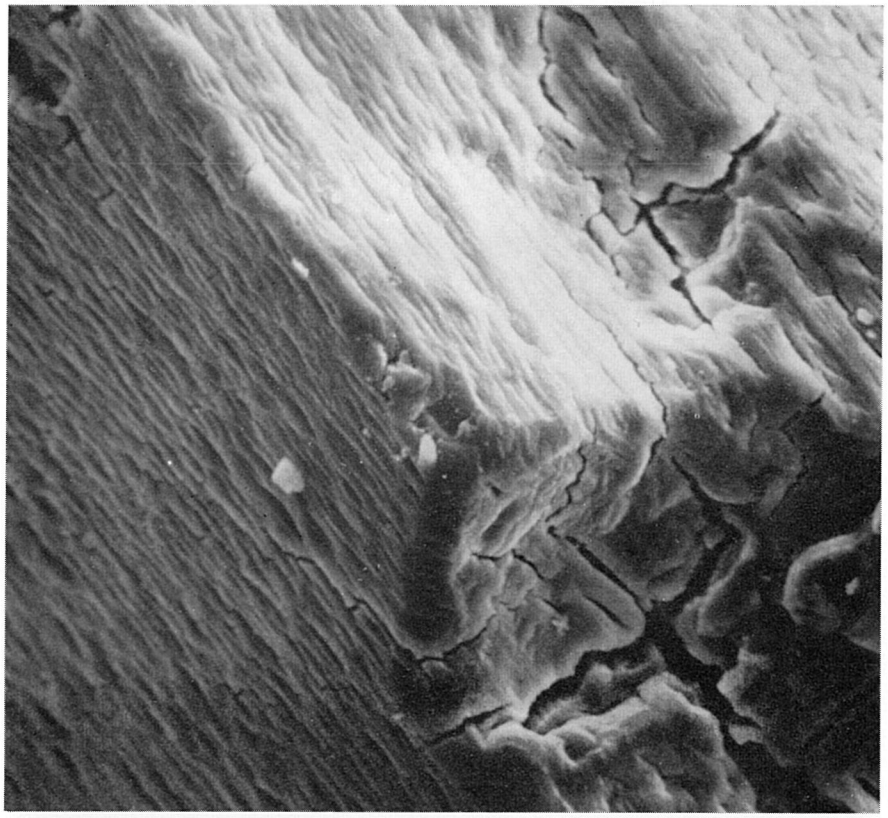


(×300)

Plate 2. Crystal of *Streptomyces sindenensis* No. 5866 (Scanning electron microscope)



(×1000)

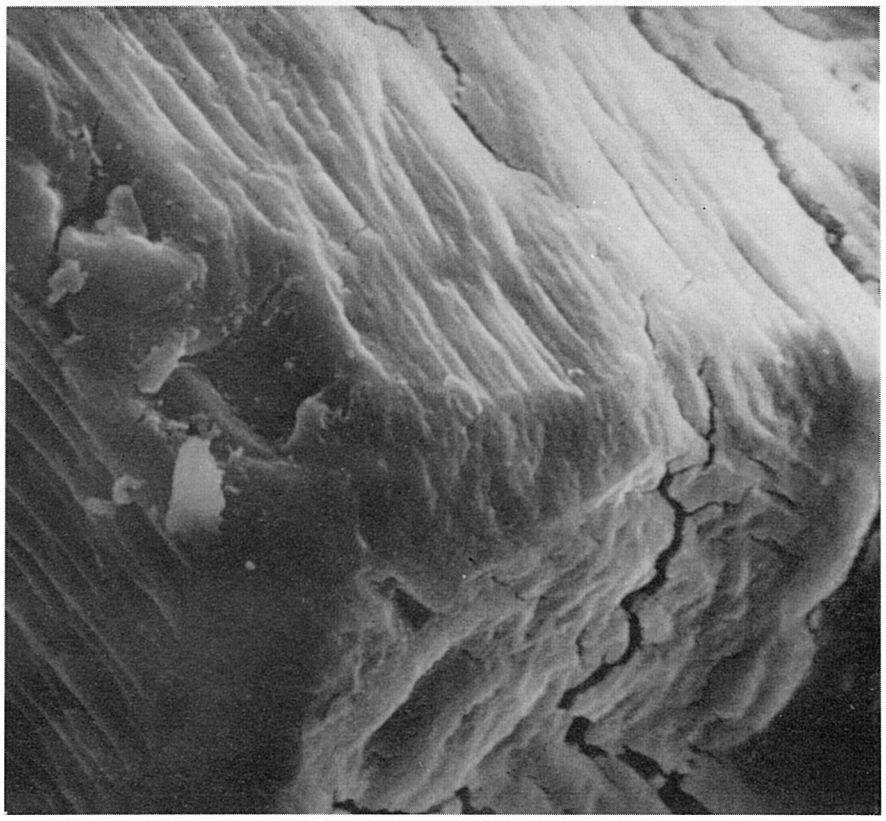


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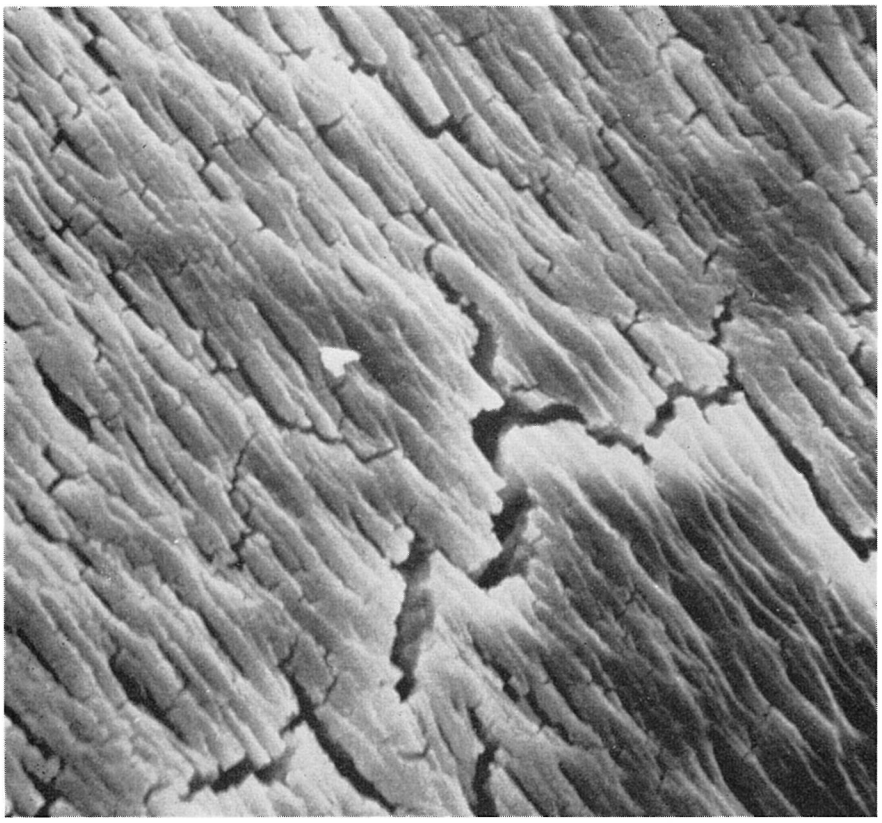
Plate 3. Crystal of *Streptomyces sindenensis* No. 5866 (Scanning electron microscope)



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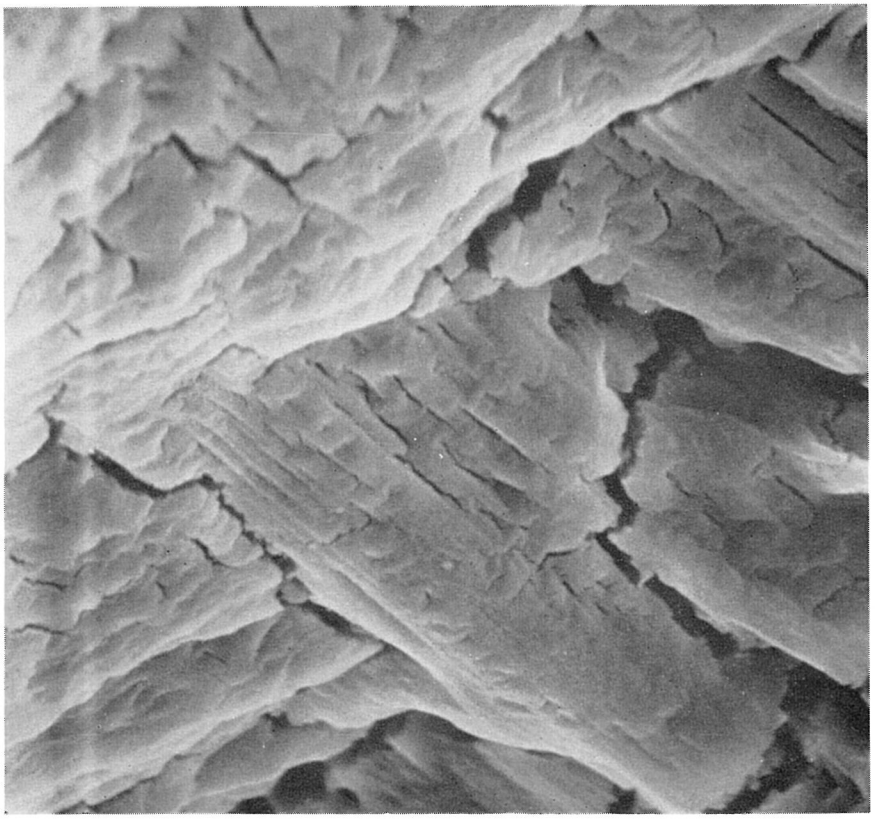

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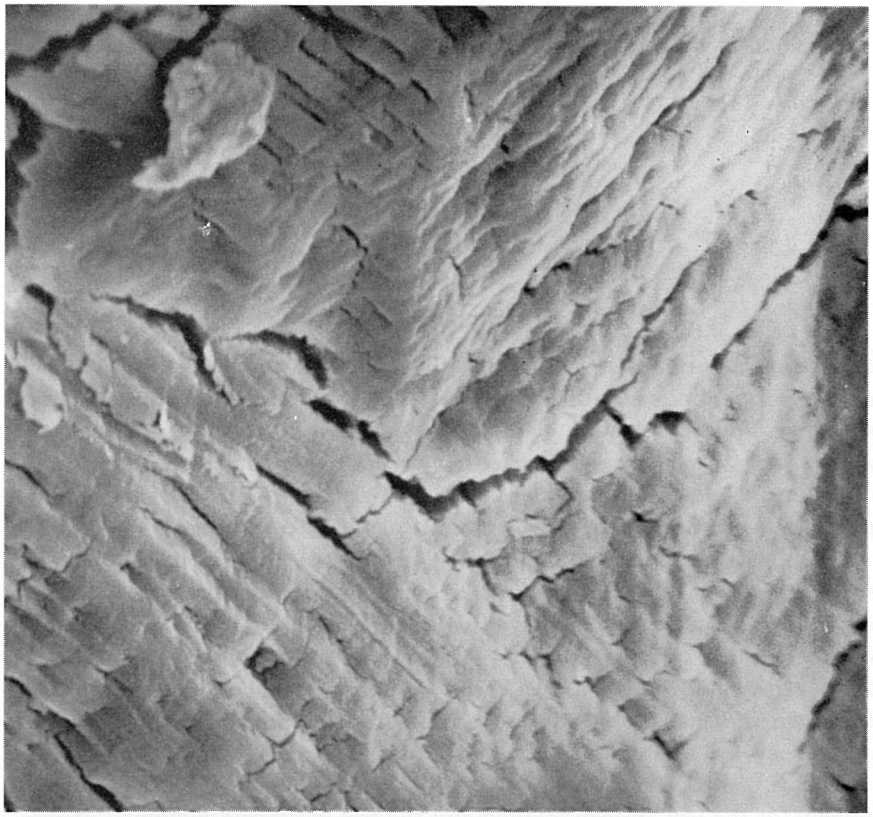
Plate 4. Crystal of *Streptomyces sindenensis* No. 5866 (Scanning electron microscope)



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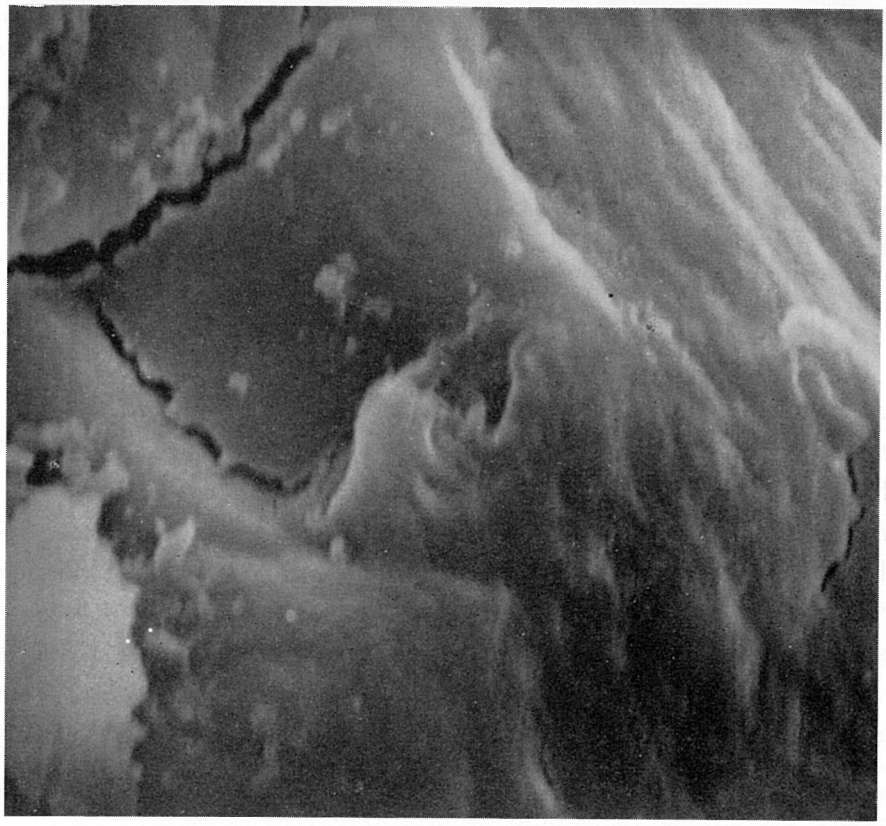

(×10000)

Plate 5. Crystal of *Streptomyces sindenensis* No. 5866 (Scanning elcctron microscope)

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LATEX AGGLUTINATION TEST FOR THREE PLANT VIRUSES*

Tatsuo YOKOYAMA

Summary

This paper reports the results of experiment on the conditions and procedures of the latex agglutination test with three selected plant viruses and the sensitivity of the test for the serological detection of these viruses. Polystyrene latex particles 0.81 μm in diameter were used for the preparation of both test antigen and test antiserum. Virus antigen was consisted of the intact virus particles either in crude sap or in the partial purified preparation by an ultracentrifuge. The favourable condition for the latex agglutination test was found to use 1% latex suspended in a phosphate-buffered saline solution, pH 5.0 for the test antigen method and pH 8.0 for the test antiserum method, respectively, followed by overnight incubation at 37 C in a water bath for the reaction against the corresponding reactant. The quantitative experiment using the latex agglutination test was also carried out. The highest dilution end-points of TMV antigen showing the positive reaction were recorded to be $1:300\times2^{10}$ with the test antigen and $1:30\times2^{9}$ with the test antiserum, respectively. Those of PVX antigen were $1:300\times2^{7}$ and $1:30\times2^{6}$, and those of PVY antigen were $1:300\times2^{5}$ and $1:30\times2^{4}$, respectively for the test antigen method and the test antiserum method. It was found that the latex agglutination test is highly sensitive for the detection of the plant viruses having rod-shaped particles, particularly with lower concentration of the antigenic protein. From the experimental evidences, it was considered that the test might be useful and specific to some other plant viruses forming fragile or small antigen-antibody complexes. Minimum amounts of TMV antigen detectable by the latex agglutination test were found 6.1×10^{-7} mg/ml with the test antigen and 4.9×10^{-5} mg/ml with the test antiserum, respectively. As the latex agglutination test proved to be simple and rapid to perform and the resultant reaction is easy to read, the test might also be suitable for both quantitative and qualitative analysis of the plant viruses.

Materials and Methods

Virus antigens. Three plant viruses having the rod-shaped particles, namely, tobacco mosaic virus (TMV), potato virus X (PVX) and potato virus Y (PVY) were used in this experiment.

TMV. An ordinary strain of TMV propagated for 10 days in sap-inoculated *Nicotiana tabacum* L. var. Samsun plants grown in a green house was used for the preparation of the virus antigen. Soon after harvesting systemically infected plants and removing the stems and petioles, the leaves were minced by passage through a domestic meat chopper. By adding 0.01 M phosphate-buffered saline solution, pH 6.98, containing 0.001 M ethylenediaminetetraacetic acid disodium salt (EDTA) to make a volume equal

* This manuscript represents a portion of a thesis submitted for the D.A. at the Hokkaido University.

to the original material (v/w), the infective sap was expressed by squeezing the minced leaves in a sterilized gauze. The clarified crude sap was prepared by low speed centrifugation at 3,000 rpm from the expressed juice previously treated with freezing and thawing. The resultant preparation was designated as "crude sap". Partially purified virus preparation was made by following procedure. An equal volume of a 1:1 n-butanol-chloroform mixture was added to the crude sap formerly prepared and the resulting mixture was shaken gently on a magnetic stirrer for 30 min. The emulsion was then broken by a low speed centrifugation for 5 min. The aqueous phase was removed in the plastic tubing and was centrifuged in a RP–30 rotor in Hitachi Model 30 P preparative centrifuge at 8,000 rpm for 30 min. The supernatant fluid was then centrifuged at 30,000 rpm for 60 min. The pellet was resuspended in a 0.01 M phosphate-buffered saline solution, pH 6.98. The virus suspension was then centrifuged to remove the insoluble materials at 10,000 rpm for 30 min. After more cycles of a differential centrifugation, final high speed pellet was resolved in the same buffer solution to make an equal concentration of the virus to that in the crude sap unless otherwise specified. This preparation was used as "purified virus suspension."

PVX. *Nicotiana tabacum* L. var. Samsun was also inoculated with PVX and harvested after 2 weeks' incubation period. Crude sap preparation of this virus was made by the same procedure as mentioned above except for the use of 0.033 M phosphate-buffered saline solution, pH 6.98, containing 0.01 M sodium diethyldithiocarbamate (Na-DIECA) instead of EDTA. Purified virus preparation was made by 2 cycles of differential centrifugation at 10,000 rpm for 30 min and at 30,000 rpm for 90 min, alternatively, in a RP 30 rotor, after clarification by adding 25% (v/v) of a 1:1 n-butanol-chloroform mixture. Final high speed pellets were resuspended in the same buffer solution to make a volume equal to that of the original crude sap.

PVY. *Nicotiana sylvestris* Speg. & Comes was used as the source material of PVY after 3 weeks' incubation period in a green house. Systemically infected leaves were ground in a meat chopper, added 0.033 M phosphate buffer, pH 6.98, containing 0.01 M Na-DIECA and expressed infectious juice through a sterilized gauze. Clarified sap was prepared by low speed centrifugation at 10,000 rpm for 30 min in a RP–30 rotor. Virus concentration of the preparation was adjusted equal to that in the infected plants (v/w). Procedure of the differential centrifugation applied to the partial purification of PVY was quite the same as for that of PVX.

Diluent. To dilute the antigens and antisera as well as latex stock suspensions, ten kinds of the salt solutions were compared to determine which diluent is most favorable on the application of the latex agglutination test. Phosphate-buffered salines, pH 5.0, pH 6.0, pH 7.0 and pH 8.0, respectively, 0.01 M in phosphate and each containing 0.85% sodium chloride; Borate-buffered salines of pH 7.0 and pH 8.0, 0.05 M sodium borate: 0.2 M boric acid and 0.005 M sodium borate and 0.02 M boric acid, respectively, each containing 0.85% sodium chloride; Veronal buffer of pH 6.0 (5–5 diethylbarbituric acid 0.828 g/l, 5–5–sodium diethylbarbiturate 0.086 g/l, sodium

chloride 8.5 g/l, magnesium chloride 0.168 g/l and calcium chloride 0.028 g/l); and 0.85% sodium chloride solution in deionized water were used.

Antiserum. Antisera against three viruses were obtained from rabbits injected intramuscularly with partially purified virus antigens two to four times at monthly intervals. One ml of concentrated virus suspension as hundred times as original concentration was mixed with an equal volume of the Freund's complete adjuvant and was used for each injection.

Latex suspension. Polystyrene latex particles 0.81 μm in diameter used in this experiment were in the form of a milky white, semifluid suspension which was available commercially as Bacto latex 0.81. After diluted to 1:4 with deionized water, it was filtered through a Toyo No. 2 filter paper. The resulting suspension henceforth will be reffered to as latex stock suspension. The sotck suspension was diluted with a diluent previously described to make suspensions of a concentration of 1, 0.5 and 0.25% of the original suspension just before use, and reffered to as the final latex suspension.

Test antigen and test antiserum. The test antigen and test antiserum were prepared by mixing 1 part of either viral antigen or antiserum with 19 parts of the final latex suspension and then incubated at various experimental conditions.

The test. Antigen was diluted serially with a diluent to make twofold series in test tubes each containing 0.5 ml of dilute antigen and then equal volume of the test antiserum at a given antibody titer in the same diluent was mixed. Reciprocally, to a series of dilute antiserum was mixed with the test antigen at a given antigen titer in the same manner. After the treatment under various incubation conditions, the results of serological reaction, unless otherwise stated, were read by the naked eye with the aid of vertically transmitted light along the long axis of the test tubes against a black background. Sometimes, a drop of the suspension containing the antigen-latex-antibody complexes was placed on a slide glass without a cover slip and examined under low power magnification ($\times 40 - \times 100$) on a microscope to read the result.

Results

General aspects of the latex agglutination test with three plant viruses

Three plant viruses, TMV, PVX and PVY, were examined by the latex agglutination test (LAT), using ordinary procedure recommended for other works on serodiagnosis for such as leptospiral soluble antigen(5). Viral antigens and antisera were mixed with final latex suspension containing 0.25% latex and kept them at room temperature for overnight adsorption. After adding test antigen or test antiserum to corresponding antiserum or antigen, respectively, test tubes were allowed to stand at 37 C in a water bath for overnight incubation. Borate-buffered saline solution of pH 8.0 was used as diluent. The grid titration using the macroagglutination test (MAT) was also carried out so as to compare the sensibility of the LAT.

1. Latex agglutination test with the test antigen method.

TMV. Comparatively spacious area indicating the field of reaction was obtained with TMV antigen in crude sap from tobacco plants which gave two small zones in the regions of both antigen and antibody excess where there was no precipitate. The highest serological titer of antigen adsorbed on the surface of latex particles, $1:300\times2^{10}$, was obtained with antiserum diluted to $1:2^{10}$. Antiserum at a given dilution exceeding this extent never reacted with the antigen of such a least amount. The detectability of virus antigen, in other words, decreased gradually when antiserum at a dilution of both above and below $1:2^{10}$ wsa used to titrate. The dilution end-point of antiserum used was determined to be $1:2^{14}$, though it was reproduceable only when titrated with antigen at a constant of $1:300\times2^{4}$. With more dilute antigen, descending end-point of antiserum was noticeable as lower as those observed with highly concentrated antigen which caused the inhibition of reaction by excess of antigen. This is shown in Table 1.

Table 1. Latex agglutination test with TMV antigen (crude sap) and antiserum.

Reciprocal of antigen dilution in test antigen 300×2^n		Reciprocal of antiserum dilution 2^n											Control
	n=	5	6	7	8	9	10	11	12	13	14	15	
n=	1	++ *	++	++	++	++	++	+	+	±	−	−	−
	2	++	++	++	++	++	++	+	+	±	−	−	−
	3	++	++	++	++	++	++	+	+	+	±	−	−
	4	++	++	++	++	++	++	+	+	+	+	±	−
	5	+	+	++	++	++	+	+	+	+	±	−	−
	6	+	+	+	+	++	+	+	+	±	−	−	−
	7	+	+	+	+	+	+	+	±	−	−	−	−
	8	±	+	+	+	+	+	±	−	−	−	−	−
	9	−	−	±	+	+	+	±	−	−	−	−	−
	10	−	−	−	−	±	+	−	−	−	−	−	−
	11	−	−	−	−	−	−	−	−	−	−	−	−
	Control	−	−	−	−	−	−	−	−	−	−	−	−

* ++, Strongly positive reaction; +, weakly positive; ±, doubtful; −, negative. Same in the following tables.

The LAT with purified TMV as test antigen showed less spacious area indicating the field of reaction with a conspicuous zone in the region of antibody excess and a small zone in the region of antigen excess where there was no precipitate. The shape of the field of recation obtained here was not essentially different from those obtained above. The difference in the size of the field of reaction may have been depended on the concentration of antigen to be compared. The serological behaviour of both type of antigens seems to be fundamentally of the same entity. The serological titer of antigen was the highest when titrated with antiserum diluted to $1:2^{11}$, indicating $1:300\times2^{7}$. The highest dilution end-point similarly obtained with the test antigen at

an antigen dilution of $1:300\times2^3$ reached up to $1:2^{14}$. This is shown in Table 2.

PVX Positive reactions were obtained to form the field of reaction a little elongated towards the region of dilute antiserum when PVX test antigen in crude sap was allowed to react against PVX antiserum. The highest dilution end-point of virus antigen in test antigen was $1:300\times2^7$ and the highest precipitation end-point of antiserum was $1:2^{11}$. The inhibition of reaction did not seem to occur. This is shown in Table 3.

Table 2. Latex agglutination test with TMV test antigen (purified virus) and antiserum.

Reciprocal of antigen dilution in test antigen 300×2^n		Reciprocal of antiserum dilution 2^n											Control
	n=	5	6	7	8	9	10	11	12	13	14	15	
n=	1	++	++	++	++	++	++	+	+	±	−	−	−
	2	+	+	++	++	++	++	++	+	+	±	−	−
	3	+	+	+	+	+	+	+	+	+	+	±	−
	4	−	±	+	+	+	+	+	+	+	±	−	−
	5	−	−	−	±	+	+	+	+	+	±	−	−
	6	−	−	−	−	±	+	+	+	±	−	−	−
	7	−	−	−	−	−	±	+	±	−	−	−	−
	8	−	−	−	−	−	−	−	−	−	−	−	−
	9	−	−	−	−	−	−	−	−	−	−	−	−
	10	−	−	−	−	−	−	−	−	−	−	−	−
	11	−	−	−	−	−	−	−	−	−	−	−	−
	Control	−	−	−	−	−	−	−	−	−	−	−	−

Table 3. Latex agglutination test with PVX test antigen (crude sap) and antiserum.

Reciprocal of antigen dilution in test antigen 300×2^n		Reciprocal of antiserum dilution 2^n											Control
	n=	3	4	5	6	7	8	9	10	11	12	13	
n=	1	+++	+++	++	++	++	++	+	+	+	±	−	−
	2	++	++	+	+	+	+	+	+	+	±	−	−
	3	++	++	+	+	+	+	+	+	±	−	−	−
	4	++	+	+	+	+	+	+	±	−	−	−	−
	5	+	+	+	+	+	±	−	−	−	−	−	−
	6	+	+	±	−	−	−	−	−	−	−	−	−
	7	+	±	−	−	−	−	−	−	−	−	−	−
	8	±	−	−	−	−	−	−	−	−	−	−	−
	9	−	−	−	−	−	−	−	−	−	−	−	−
	10	−	−	−	−	−	−	−	−	−	−	−	−
	11	−	−	−	−	−	−	−	−	−	−	−	−
	Control	−	−	−	−	−	−	−	−	−	−	−	−

On the other hand, as shown in Table 4, within a significantly narrow area were positive reaction observed when PVX test antigen in purified from was titrated with antiserum against PVX using LAT, though the field of reaction was similar in its shape as compared with those obtained in the preceeding experiment. The precipitin end-point of this antiserum was determined as $1:2^7$ and the serological titer of antigen was $1:300\times 2^4$ against antiserum diluted $1:2^3$.

PVY. PVY, both in crude sap and purified virus suspension, was also titrated by the test antigen method of the LAT. The test antigen prepared from PVY in crude sap

Table 4. Latex agglutination test with PVX antigen (purified virus) and antiserum.

Reciprocal of antigen dilution in test antigen 300×2^n	Reciprocal of antiserum dilution 2^n											Control
n=	3	4	5	6	7	8	9	10	11	12	13	
1	+	+	+	+	+	±	−	−	−	−	−	−
2	+	+	+	+	+	±	−	−	−	−	−	−
3	+	+	±	−	−	−	−	−	−	−	−	−
4	+	±	−	−	−	−	−	−	−	−	−	−
5	±	−	−	−	−	−	−	−	−	−	−	−
6	−	−	−	−	−	−	−	−	−	−	−	−
7	−	−	−	−	−	−	−	−	−	−	−	−
8	−	−	−	−	−	−	−	−	−	−	−	−
9	−	−	−	−	−	−	−	−	−	−	−	−
10	−	−	−	−	−	−	−	−	−	−	−	−
11	−	−	−	−	−	−	−	−	−	−	−	−
Control	−	−	−	−	−	−	−	−	−	−	−	−

Table 5. Latex agglutination test with PVY test antigen (crude sap) and antiserum.

Reciprocal of antigen dilution in test antigen 300×2^n	Reciprocal of antiserum dilution 2^n											Control
n=	3	4	5	6	7	8	9	10	11	12	13	
1	⧺	⧺	⧺	+	+	±	−	−	−	−	−	−
2	⧺	⧺	+	+	±	−	−	−	−	−	−	−
3	⧺	+	±	−	−	−	−	−	−	−	−	−
4	+	+	±	−	−	−	−	−	−	−	−	−
5	+	±	−	−	−	−	−	−	−	−	−	−
6	±	−	−	−	−	−	−	−	−	−	−	−
7	−	−	−	−	−	−	−	−	−	−	−	−
8	−	−	−	−	−	−	−	−	−	−	−	−
9	−	−	−	−	−	−	−	−	−	−	−	−
10	−	−	−	−	−	−	−	−	−	−	−	−
11	−	−	−	−	−	−	−	−	−	−	−	−
Control	−	−	−	−	−	−	−	−	−	−	−	−

was detected serologically with antiserum against PVY at a constant dilution of $1:2^3$ even when infectious sap used for adsorption was diluted up to $1:300\times2^5$. The highest precipitation end-point of antiserum thus titrated with the test antigen at a virus dilution of $1:300\times2$ was $1:2^7$. As shown in Table 5, the field of reaction only situated in the regions of both antigen and antiserum of high content. Positive reaction was obtained only with antiserum diluted to $1:2^4$, when antigen in purified virus suspension at a dilution of $1:300\times2$ was adsorbed on latex particles and allowed to react against antiserum to PVY. The serological titer as determined by this method, so to speak, was $1:300\times2$. This is shown in Table 6.

Table 6. Latex agglutination test with PVY test antigen (purified virus) and antiserum.

Reciprocal of antigen dilution in test antigen 300×2^n		Reciprocal of antiserum dilution 2^n											Control
	n=	3	4	5	6	7	8	9	10	11	12	13	
n=	1	±	+	±	−	−	−	−	−	−	−	−	−
	2	−	−	−	−	−	−	−	−	−	−	−	−
	3	−	−	−	−	−	−	−	−	−	−	−	−
	4	−	−	−	−	−	−	−	−	−	−	−	−
	5	−	−	−	−	−	−	−	−	−	−	−	−
	6	−	−	−	−	−	−	−	−	−	−	−	−
	7	−	−	−	−	−	−	−	−	−	−	−	−
	8	−	−	−	−	−	−	−	−	−	−	−	−
	9	−	−	−	−	−	−	−	−	−	−	−	−
	10	−	−	−	−	−	−	−	−	−	−	−	−
	11	−	−	−	−	−	−	−	−	−	−	−	−
	Control	−	−	−	−	−	−	−	−	−	−	−	−

2. Latex agglutination test with the test antiserum method.

TMV. The highest serological titer of TMV antigen, $1:30\times2^8$, was exceptionally obtained with test antiserum at an antibody dilution of $1:10\times2^5$. TMV antigen of $1:30\times2^7$, however was widely detected by the test antiserum consisting antibody at several steps of serial dilution when box titration was made using antigen in crude sap. The inhibition of the reaction by excess of antigen occurred markedly. The detectability of antigen, especially of that of high content, was comparatively lower when titrated with test antiserum at a constant antibody dilution of $1:10\times2^4$ than when titrated with test antiserum of $1:10\times2^5$. The precipitation end-point of the test antiserum was $1:10\times2^{10}$ in its antibody dilution when use was made with antigen diluted to $1:30\times2^5$. This is shown in Table 7. On the other hand, with TMV antigen in purified virus suspension, almost the same tendency of precipitation behaviour in forming the field of reaction, but a little spacious in its size, was observed in the grid titration by the test antiserum method. In contrast to the preceeding observa-

tion, the highest detectability of antigen was ascertained with test antiserum at a constant antibody dilution of $1:10\times2^4$, and the highest serological titer of antigen, $1:30\times2^9$, was determined in this system. Antigen diluted to $1:30\times2^8$, however, was more widely detectable with a series of test antiserum containing antibody of various two fold dilution from $1:10\times2^4$ to $1:10\times2^7$. The highest precipitation end-point of test antiserum reached up to $1:10\times2^{11}$ in its antibody dilution when titrated only with antigen at $1:30\times2^5$. This is shown in Table 8.

Table 7. Latex agglutination test with TMV antigen (crude sap) and test antiserum.

Reciprocal of antigen dilution 30×2^n	Reciprocal of antibody dilution in test antiserum 10×2^n											Control
n=	4	5	6	7	8	9	10	11	12	13	14	
0	−	±	−	−	−	−	−	−	−	−	−	−
1	−	+	±	−	−	−	−	−	−	−	−	−
2	+	+	+	±	−	−	−	−	−	−	−	−
3	+	+	+	+	±	±	−	−	−	−	−	−
4	+	‡	+	+	+	+	±	−	−	−	−	−
5	+	‡	‡	‡	+	+	+	±	−	−	−	−
6	+	‡	+	+	+	+	+	−	−	−	−	−
7	+	+	+	+	+	+	±	−	−	−	−	−
8	−	+	±	±	±	±	−	−	−	−	−	−
9	−	±	−	−	−	−	−	−	−	−	−	−
10	−	−	−	−	−	−	−	−	−	−	−	−
Control	−	−	−	−	−	−	−	−	−	−	−	−

Table 8. Latex agglutination test with TMV antigen (purified virus) and test antiserum.

Reciprocal of antigen dilution 30×2^n	Reciprocal of antibody dilution in test antiserum 10×2^n											Control
n=	4	5	6	7	8	9	10	11	12	13	14	
0	−	±	−	−	−	−	−	−	−	−	−	−
1	+	+	±	±	−	−	−	−	−	−	−	−
2	‡	+	+	+	±	±	−	−	−	−	−	−
3	‡	‡	+	+	+	+	±	−	−	−	−	−
4	‡	‡	‡	‡	‡	‡	+	±	−	−	−	−
5	+	‡	‡	+	+	+	+	+	−	−	−	−
6	+	+	+	+	+	+	+	±	−	−	−	−
7	+	+	+	+	+	+	±	−	−	−	−	−
8	+	+	+	+	+	±	−	−	−	−	−	−
9	+	±	±	±	±	−	−	−	−	−	−	−
10	±	−	−	−	−	−	−	−	−	−	−	−
Control	−	−	−	−	−	−	−	−	−	−	−	−

PVX. The field of reaction appeared almost in square shape except for the remarkable lack of positive reaction in the region of antigen excess, when use was made with test antiserum to PVX in order to detect PVX antigen in crude sap. The detectability of antigen in this method, as it were, seemed equivalent even if titrated with test antiserum of different antibody content within appropriate extent. The dilution endpoint of the antigen thus obtained was $1:30\times2^{6}$ and serological titer of the test antiserum as determined by this antibody dilution was $1:10\times2^{11}$. This is shown in Table 9. It was found that almost the same pattern of the field of reaction was also obtainable when PVX antigen in purified form was titrated with the test antiserum, though it

Table 9. Latex agglutination test with PVX antigen (crude sap) and test antiserum.

Reciprocal of antigen dilution 30×2^{n}	Reciprocal of antibody dilution in test antiserum 10×2^{n}											Control
n=	4	5	6	7	8	9	10	11	12	13	14	
0	⧺	⧺	⧺	⧺	+	−	−	−	−	−	−	−
1	⧺	⧺	⧺	⧺	⧺	+	±	−	−	−	−	−
2	⧺	⧺	⧺	⧺	⧺	+	+	±	−	−	−	−
3	⧺	⧺	⧺	⧺	⧺	⧺	+	+	±	−	−	−
4	⧺	⧺	⧺	⧺	+	⧺	+	±	−	−	−	−
5	+	+	+	+	+	+	+	−	−	−	−	−
6	+	+	+	+	+	+	±	−	−	−	−	−
7	±	±	±	±	±	±	−	−	−	−	−	−
8	−	−	−	−	−	−	−	−	−	−	−	−
9	−	−	−	−	−	−	−	−	−	−	−	−
10	−	−	−	−	−	−	−	−	−	−	−	−
Control	−	−	−	−	−	−	−	−	−	−	−	−

Table 10. Latex agglutination test with PVX antigen (purified virus) and test antiserum.

Reciprocal of antigen dilution 30×2^{n}	Reciprocal of antibody dilution in test antiserum 10×2^{n}											Control
n=	4	5	6	7	8	9	10	11	12	13	14	
0	⧺	⧺	⧺	⧺	−	−	−	−	−	−	−	−
1	⧺	⧺	⧺	⧺	±	−	−	−	−	−	−	−
2	⧺	⧺	⧺	⧺	+	±	−	−	−	−	−	−
3	⧺	⧺	+	+	+	+	±	−	−	−	−	−
4	⧺	+	+	+	+	±	−	−	−	−	−	−
5	+	+	+	+	+	−	−	−	−	−	−	−
6	+	±	±	±	±	−	−	−	−	−	−	−
7	±	−	−	−	−	−	−	−	−	−	−	−
8	−	−	−	−	−	−	−	−	−	−	−	−
9	−	−	−	−	−	−	−	−	−	−	−	−
10	−	−	−	−	−	−	−	−	−	−	−	−
Control	−	−	−	−	−	−	−	−	−	−	−	−

had an unlike uniformity in its size as shown in Table 10. There was also a small zone in the region of antigen excess where there was no precipitate and the highest precipitation end-point of the test antiserum, in this respect, was $1:10\times2^{9}$ in its antibody dilution titrating with antigen at a constant dilution of $1:30\times2^{3}$. The dilution end-point of antigen as determined by this method with test antiserum of a constant antibody dilution at $1:10\times2^{4}$ was $1:30\times2^{6}$.

PVY. There was some resemblances in the shape and size of the field of reaction obtained from the grid titration using the test antiserum method with PVY antigen in crude sap as compared with those obtained using test antigen method with the same

Table 11. Latex agglutination test with PVY antigen (crude sap) and test antiserum.

Reciprocal of antigen dilution 30×2^{n}	Reciprocal of antibody dilution in test antiserum 10×2^{n}											Control
n=	4	5	6	7	8	9	10	11	12	13	14	
0	++	++	++	++	+	+	±	−	−	−	−	−
1	++	+	+	+	+	±	−	−	−	−	−	−
2	++	+	+	+	±	−	−	−	−	−	−	−
3	+	+	+	±	−	−	−	−	−	−	−	−
4	+	±	±	−	−	−	−	−	−	−	−	−
5	±	−	−	−	−	−	−	−	−	−	−	−
6	−	−	−	−	−	−	−	−	−	−	−	−
7	−	−	−	−	−	−	−	−	−	−	−	−
8	−	−	−	−	−	−	−	−	−	−	−	−
9	−	−	−	−	−	−	−	−	−	−	−	−
10	−	−	−	−	−	−	−	−	−	−	−	−
Control	−	−	−	−	−	−	−	−	−	−	−	−

Table 12. Latex agglutination test with PVY antigen (purified virus) and test antiserum.

Reciprocalof antigen dilution 30×2^{n}	Reciprocal of antibody dilution in test antiserum 10×2^{n}											Control
n=	4	5	6	7	8	9	10	11	12	13	14	
0	−	−	−	−	−	−	−	−	−	−	−	−
1	−	−	−	−	−	−	−	−	−	−	−	−
2	−	−	−	−	−	−	−	−	−	−	−	−
3	−	−	−	−	−	−	−	−	−	−	−	−
4	−	−	−	−	−	−	−	−	−	−	−	−
5	−	−	−	−	−	−	−	−	−	−	−	−
6	−	−	−	−	−	−	−	−	−	−	−	−
7	−	−	−	−	−	−	−	−	−	−	−	−
8	−	−	−	−	−	−	−	−	−	−	−	−
9	−	−	−	−	−	−	−	−	−	−	−	−
10	−	−	−	−	−	−	−	−	−	−	−	−
Control	−	−	−	−	−	−	−	−	−	−	−	−

antigen. The highest dilution end-point of antigen showed a positive reaction was $1:30\times2^4$ when use was made with test antiserum to PVY in crude sap at a constant antibody dilution of $1:10\times2^4$. On the other hand, the highest precipitation end-point of test antiserum was $1:10\times2^9$ in its antibody content when titrated with antigen at a constant dilution of 1:30. This is shown in Table 11. While the grid titration by the LAT using the test antiserum method revealed negative result with PVY antigen in purified virus suspension, in which virus concentration of significantly smaller amount was thought to have been involved. This is shown in Table 12.

Table 13. Macroagglutination test with TMV antigen (crude sap) and antiserum.

Reciprocal of antigen dilution 30×2^n		Reciprocal of antiserum dilution 2^n											Control
	n=	3	4	5	6	7	8	9	10	11	12	13	
n=	0	++	++	+	+	+	+	+	±	−	−	−	−
	1	++	++	+	+	+	+	+	±	−	−	−	−
	2	+	+	+	+	+	+	+	±	−	−	−	−
	3	+	+	+	+	+	+	±	−	−	−	−	−
	4	+	+	+	+	+	±	−	−	−	−	−	−
	5	+	+	+	+	±	−	−	−	−	−	−	−
	6	+	+	+	±	−	−	−	−	−	−	−	−
	7	+	±	−	−	−	−	−	−	−	−	−	−
	8	±	−	−	−	−	−	−	−	−	−	−	−
	9	−	−	−	−	−	−	−	−	−	−	−	−
	10	−	−	−	−	−	−	−	−	−	−	−	−
	Control	−	−	−	−	−	−	−	−	−	−	−	−

Table 14. Macroagglutination test with TMV antigen (purified virus) and antiserum.

Reciprocal of antigen dilution 30×2^n		Reciprocal of antiserum dilution 2^n											Control
	n=	3	4	5	6	7	8	9	10	11	12	13	
n=	0	++	++	++	++	+	+	+	±	−	−	−	−
	1	++	++	++	+	+	+	+	±	−	−	−	−
	2	+	+	+	+	+	+	+	±	−	−	−	−
	3	+	+	+	+	+	+	±	−	−	−	−	−
	4	+	+	+	+	+	+	±	−	−	−	−	−
	5	+	+	+	+	+	±	−	−	−	−	−	−
	6	±	+	+	+	±	−	−	−	−	−	−	−
	7	−	±	+	±	−	−	−	−	−	−	−	−
	8	−	−	±	−	−	−	−	−	−	−	−	−
	9	−	−	−	−	−	−	−	−	−	−	−	−
	10	−	−	−	−	−	−	−	−	−	−	−	−
	Control	−	−	−	−	−	−	−	−	−	−	−	−

For comparison of the superior sensibility of the LAT beyond the traditional MAT, results of the grid titration of the three plant viruses under consideration were also shown in Tables 13–18.

Determination of the favourable condition for the latex agglutination test with TMV antigen

Several factors affecting the sensibility and specificity of the latex agglutination test were examined to determine the favourable condition for the test using TMV antigen and the corresponding antiserum. For a control, macroagglutination test with the same preparations used in the experiment were carried out using box titration. Pattern of the field of reaction thus obtained is shown in Table 19.

Table 15. Macroagglutination test with PVX antigen (crude sap) and antiserum.

Reciprocal of antigen dilution 30×2^n		Reciprocal of antiserum dilution 2^n											Control
	n=	3	4	5	6	7	8	9	10	11	12	13	
n=	0	##	+	+	+	+	+	+	+	±	−	−	−
	1	##	+	+	+	+	+	+	±	−	−	−	−
	2	+	+	+	+	±	−	−	−	−	−	−	−
	3	+	+	+	±	−	−	−	−	−	−	−	−
	4	+	+	±	−	−	−	−	−	−	−	−	−
	5	+	±	−	−	−	−	−	−	−	−	−	−
	6	±	−	−	−	−	−	−	−	−	−	−	−
	7	−	−	−	−	−	−	−	−	−	−	−	−
	8	−	−	−	−	−	−	−	−	−	−	−	−
	9	−	−	−	−	−	−	−	−	−	−	−	−
	10	−	−	−	−	−	−	−	−	−	−	−	−
	Control	−	−	−	−	−	−	−	−	−	−	−	−

Table 16. Macroagglutination test with PVX antigen (purified virus) and antiserum.

Reciprocal of antigen dilution 30×2^n		Reciprocal of antiserum dilution 2^n											Control
	n=	3	4	5	6	7	8	9	10	11	12	13	
n=	0	##	+	+	+	+	+	+	±	−	−	−	−
	1	+	+	+	+	±	−	−	−	−	−	−	−
	2	+	+	±	−	−	−	−	−	−	−	−	−
	3	+	+	±	−	−	−	−	−	−	−	−	−
	4	+	+	±	−	−	−	−	−	−	−	−	−
	5	+	±	−	−	−	−	−	−	−	−	−	−
	6	±	−	−	−	−	−	−	−	−	−	−	−
	7	−	−	−	−	−	−	−	−	−	−	−	−
	8	−	−	−	−	−	−	−	−	−	−	−	−
	9	−	−	−	−	−	−	−	−	−	−	−	−
	10	−	−	−	−	−	−	−	−	−	−	−	−
	Control	−	−	−	−	−	−	−	−	−	−	−	−

1. Determination of the suitable diluent for the test.

Ten different diluents previously described were compared with each other to tell what kind of electrolyte or what value of pH is the most suitable for the test, respectively.

The latex stock suspension was diluted with a diluent to be tested to make a solution of 1:100. The final latex suspension thus obtained were mixed with 1/20 volume of either virus antigen diluted with the same diluent to make a ten-fold serial dilution from 10^2 to 10^6, or antiserum samely diluted to make a solution of 1:1, 1:5, 1:10, 1:50

Table 17. Macroagglutination test with PVY antigen (crude sap) and antiserum.

Reciprocal of antigen dilution 30×2^n		Reciprocal of antiserum dilution 2^n											Control
	n=	3	4	5	6	7	8	9	10	11	12	13	
n=	0	+	+	±	−	−	−	−	−	−	−	−	−
	1	+	+	±	−	−	−	−	−	−	−	−	−
	2	+	±	−	−	−	−	−	−	−	−	−	−
	3	+	±	−	−	−	−	−	−	−	−	−	−
	4	±	−	−	−	−	−	−	−	−	−	−	−
	5	−	−	−	−	−	−	−	−	−	−	−	−
	6	−	−	−	−	−	−	−	−	−	−	−	−
	7	−	−	−	−	−	−	−	−	−	−	−	−
	8	−	−	−	−	−	−	−	−	−	−	−	−
	9	−	−	−	−	−	−	−	−	−	−	−	−
	10	−	−	−	−	−	−	−	−	−	−	−	−
	Control	−	−	−	−	−	−	−	−	−	−	−	−

Table 18. Macroagglutination test with PVY antigen (purified virus) and antiserum.

Reciprocal of antigen dilution 30×2^n		Reciprocal of antiserum dilution 2^n											Control
	n=	3	4	5	6	7	8	9	10	11	12	13	
n=	0	−*	−	−	−	−	−	−	−	−	−	−	−
	1	−	−	−	−	−	−	−	−	−	−	−	−
	2	−	−	−	−	−	−	−	−	−	−	−	−
	3	−	−	−	−	−	−	−	−	−	−	−	−
	4	−	−	−	−	−	−	−	−	−	−	−	−
	5	−	−	−	−	−	−	−	−	−	−	−	−
	6	−	−	−	−	−	−	−	−	−	−	−	−
	7	−	−	−	−	−	−	−	−	−	−	−	−
	8	−	−	−	−	−	−	−	−	−	−	−	−
	9	−	−	−	−	−	−	−	−	−	−	−	−
	10	−	−	−	−	−	−	−	−	−	−	−	−
	Control	−	−	−	−	−	−	−	−	−	−	−	−

* Positive reaction was obtained with antigen diluted to 1 : 2 and antiserum diluted to 1 : 8.

Table 19. Macroagglutination test with TMV antigen (purified virus) and antiserum.

Reciprocal of antigen dilution 100×2^n	Reciprocal of antiserum dilution 2^n											Control
n=	3	4	5	6	7	8	9	10	11	12	13	
0	##	##	##	##	+	+	−	−	−	−	−	−
1	##	##	##	##	##	##	±	−	−	−	−	−
2	##	##	+	+	+	+	+	±	−	−	−	−
3	##	+	+	+	+	+	+	+	−	−	−	−
4	+	+	+	+	+	+	+	±	−	−	−	−
5	+	+	+	±	±	±	±	−	−	−	−	−
6	+	±	±	−	−	−	−	−	−	−	−	−
7	±	−	−	−	−	−	−	−	−	−	−	−
8	−	−	−	−	−	−	−	−	−	−	−	−
9	−	−	−	−	−	−	−	−	−	−	−	−
10	−	−	−	−	−	−	−	−	−	−	−	−
Control	−	−	−	−	−	−	−	−	−	−	−	−

and 1:100, respectively. As a control, final latex solution was mixed with 1/20 volume of the same diluent as used to dilute the antigen or antiserum in the same test. Thus, both antigen and antiserum were prepared against ten different kinds of diluents and tested against antiserum and antigen, respectively, diluted with same buffer in serial row between 100×2^0 to 100×2^{10}. Of these diluents used, the phosphate-buffered saline solution, 0.01 M, pH 5.0, was found to be the most suitable one for the preparation of the test antigen as well as the antiserum (Plates I & II). Data are shown in Tables 20 and 21. It was also confirmed that the phosphate-buffered saline solution, 0.01 M, pH 8.0, is the most favourable diluent to obtain the highest sensitivity and specificity for the preparation of the test antiserum as well as the antigen dilution (Plates III & IV). Veronal-buffered saline solution, pH 6.0, was also found favourable (Plate V). Data also shown in Tables 22 and 23.

2. Determination of the optimal temperature and time upon adsorption procedure.

The optimal temperature and time upon the adsorption were determined by testing antigen or antiserum mixed with latex suspension at 0 C in cold room, at room temperature and at 37 C in water bath, respectively, for 2 hr or overnight, followed by making react with the corresponding antiserum or antigen. The concentration of a final latex suspension was 1:200 and those of antigen and antiserum used for adsorption were 1:100 and 1:10, respectively. Diluents used were phosphate-buffered saline solution of pH 5.0 for the test antigen and of pH 8.0 for the test antiserum, respectively. Incubation was carried out at 37 C for 2 hr in a water bath with subsequent overnight standing at 0 C in a cold room. The results are shown in Tables 24 and 25 indicating the sensibility of the test is irrespective of the temperature and time required for the adsorption of the antigen as well as antiserum on the biologically inert surface of the latex particles.

Table 20. Determination of the suitable diluent for preparation of the test antigen and for dilution of antiserum (1).

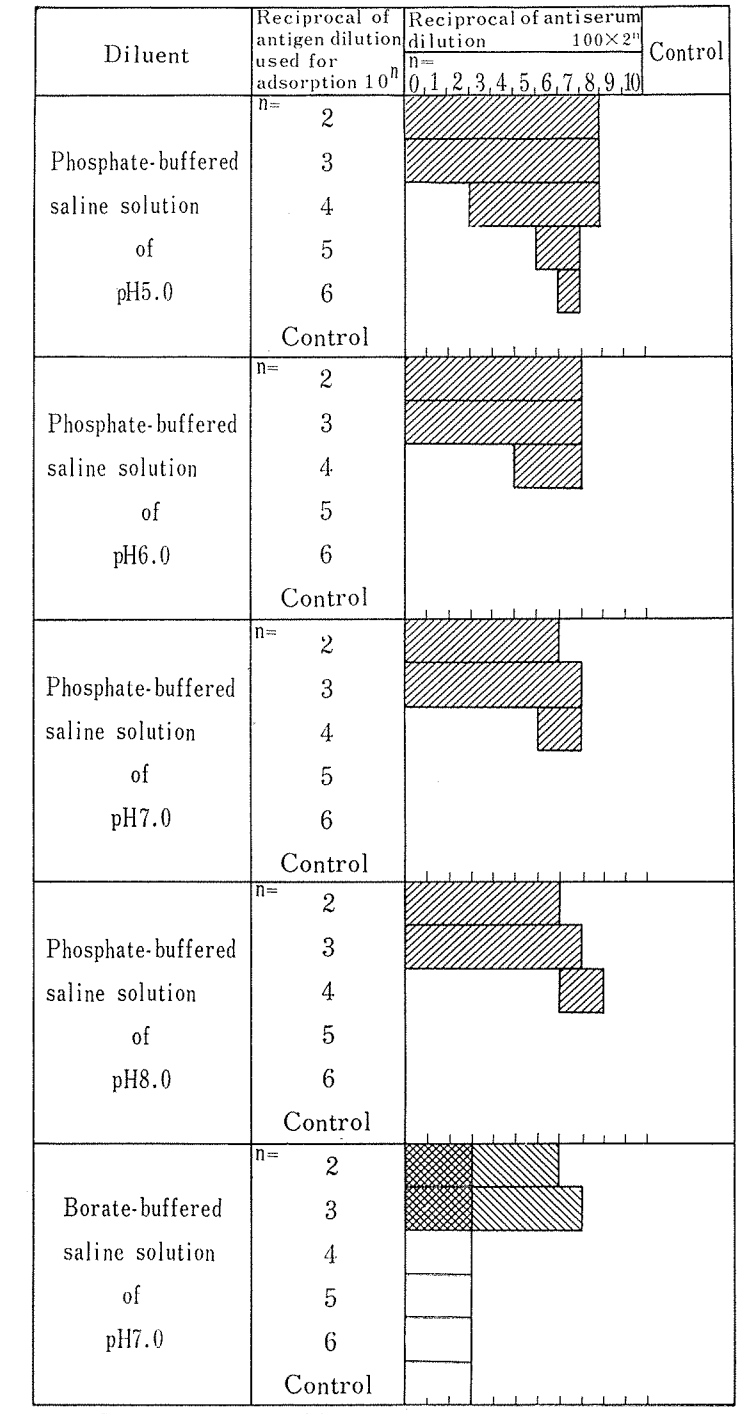


Table 21. Determination of the suitable diluent for preparation of the test antigen and for dilution of antiserum (2).

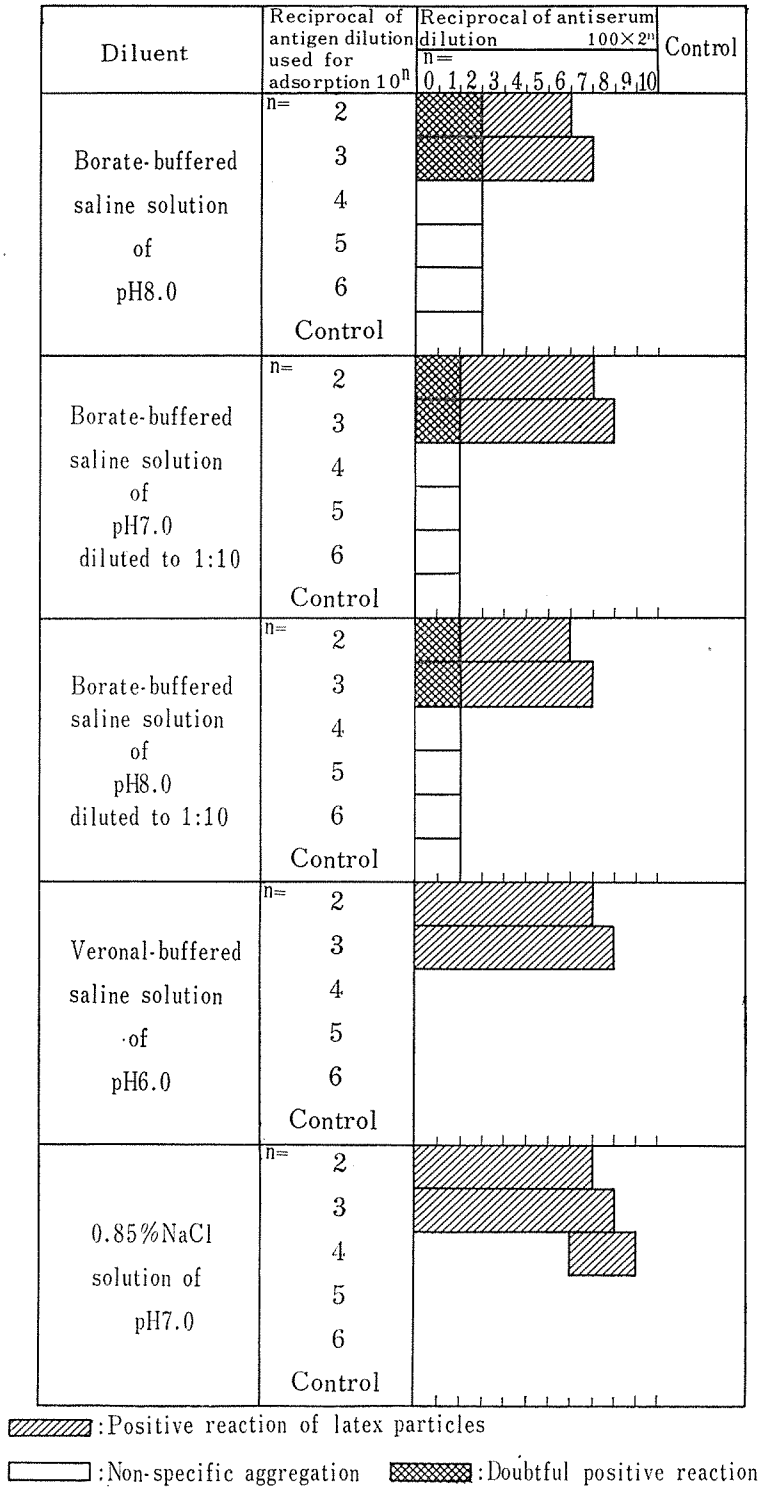
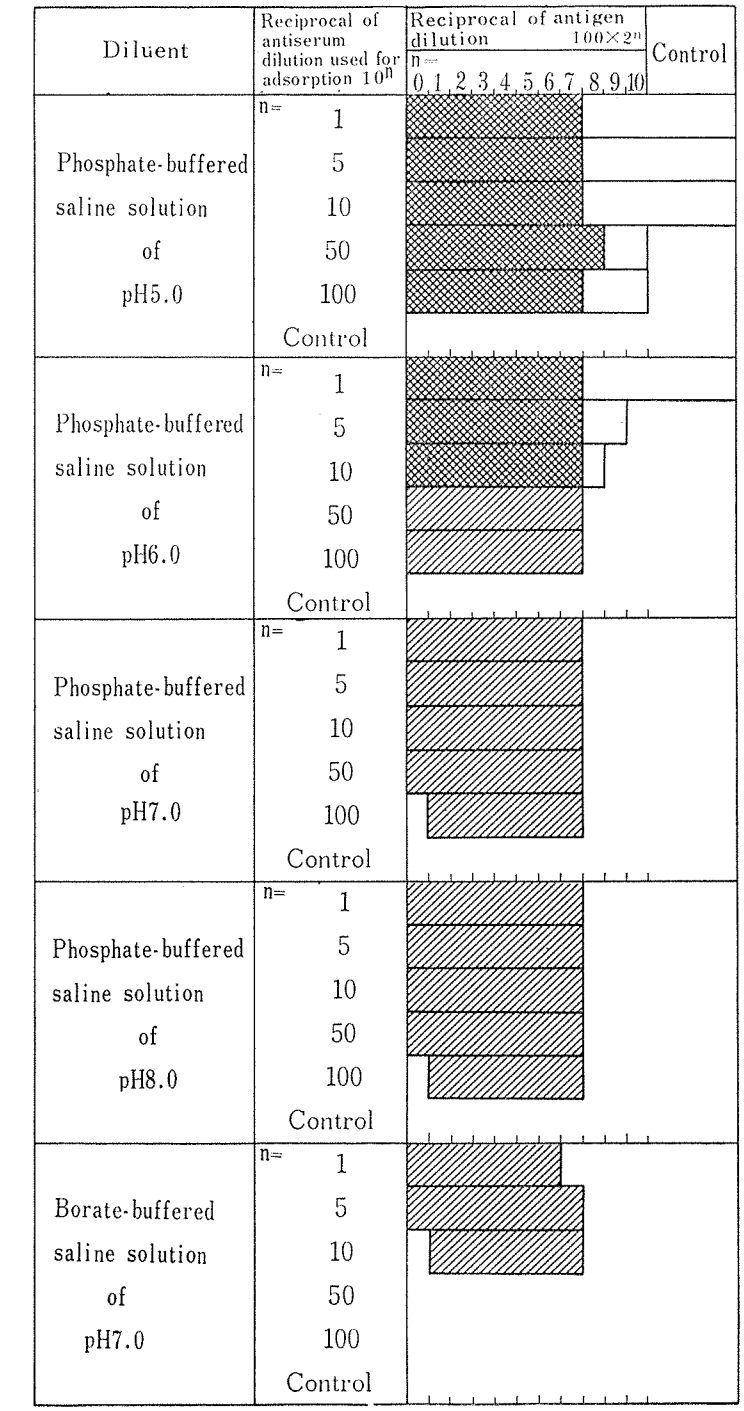


Table 22. Determination of the suitable diluent for preparation of the test antiserum and for dilution of antigen (1).



▨: Positive reaction of latex particles

□: Non-specific aggregation ▩: Doubtful positive reaction

Table 23. Determination of the suitable diluent for preparation of the test antiserum and for dilution of antigen (2).

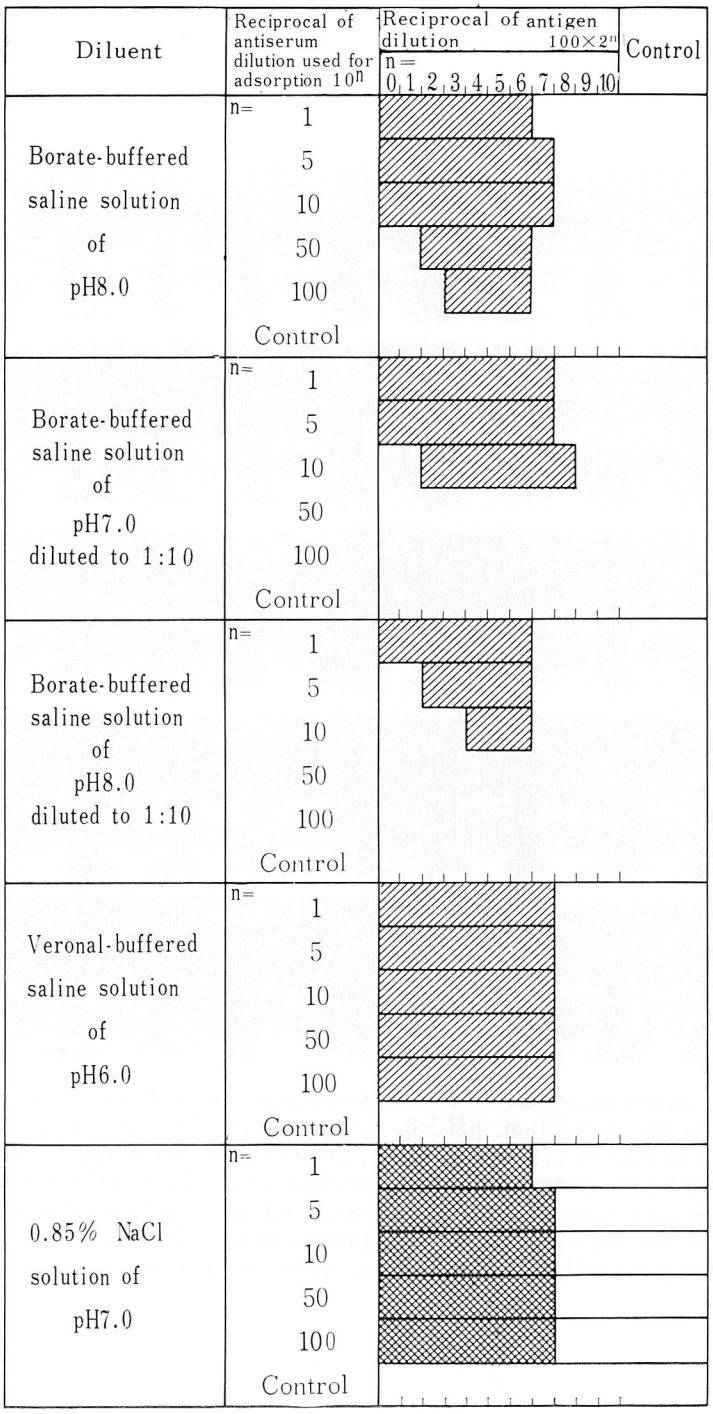


Plate I. Latex agglutination test with TMV test antigen and antiserum. A 0.01 M phosphate-buffered saline solution, pH 5.0, containing 1% latex was used as diluent. TMV antigen diluted to 1:100, 1:1,000, 1:10,000 and 1:100,000 (from upper to lower) was used for the preparation of the test antigen, respectively, and latex suspension of the same diluent without TMV antigen was used as a control for the reaction. Antiserum used for the test was diluted from 1:100 to $1:100\times2^{10}$ (left to right). The last tubes containing no antibody were added as control for the test.

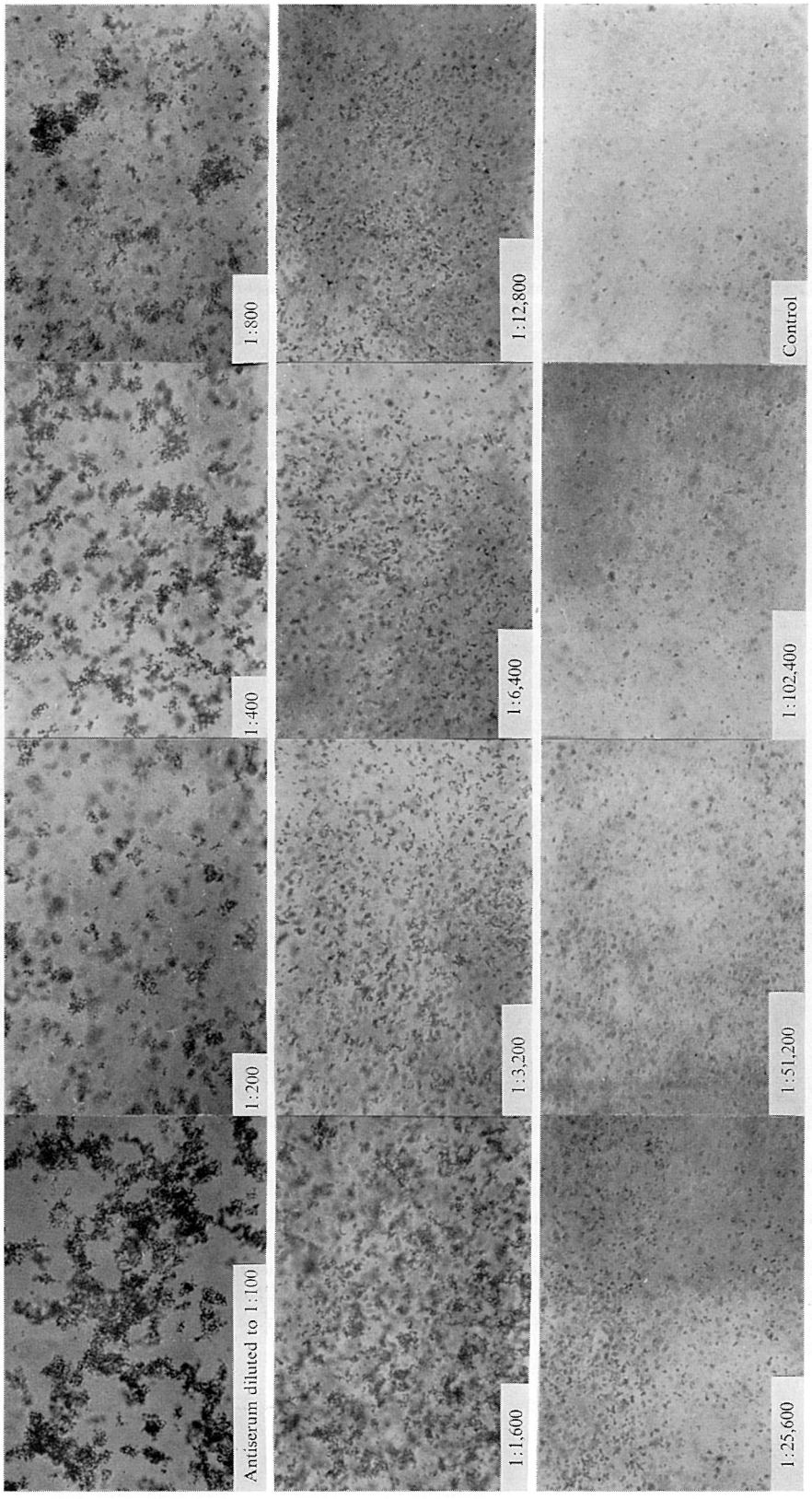


Plate II. Latex agglutination test with test antigen. Observation under a microscope.
Diluent: 0.01 M phosphate-buffered saline solution, pH 5.0, containing 1% latex.
TMV antigen adsorbed on the latex: 1:100

Plate III. Latex agglutination test with TMV antigen and test antiserum.
A 0.01 M phosphate buffered saline solution, pH 8.0, containing 1% latex was used as diluent. TMV antiserum diluted to 1:1, 1:5, 1:10, 1:50 and 1:100 (from upper to lower) was used for the preparation of the test antiserum, respectively, and latex suspension of the same diluent without TMV antiserum was used as a control for the reaction. Antigen used for the test was diluted from 1:100 to $1:100\times 2^{10}$ (left to right). The last tubes containing no antigen were added as a control for the test.

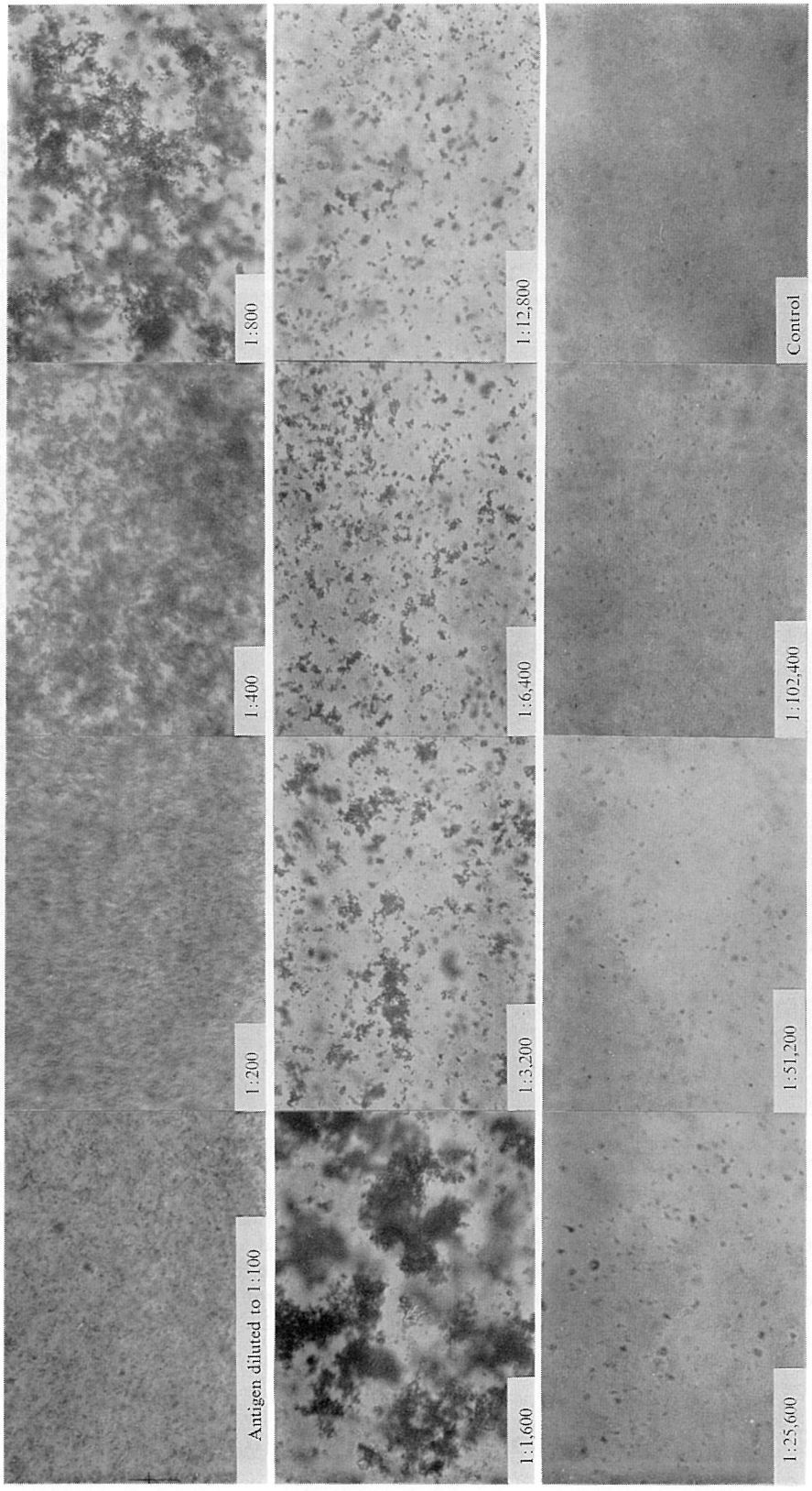


Plate IV. Latex agglutination test with TMV test antiserum. Observation under a microscope. Diluent: 0.01 M phosphate-buffered saline solution, pH 8.0, containing 1% latex. TMV antiserum adsorbed on the latex: 1:100.

Plate V. Latex agglutination test with TMV antigen and antiserum.
Veronal-buffered saline solution, pH 6.0, containing 1% latex was used as diluent. TMV antiserum diluted to 1:1, 1:5, 1:10, 1:50 and 1:100 (from upper to lower) was used for the preparation of the test antiserum, respectively, and latex suspension of the same diluent with out TMV antiserum was used as a control for the reaction. Antigen used for the test was diluted from 1:100 to $1:100\times 2^{10}$ (left to right). The last tubes containing no antigen were added as a control for the reaction.

3. Determination of optimal concentration of latex, antigen and antiserum for the test.

To determine the influence of optimal concentration of latex suspension, antigen and antiserum used for adsorption, latex stock suspension was diluted to make a solution of 1:100, 1:200 and 1:400, respectively, and mixed with a 1/20 volume of antigen diluted to 1:100 and 1:200, or antiserum diluted to 1:10 and 1:100, respectively. Diluents used for the test were 0.01 M phosphate-buffered saline solution of pH 5.0 for the test antigen and of pH 8.0 for the test antiserum. Overnight incubation at room temperature was carried out for adsorption adsorption and 0.5 ml of the test antigen

Table 24. Influence of temperature and time upon adsorption with antigen.

Temperature	Time	Reciprocal of antiserum dilution 100×2^n (n = 0 1 2 3 4 5 6 7 8 9 10)	Control
0 C	2 hr		
	overnight		
20 C	2 hr		
	overnight		
37 C	2 hr		
	overnight		

□ : Positive reaction at 37 C for 2 hr.
■ : Positive reaction at 37 C for 2 hr and subsequent overnight incubation at 0 C.

Table 25. Influence of temperature and time upon adsorption with antiserum.

Temperature	Time	Reciprocal of antiserum dilution 100×2^n (n = 0 1 2 3 4 5 6 7 8 9 10)	Control
0 C	2 hr		
	overnight		
20 C	2 hr		
	overnight		
37 C	2 hr		
	overnight		

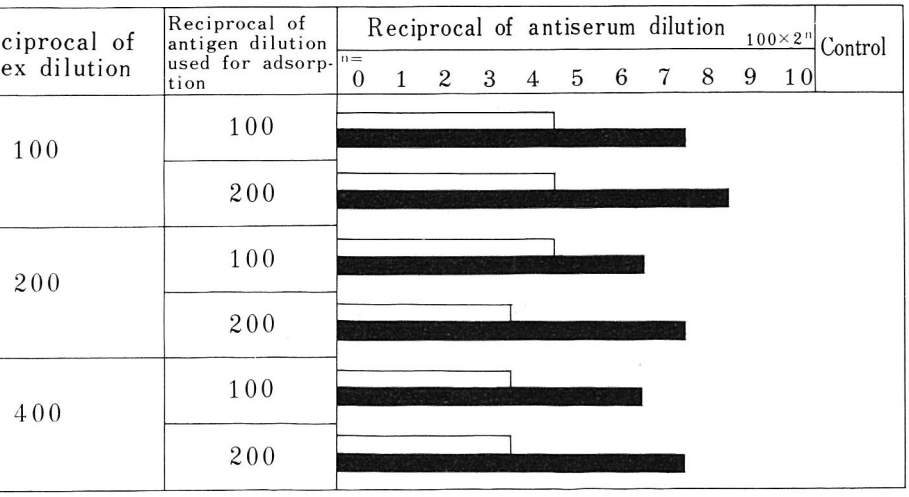
■ : Positive reaction at 37 C for 2hr.

or test antiserum thus prepared were mixed with an equal volume of the corresponding reactants in a serial dilution by a factor of two. After overnight incubation followed by the incubation in a water bath at 37 C for 2 hr, results of the test were recorded macroscopically and are shown in Tables 26 and 27.

When the test was carried out by using the test antigen method, higher sensitivity and specificity of the reaction were obtained at higher concentration of latex particles. However, the results obtained with the use of lower concentration of latex were more clear-cut in the region of end-point showing positive reaction. The better result was obtained with antigen diluted 1:200 rather than 1:100 for adsorption (Plate VI). On

Table 26. Determination of suitable concentration of latex and antigen for adsorption.

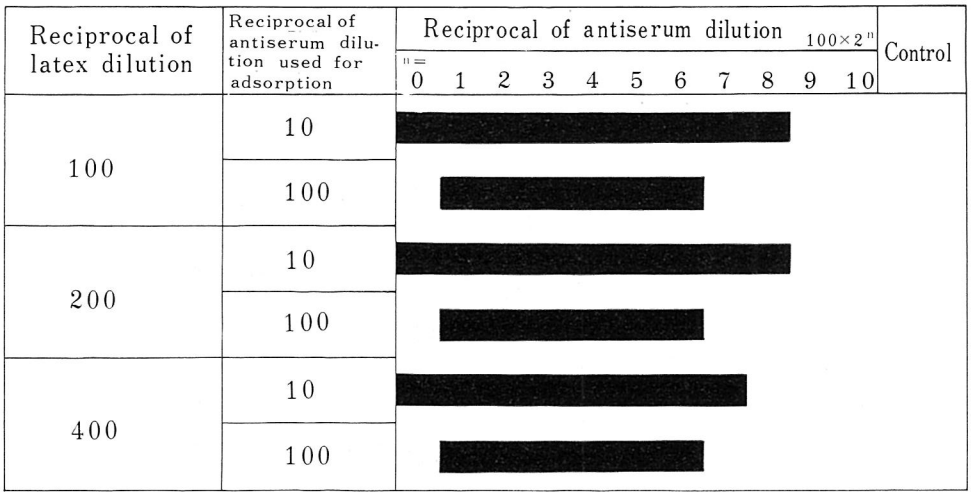
Reciprocal of latex dilution	Reciprocal of antigen dilution used for adsorption	Reciprocal of antiserum dilution 100×2^n (n = 0 1 2 3 4 5 6 7 8 9 10)	Control
100	100		
	200		
200	100		
	200		
400	100		
	200		



□: Positive reaction at 37C for 2hr.
■: Positive reaction at 37C for 2hr and subsequent overnight incubation at 0C.

Table 27. Determination of suitable concentration of latex and antiserum for adsorption.

Reciprocal of latex dilution	Reciprocal of antiserum dilution used for adsorption	Reciprocal of antiserum dilution 100×2^n (n = 0 1 2 3 4 5 6 7 8 9 10)	Control
100	10		
	100		
200	10		
	100		
400	10		
	100		



■: Positive reaction at 37C for 2hr.

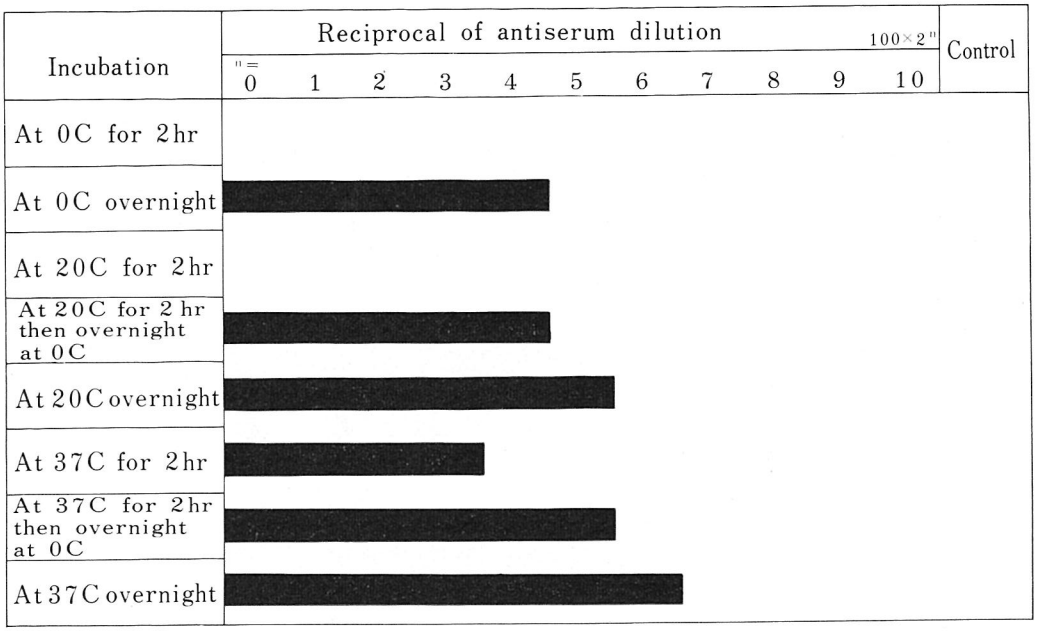
the other hand, it was found that there was no significant difference among the end-points showing positive reaction, when use was made with the latex solutions diluted to 1:100, 1:200 and 1:400, respectively, for adsorption of the antiserum. For the antiserum diluted to 1:10 the lower concentration of the latex gave somewhat more clear-cut results when the adsorption and subsequent agglutination test were carried out. With the antiserum diluted to 1:100 it was found that the inhibition by antigen in excess occurred against the antigen diluted to 1:100.

Plate VI. Influence of concentration of latex and TMV antigen used for adsorption upon the test.
A 0.01 M phosphate-buffered saline solution, pH 5.0, containing latex diluted to 1:100 (upper two), 1:200 (middle two) and 1:400 (lower two), respectively, was used for the preparation of the test antigen. Antiserum diluted from 1:100 to $1:100\times2^{10}$ (left to right) was used for the test. The last tubes containing no antibody were added as a control for the reaction.

4. Determination of the favourable condition for the test incubation.

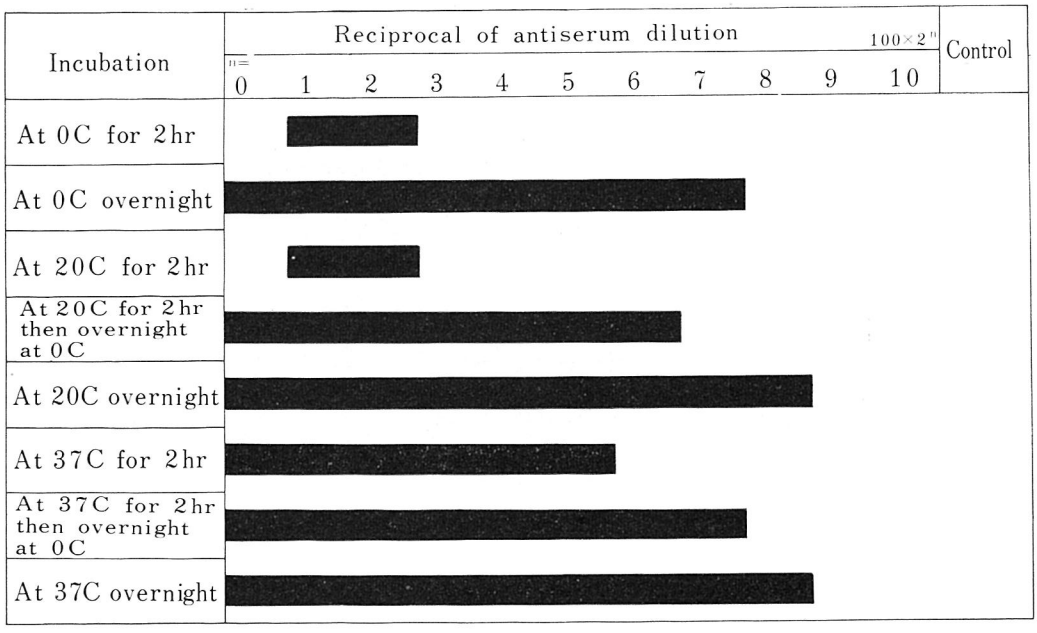
TMV antigen diluted to 1:100 was mixed with a 19 parts of the latex suspension diluted to 1:200 with 0.01 M phosphate-buffered saline solution of pH 5.0 and then allowed to stand for overnight incubation at room temperature for adsorption. The same procedure was applied to TMV antiserum diluted to 1:100 using the same buffer

Table 28. Influence of incubation upon the test with test antigen.



: Positive reaction

Table 29. Influence of incubation upon the test with antiserum.



: Positive reaction

solution of pH 8.0. A 0.5 ml of the test antigen or test antiserum thus prepared was mixed with the corresponding reactant serially diluted from 100×2^{0} to 100×2^{10} in an equal volume in test tubes, and then kept at 0 C, at room temperature (ca 20 C) and at 37 C, respectively for various incubation periods. Overnight incubation in water bath at 37 C found to be the most favourable for both test antigen and test antiserum methods, and incubation at 37 C for 2 hr with subsequent overnight preservation at 0 C or overnight incubation at room temperature also proved to be useful for both tests. Incubation for 2 hr at 0 C or at room temperature was found to be unsatisfactory as well as at 37 C. These results are shown in Tables 28 and 29 and also in Plates VII and VIII, respectively.

Quantitative studies on the sensitivity of the latex agglutination test as compared with other serological tests

The results obtained with latex agglutination test (LAT) were compared with those of other serological techniques such as macroagglutination test (MAT), microagglutination test (MIT) and ring-interface precipitin test (RIT). The concentration of TMV protein used in this experiment was adjusted to 10 mg/ml in its dry weight. Latex stock suspension was diluted to 1:400 with the phosphate-buffered saline solution of pH 5.0 for the test antigen and of pH 8.0 for the test antiserum, respectively. Overnight adsorption at room temperature was applied in this test for the convenience' sake. Incubation for the reaction was carried out for 2 hr in a water bath at 37 C with subsequent overnight standing at 0 C. Procedures for other serological techniques were of ordinary use. The resultant aggregates were observed by either macroscopically (–MA) or microscopically (–MI), or by both macroscopically and microscopically.

The result of a grid titration by the LAT with TMV test antigen in purified virus suspension and its corresponding antiserum detecting microscopically is shown in Table 30. Positive reactions were obtained with the test antigen that had been adsorbed with the antigen diluted to $1:1000\times2^{14}$ against the antiserum diluted from 1:200 to $1:100\times2^{5}$. However, with the antiserum diluted to $1:100\times2^{7}$, positive reactions were only found against the test antigens which contained TMV antigen at an initial dilution of $1:1000\times2^{8}$ to $1:1000\times2^{12}$.

On the other hand, as shown in Table 31, the sensitivity of the LAT-MI with the test antiserum was found a little less than that of the LAT-MI. It was found, using the test antiserum prepared from TMV antiseru diluted to $1:500\times2^{2}$, that TMV antigen was able to detect serologically at an initial antigen dilution of $1:100\times2^{11}$. TMV antigens at an initial antigen dilution of $1:100\times2^{10}$ and $1:100\times2^{9}$, respectively, showed the positive reaction against the test antiserum consisting the antibody at an initial dilution of 1:500 to $1:500\times2^{3}$ and 1:500 to $1:500\times2^{7}$. With the test antiserum prepared from antiserum diluted to $1:500\times2^{2}$ or more, it was found that the inhibition by antigen excess where there was no precipitate took place, indicating unsatisfactory

Plate VII. Influence of incubation upon the test with TMV test antigen and antiserum.
From upper to bottom: After overnight incubation at 0 C, at 20 C and at 37 C, and after incubation at 20 C for 2 hr and 37 C for 2 hr with subsequent overnight refrigeration.
Antigen diluted to 1:100 was adsorbed on latex diluted to 1:200 in 0.01 M phosphate-buffered saline solution, pH 5.0. Antiserum used for the test was diluted from 1:100 to $1:100\times2^{10}$ (left to right). The last tubes containing no antibody were added as a control for the test.

Plate VIII. Influence of incubation upon the test with TMV antigen and test antiserum.
From upper to bottom: After overnight incubation at 0 C, at 20 C and at 37 C, and after incubation t at 20 C for 2 hr and at 37 C for 2 hr with subsequent overnight refrigeration.
Antiserum diluted to 1:100 was adsorbed on latex diluted to 1:200 in 0.01 M phosphate-buffered saline solution, pH 8.0. Antigen used for the test was diluted from 1:100 to $1:100 \times 2^{10}$ (left to right). The last tubes containing no antigen were added as a control for the test.

use of the test antiserum with a low antibody concentration for the detection of TMV antigen.

Table 32 shows the grid titration by the RIT with TMV antigen in purified virus suspension and its corresponding antiserum. The results obtained from the grid titration by the MAT with the same antigen and antiserum detecting macroscopically

Table 30. Latex agglutination test with TMV test antigen and antiserum*.

Reciprocal of initial antigen dilution in the test antigen 1000×2^n	Reciprocal of antiserum dilution** 100×2^n											Control
n=	0	1	2	3	4	5	6	7	8	9	10	
5	+	+	++	++	++	+	+	−	−	−	−	−
6	+	+	+	++	++	+	+	−	−	−	−	−
7	+	+	+	+	++	++	+	±	−	−	−	−
8	+	+	+	+	+	++	+	+	±	−	−	−
9	+	+	+	+	+	+	++	+	±	−	−	−
10	+	+	+	+	+	+	+	+	±	−	−	−
11	+	+	+	+	+	+	+	+	−	−	−	−
12	+	+	+	+	+	+	+	+	−	−	−	−
13	+	+	+	+	+	+	+	−	−	−	−	−
14	±	+	+	+	+	+	±	−	−	−	−	−
15	−	−	−	−	±	±	−	−	−	−	−	−
Control	−	−	−	−	−	−	−	−	−	−	−	−

* Reading was made microscopically
** Initial antiserum dilution used for reaction

Table 31. Latex agglutination test with TMV antigen and test antiserum*.

Reciprocal of antigen dilution*** 100×2^n	Reciprocal of antibody dilution** 500×2^n											Control
n=	0	1	2	3	4	5	6	7	8	9	10	
2	+	+	±	−	−	−	−	−	−	−	−	−
3	+	+	+	+	±	−	−	−	−	−	−	−
4	++	++	++	+	+	±	−	−	−	−	−	−
5	++	++	++	++	+	+	±	−	−	−	−	−
6	++	++	++	++	++	+	+	±	−	−	−	−
7	++	++	++	++	+	+	+	+	±	−	−	−
8	++	++	++	+	+	+	+	+	+	±	−	−
9	++	++	++	+	+	+	+	+	±	−	−	−
10	+	+	++	+	±	±	±	±	−	−	−	−
11	±	±	+	±	−	−	−	−	−	−	−	−
12	−	−	±	−	−	−	−	−	−	−	−	−
Control	−	−	−	−	−	−	−	−	−	−	−	−

* Reading was made microscopically
** Initial antibody dilution in the test antiserum
*** Initial antigen dilution used for reaction

(MAT-MA) and microscopically (MIT-MI) are also shown in Tables 33 and 34, respectively. Summarized datas obtained from these comparative titrations showed the evidence of high sensitivity of the LAT with the test antigen. It was estimated from the results shown in Table 35 that the serological titers of TMV antigen were $1:10\times2^{8}$, $1:10\times2^{10}$, $1:10\times2^{10}$, $1:1000\times2^{14}$ and $1:100\times2^{11}$, respectively, at an initial dilution end-point showing positive reaction, when titrations were made by the MAT-MA, MAT-MI, RIT, LAT with test antigen and LAT with test antiserum. The least amounts of TMV antigen serologically detectable, in this respect, were found

Table 32. Ring-interface precipitin test with TMV antigen*.

Reciprocal of antigen dilution 10×2^{n}		Reciprocal of antiserum dilution 2^{n}											Control
	n=	3	4	5	6	7	8	9	10	11	12	13	
n=	0	+	+	+	±	−	−	−	−	−	−	−	−
	1	+	+	+	+	±	−	−	−	−	−	−	−
	2	+	+	+	+	+	±	−	−	−	−	−	−
	3	+	+	+	+	+	+	±	−	−	−	−	−
	4	+	+	+	+	+	+	+	±	−	−	−	−
	5	+	+	+	+	+	+	+	+	−	−	−	−
	6	+	+	+	+	+	+	+	±	−	−	−	−
	7	+	+	+	+	+	+	±	−	−	−	−	−
	8	±	+	+	+	+	±	−	−	−	−	−	−
	9	−	+	+	+	±	−	−	−	−	−	−	−
	10	−	+	+	±	−	−	−	−	−	−	−	−
	Control	−	−	−	−	−	−	−	−	−	−	−	−

* Reading was made macroscopically (MA)

Table 33. Macroagglutination test with TMV antigen*.

Reciprocal of antigen dilution 10×2^{n}		Reciprocal of antiserum dilution 2^{n}											Control
	n=	3	4	5	6	7	8	9	10	11	12	13	
n=	0	±	±	−	−	−	−	−	−	−	−	−	−
	1	++	+	±	−	−	−	−	−	−	−	−	−
	2	++	++	+	±	−	−	−	−	−	−	−	−
	3	++	++	++	+	±	−	−	−	−	−	−	−
	4	++	++	++	+	+	±	−	−	−	−	−	−
	5	++	++	++	+	+	±	−	−	−	−	−	−
	6	+	++	+	+	+	±	−	−	−	−	−	−
	7	+	+	+	+	±	−	−	−	−	−	−	−
	8	±	+	+	±	−	−	−	−	−	−	−	−
	9	−	±	±	−	−	−	−	−	−	−	−	−
	10	−	−	−	−	−	−	−	−	−	−	−	−
	Control	−	−	−	−	−	−	−	−	−	−	−	−

* Reading was made macroscopically (MA)

Table 34. Macroagglutination test with TMV antigen*.

Reciprocal of antigen dilution 10×2^n		Reciprocal of antiserum dilution 2^n											Control
	n=	3	4	5	6	7	8	9	10	11	12	13	
n=	0	±	±	−	−	−	−	−	−	−	−	−	−
	1	++	+	±	±	−	−	−	−	−	−	−	−
	2	++	++	+	+	±	−	−	−	−	−	−	−
	3	++	++	++	+	+	±	−	−	−	−	−	−
	4	++	++	++	++	+	+	±	−	−	−	−	−
	5	++	++	++	++	++	+	+	±	−	−	−	−
	6	+	++	++	+	+	+	+	±	−	−	−	−
	7	+	+	+	+	+	+	±	−	−	−	−	−
	8	+	+	+	+	+	±	−	−	−	−	−	−
	9	+	+	+	+	±	−	−	−	−	−	−	−
	10	±	+	+	±	−	−	−	−	−	−	−	−
	Control	−	−	−	−	−	−	−	−	−	−	−	−

* Reading was made microscopically (MI)

Table 35. Comparison of different serological tests.

Serological tests used	Macroagglutination test (MAT)		Ring-interface precipitin test (RIT)	Latex agglutination test (LAT) with	
				test antigen	test antiserum
Method of reading of reaction used	MA**	MI***	MA**	MI***	
Reciprocal of initial dilution end-point of antigen that showed positive reaction	10×2^8	10×2^{10}	10×2^{10}	1000×2^{14}	100×2^{11}
Least amount of TMV antigen serologically detectable mg/ml*	3.9×10^{-3}	9.8×10^{-4}	9.8×10^{-4}	6.1×10^{-7}	4.9×10^{-5}

* Calculation was made on the basis of original concentration of TMV protein (10 mg/ml) in purified virus suspension used for the serological tests and a multiple in dilution.
** Reading was carried out using a agglutinoscope or with the naked eye.
*** Reading was carried out using a microscope.

3.9×10^{-3}, 9.8×10^{-4}, 9.8×10^{-4}, 6.1×10^{-7} and 4.9×10^{-5} mg/ml at the initial concentration in the antigen preparation, as determined respectively by the MAT-MA, MAT-MI, RIT, LAT with test antigen and LAT with test antiserum.

Discussion and Conclusion

For the detection of the virus antigen in the infected plants or for the diagnosis of the plant virus diseases, the serological methods are generally recommended mainly because of the simplicity and rapidness of its performance as well as its high specificity. On the other hand, most of these serological tests such as the ordinary MAT usually show undefined results at the region of the dilution end-point showing a positive reac-

tion. It is one of the disadvantages of the serological methods that the positive reaction can not be distinguished precisely by the ordinary reading method, if the clusters of the serological aggregates are comparatively small in size and few in number. From this standpoint, the LAT proved to be useful for the detection of some plant viruses because the procedure to be applied is almost the same as that of the MAT using the test tubes and the reaction is sensitive and easy to read.

Some investigators(3,4,5,9,12,13,16), who applied the latex agglutination to the serological diagnosis of such as rhumatoid arthritis, histoplasmosis and leptospirosis, etc., defined the test as it is essentially precipitation reaction between soluble antigen coated on the surface of the biologically inert latex particles and the corresponding antiserum. Muraschi(9) reported the latex agglutination test with leptospiral antigen. He used the formolized leptospiral preparation as the test antigen consisted of whole leptospiral organisms as seen by dark-field examination in a microscope. After aggregation with the corresponding antiserum, the precipitates were found to be consisted of intact leptospirae gathered together and complexed with scattered individual latex particles, leaving the most of the latex particles floating freely in the medium. According to Kelen and Labzoffsky(5), in Muraschi's formolized antigen only whole leptospirae are responsible for the reaction and the latex particles do not play as an essential contributor in the LAT. They also described the LAT using the pyridine-treated leptospiral antigen absence of visible leptospirae organisms, fragments or granules and refered to as the soluble antigen.

Intact plant virus antigens used in our experiments, in this respect, are reasonably considered as a kind of the soluble antigens in LAT, though it is very difficult to prove the accurate evidence whether antigen as well as antibody is certainly adsorbed on the surface of the latex particles or only attached to them. The facts appeared in these experiments, however, will afford the explanation to these problems indirectly.

It seems likely that the latex particles itself are responsible for the reaction instead of the intermigration of the latex particles into the antigen-antibody complexes taking place. The possible reason is that there is no individual latex particle in the positive tubes, or very rare if present, in spite of the fact being presence of evenly dispersed individual latex particles in the negative tubes as examined under a microscope. If the latex particles are intermingled non-specifically with in the antigen-antibody complexes, then freely dispersed individual latex particles might be remained, more or less, in the diluent even in the positive tubes. Furthermore, in the LAT, the large groups of the antigen-antibody complexes usually have a tendency easy to scatter in fine pieces composed of a few latex particles when they are shaken or stirred, freely dispersed individual latex particles being scarcely detectable even under these circumstances. On the other hand, such a dispersion of aggregates is rare to occur in the ordinary MAT. In this respect, it seems probably that the antigen-latex-antibody complexes in the LAT are generally more fragile than those of the antigen-antibody.

The LAT with three plant viruses revealed the advantage in detecting viral anti-

gens with comparatively high sensitivity. The virus antigens could be detected more sensitively by the test antigen method of the LAT than by the test antiserum method. The highest dilution end-points of antigens which gave positive reaction in the test antigen method were approximately 40, 20, and 20 times as much higher as those obtained by the test antiserum method, for TMV, PVX and PVY, respectively, in a crude sap. While the highest dilution end-points of a purified virus suspension in the same test were approximately 80, 2.5 and at least 200 times as much higher, respectively for TMV, PVX and PVY, as those obtained by the test antiserum method. The test antiserum method of the LAT, however, have an adequate sensitivity in detecting moderate concentration of the virus antigens as well as having an advantage in economy of the amount of antiserum required, though it is insensitive in detecting the least amount of viral antigens which may be detected when use was made with the test antigen method. Occasionally, especially with too much dilute antibody content, a strong inhibition of the reaction by excess of antigen took place when use was made with the test antiserum method. The best application of the test antiserum method, in this respect, is to the use of the test antiserum containing antibody as high titer as possible.

Several attempts so as to determine the favourable conditions for the routine works revealed some essential serological behaviour of the plant virus antigens in the LAT. TMV antigen required phosphate-buffered saline solution as diluent for the test, preferably of high hydrogen-ion and of low hydrogen-ion, respectively, for the test antigen method and for the test antiserum method. Borate-buffered saline solution was not necessarily considered as a suitable diluent for the tests. Singer and Plotz(16) have argued that the non-specific aggregation between the latex particles and antibody protein was found at pH 5.5 to 8 and the application of the LAT at pH values between 5.5 and 8 was inadequate for the practical use. They also reported the non-specific aggregation of the latex in the diluents such as phosphate-buffer of pH 8 and Veronal-buffer of pH 8 to 9 and showed that the most favourable diluent for the test was the borate-buffered saline solution of pH 8.2 to 9. Borate-buffered saline of pH 8 was also found as the most favourite diluent for the test antigen method of the LAT with Leptospirae(5). These results are quite contrary to the result obtained from the test antigen method of the LAY with TMV, in which evidence was shown indicating an useless of the borate-buffered saline solution and superiority of the phosphate-buffered saline solution.

According to Singer and Plotz(16), NaCl had a favourable effect on the reaction when applied at the cencentration of 0.31 to 1.25%, however, $MgCl_2$ and $CaCl_2$ were found to be inefficient on the reaction in the LAT. For TMV antigen, it was found that 0.85% NaCl solution of pH 7.0 in a deionized water was effective for the test antigen method, but was inadequate for the test antiserum method because of taking place non-specific aggregation.

The concentration of the latex suspension with which virus antigen or antiserum

was mixed to make either the test antigen or test antiserum seemed clearly to affect on the detectability of the resultant reactions. Generally speaking, the difference in the sensitivity was primarily caused by the distinctness of the positive reaction occurred in an antigen-antibody-mixture near the end-point. With a certain limit of the latex concentration, serological end-point was distinguishable more clearly with a dilute latex suspension than with a cencentrated one. Generally, the concentration of the latex suspension was found favourable at 0.5 or 1% than at 0.25% to give a clear aggregation. However, the best concentration of the latex may depend on the concentration of the antigens and antibody used. The sensitivity in detecting the TMV antigen was not affected significantly by the concentration of the latex suspension when use was made with the test antigen method. Singer and Plotz(16) have reported 1% of the latex was the most favourable, while others(5) argued that 0.6% of the latex suspension which was equivalent to OD 0.30 at 665 nm was optimal concentration for the LAT.

It was also shown that the concentration of both the antigen and antiserum adsorpted on the latex particles gave an effect proportionally on the sensitivity of the tests. For examples, detectability obtained from the test antigen method was higher when use was made with test antigen containing virus antigen of 0.05 mg/ml than with 0.1 mg/ml, whereas those obtained from the test antiserum method was higher when use was made with test antiserum at an antibody dilution of 1:10 than with antiserum of 1:100. In other words, it was most suggestive that both the antigen and antibody would react most sensitively only when they were adsorbed on the latex particles at the most favourable ratio to the corresponding reactants, though it has been already proved by many other experiments. Using Leptospira canicola as the antigen, Kelen and Labzoffski have found that the optimal concentration of the the antigen was 7% and self aggregation usually associated with the higher concentration of the antigen(5). They also confirmed that comparable sensitivity was able to receive from the test with 5% solution of the latex and this concentration was practically favourable for economy of the antigen.

In no instance the temperature and time applied for the adsorption of the antigens and antisera on the latex particles has shown any recognizable effect on the sensitivity of the test. On the contrary to this, Kelen and Labzoffski(5) have reported that the temperature and time for the adsorption procedure had an effect on the resultant sensitivity and showed that overnight incubation at 22 C was most suitable to give the highest sensitivity. They found that short incubation was preferable around the high temperature zone and reversely long incubation was preferable around the low temperature zone.

On the other hand, the best incubation for the serological reaction was found overnight standing in a water both at 37 C. This corresponds well with the result obtained by Kelen and Labzoffski(5). Incubation at 56 C, for 2 hr, was reported as the optimal condition for the serological diagnosis of rhumatiod arthritis.

When readings of the results of the serological reactions were made with the naked eye or by the use of an agglutinoscope, well-developed bulky aggregates or dispersed granular precipitates were observed in the positive tubes, whereas mixtures in the negative tubes remained as the homologous latex suspension showing opalescent appearance. Fine granules homologously scattered in the mixture indicate a macroscopical agglutination end-point and is designated as ±. Microscopical observation of the doubltfull reaction, however, have revealed distinct aggregates, though small and few, of the positive reaction mixed with free latex particles. Kelen and Labzoffski have difined the positive reaction as a reaction in which more than 50% of the latex particles was aggregated(5).

As shown already, the highest dilution end-points of the antigens which showed the positive reaction were found $1:300\times2^{10}$, $1:300\times2^{7}$ and $1:300\times2^{5}$, respectively for TMV, PVX and PVY in the test antigens. On the other hand, those of the antigens which showed the positive reaction were $1:30\times2^{9}$, $1:30\times2^{6}$ and $1:30\times2^{4}$, respectively for TMV, PVX and PVY when use was made with the corresponding test antiserum. Thus, the LAT proved to be superior and usefull method for the detection of the plant viruses having the rod-shaped particles, not only because of its high sensibility, but also simpleness and rapidness of the procedure. Unless the auto-agglutination of the latex particles may occur under an unfavourable condition for the test, it is considered that the LAT might be an useful and rapid method for the diagnosis and the quantitative assay of many other plant viruses.

Adsorption of the virus antigen or antiserum on the sheep red blood cells and subsequent serological agglutination test have been reported for several plant viruses (6,7,8,14,15,17). The use of the latex particles for the serological test was recently reported by some investigators indicating the usefulness of the test for the serological detection of the plant viruses(1,2). Very recently, the carbon agglutination test using the carbon particles was reported for the identification of the *Leptospira* strains(10,11).

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DESCRIPTIVE CATALOGUE OF IFO FUNGUS COLLECTION IV.

In the routine work of identification of fungi and in consulting the list of fungi preserved in the IFO culture collection, many species have been appeared for the first record from Japan. The object of this series of paper is to provide descriptions of fungi preserved or newly deposited in the IFO culture collection or/and in the herbarium of IFO and to contribute to the knowledge of the fungus flora of Japan. All of those described in this paper have not been recorded yet or only insufficiently been described from Japan. New taxa will be described in original papers or submitted to other mycological journals. Author(s) of respective taxon is shown in the bracket.

35. Acrodictys erecta (Ellis & Everh.) M.B. Ellis (Pl. 1, A-B) Hyphomycetes
Mycol. Pap. CMI **79:** 12 (1961).

Conidiophores erect, simple, septate, light brown, 3–5 μm wide, up to 30 μm high, bearing a single conidium. Conidia subglobose to ellipsoidal, dark brown, smooth, transversely 4–5-septate, longitudinally 1–2-septate, 20–30$\times$15–20 μm, base pale brown, 2.5–5$\times$3–4 μm.

Hab. On the culm of *Saccharum officinarum* L. Onoaida, Yakushima, Kagoshima, May 29, 1970, T. Yokoyama 4505–29–82 (IFO. H–11661)

[T. Yokoyama]

36. Bispora betulina (Corda) Hughes (Pl. 2, A-B) Hyphomycetes
Canad. J. Bot. **36:** 740 (1958).

Conidiophores mononematous, septate, abruptly constricted at a septum, variable in length, 2–4 μm wide, smooth or often roughened, dark brown. Conidia blastic, catenate in a fragile acropetal chain, 1-septate, occasionally 2-septate, ellipsoid, pale olive brown, 11–18(—19)$\times$5–6 μm.

Growth on malt agar rapid, spreading, woolly, dark olive green to almost black; reverse same colored.

Hab. Isolated by T. Ito from dead banana-leaf, Minoo, Osaka Pref., Aug. 24, 1972, T. Ito S47–7 (IFO 30002).

This species is common on dead plant materials in Japan.

[K. Tubaki & T. Ito]

37. Blastophorum truncatum Matsushima (Pl. 1, C-E) Hyphomycetes
In Microfungi of the Solomon Islands and Papua-New Guinea, p. 18 (1971).

Conidiophores simple, erect, septate, dark brown at the base, paler and subhyaline towards the fertile end, smooth, 50–120×3–5 μm, apex sometimes irregularly swollen. Conidia cylindrical, truncate at the base, obtusely rounded at the apical end, hyaline, smooth, 2-septate, 12–14×2.5–3 μm.

Growth on malt agar moderate, finely floccose, white, then grayish ochraceous, reverse reddish brown. Growth on potato sucrose agar as on malt agar, gray to silver gray, reverse pale grayish brown to sepia.

Hab. On the fallen leaves of *Castanopsis cuspidata* Schottky var. *sieboldii* (Nakai) Nakai. Funaura, Iriomote, Okinawa, June 2, 1973, T. Yokoyama RI–31–1 (IFO. H–11662). On the fallen leaves of *Machilus thunbergii* Sieb. & Zucc., Gyokusendo Park, Okinawa, June 12, 1973, T. Yokoyama RI–75–4 (IFO 30014 and IFO H–11663)

This fungus is very common in *Castanopsis*-forest in Japan.

[T. Yokoyama]

38. Brachydesmiella biseptata Arnaud ex Hughes

(Pl. 2, C-D) Hyphomycetes

Arnaud, Bull. soc. mycol. France **69:** 265 (1950); Hughes, Can. J. Bot., **39:** 1095 (1961); Nicot, Bull. Soc. mycol. France **86:** 705 (1971).

Colonies effuse, black and shiny. Conidiophores macronematous, mononematous, crowded, usually unbranched, straight or irregularly geniculate, hyaline to pale brown, continuous or septate, up to 50 μm long, 4 μm wide at base, gradually expanding to 5–8 μm wide towards apex. Conidiogenous cells terminal, mono- or polytretic, with brown conidial scar. Conidia develop from a pore at apex of conidiogenous cell, more or less oval with rounded apex, 1(2)-septate, with upper cell quite large, thick walled, smooth, dark brown to almost black; basal cell cylindrical, pale brown, flattened at basal scar. Conidia measure 26–38×12–18 μm.

Hab. On the reverse side of bark of *Fagus crenata* Bl., Mt. Fuji, Yamanashi Pref., July 8, 1972, K. Tubaki 200–3–2 (IFO. H–11658).

Conidia of the present fungus are typically 2-septate and the central cell is very large according to the original description. However, as described by Hughes, abnormal conidia which lack the small distal cell are not uncommon. As shown in the plate, the conidia of the present Japanese collection consist mainly of the abnormal cells which lack the small distal cell and are same with those found in the specimen DAOM 93848, kindly offered from Dr. S.J. Hughes, to whom I owe many thanks.

[K. Tubaki]

39. Codinaea gonytrichodes Schearer & Crane

(Pl. 3, A-D; Pl. 4, D-E) Hyphomycetes

Mycologia **63:** 245 (1971).

Conidiophores simple or occasionally branched, straight or flexuous, scattered or

aggregated into groups, 150–250 μm high, 4–6 μm wide, swollen up to 6–10 μm at the darker base, paler to subhyaline at apex, dark brown, thick-walled, smooth, 5–12-septate, bearing 3–5 whorls of polyphialides at a septum, with a simple terminal phialide or a terminal polyphialide with sympodially produced collarettes at the distal end of the conidiophores. Polyphialides subhyaline to very pale brown, 10–15 $\times$ 3–5 μm, simple with a terminal collarettes, then elongated up to 50 μm long and septate, finally a succession of collarettes develop in the manner same as the distal end of the conidiophores. Phialoconidia aggregated in mucilaginous mass at the whorls, lunate, continuous, hyaline, smooth, 12–15 $\times$ 2–2.5 μm, furnished with a single filiform setula at both ends, setula straight or slightly curved, 5–12 μm.

Growth on malt agar rapid, velvety, gray to fuliginous brown, reverse almost blackish brown. Growth on potato sucrose agar as on malt agar, dark mouse gray, reverse black.

Hab. On the fallen leaves of *Castanopsis cuspidata* Schottky. Taniyashiki, Ochi-cho, Agawa, Kochi, Oct. 10, 1972, T. Yokoyama 4720–33–8 (IFO 9646 and IFO. H–11664)

This is the second described report of the occurrence of this fungus which was originally found in the North America.

[T. Yokoyama]

40. Cryptophiale guadalcanalense Matsushima (Pl. 4, A-C) Hyphomycetes In Microfungi of the Solomon Islands and Papua-New Guinea, p. 15 (1971).

Fertile setae solitary, erect, smooth, thick-walled, dark brown to blackish brown, septate, 150–200 μm high, 6–8 μm thick, 2–3 times dichotomously branched at the distal end. Phialide aggregated into cylindrical cluster surrounding the upper portion of fertile seta including branches, 60–80 $\times$ 14–16 μm, pale brown. Phialoconidia 1-septate, hyaline, curved, rounded at the base, pointed but not bent at the apex, 16–22 $\times$ 1.5–2 μm.

Growth on malt agar moderate, floccose, grayish brown, reverse dark brown to almost black. Growth on potato sucrose agar as on malt agar, but more restricted, velvety to finely floccose, grayish brown, reverse black.

Hab. On the fallen leaves of *Castanopsis cuspidata* Schottky var. *sieboldii* (Nakai) Nakai. Mt. Omoto, Ishigaki, Okinawa, May 31, 1973, T. Yokoyama RI–5–10 (IFO 30029 and IFO. H–11665); Iriomote, Okinawa, June 7, 1973, T. Yokoyama RI–64–9 (IFO 30030 and IFO. H–11666).

The present fungus is not uncommon in the South Japan. Several isolates have been obtained from Okinawa, Ogasawara, Kagoshima and other localities.

[T. Yokoyama]

41. Dendryphion comosum Wallroth (Pl. 5, A-C) Hyphomycetes
Fl. crypt. Germ. **2**: 300 (1833); Ellis, in Dematiaceous Hyphomycetes, p. 503 (1971).

Colonies on the host dark reddish brown to almost black, velvety, spreading on and encircling host substrate, with numerous conidiophores close together. Hyphae pale brown, slender, 2–3 μm in diam. Stroma often developed, superficial, dark brown, 40–60 μm wide. Conidiophores macronematous, arising singly or in small groups, branched at apex. Stipe straight, cylindrical, swollen at base, many-septated, 70–320 μm long or more, 8–12 μm wide at base, 6–8 μm wide near apex, dark brown, paler apex. Branches singly or in pairs developed at apex of conidiophore, two or three in series, short, septate, reddish brown, 4–6 μm wide. Conidiogenous cells terminal or intercalary on branches, monotretic or polytretic, with distinct scars. Conidia almost cylindrical, often slightly curved, 1–7-septate, slightly constricted at septa, brown to dark brown, very minutely verrucose, with round apex and indistinct scar, 20–36$\times$6.0–8.5 μm; branched conidia common, cheiroid.

Hab. On dead stem of herbaceous plant, Minoo, Osaka Pref., Aug. 25, 1973, K. Tubaki 199–5 (IFO. H–11657).

Morphological characteristics of the present collection are very close to the description given by Ellis except surface nature of the conidia. Conidia of the present collection are very minutely verrucose differing from those illustrated by Ellis. This species is not uncommon on the dead stem of herbaceous plant in Japan.

[K. Tubaki]

42. Didymobotryum rigidum (Berk. & Br.) Saccardo
(Pl. 5, D-E) Hyphomycetes
Syll. Fung. **4**: 627 (1886); Ellis, in Dematiaceous Hyphomycetes, p. 382 (1971).

Colonies on the host scattered, velvety, restrict, dark brown to almost black. Synnemata dark blackish brown, up to 1.5 mm high, mostly 700–900 μm long, 60–180 μm wide at base, tapering gradually to 26–40 μm wide in middle, expanded slightly at apex measuring 60–100(–120) μm wide; separate thread 2–3 μm wide. Conidiogenous cells monotretic, clavate, swollen to 4–5 μm. Conidia tretic, catenate, dry, acrogenous, broadly ellipsoidal or cylindrical, with a dark band at a septum and a polar apical pore, slightly constricted at a septum, (12–)15–18$\times$5–6 μm, dark brown.

Hab. On dead bamboo-culm (*Phyllostachys* sp.), Sata, Kagoshima Pref., July 27, 1974, K. Tubaki 214–21 (IFO. H–11660).

This species is fairly common in Japan. Although I haven't seen the type specimen, *D. kusanoi* P. Hennings (Engl. Jarb., **32**: 45, 1902; Hino & Katsumoto, in Icon. Fung. Bamb. Jap., p. 261, 1961) will probably be synonymous with the present species.

[K. Tubaki]

43. Leptodiscella africana (Papend.) Papend. (Pl. 9, A-B) Hyphomycetes
Trans. Brit. mycol. Soc. **53**: 145 (1969).

Hyphae septate, hyaline, 1.5–2.0 μm wide, often aggregated in a ropy bundles with fragment anastomosis. Conidiophores macronematous, mononematous, sinple or branched, cells globose or irregular, 1.5–1.7 μm wide, often aggregated bearing conidia in abundance. Conidiogenous cells terminal, polyblastic, determinate, nearly globose or irregular in shape, 2–3 μm wide at base, up to 10 μm wide at middle, hyaline. Conidia blastic, borne on terminal cells or rarely directly on hyphae, cylindrical with round ends, smooth-walled, thin-walled, medianly 1-septate, sometimes constricted slightly at a septum, bearing a single, straight or curved filamentous setula sub-laterally at each end, 10–13 $\times$ 2–3 μm, hyaline or faintly colored; often becoming darker and longer with a dark band at a septum.

On potato-carrot agar, growth rapid, spreading, flat and thin, bearing conidia in abundance, but the conidia are variable in length.

Hab. Isolated by T. Ito from aquatic sediment of Lake Sengari, Hyogo Pref., June 20, 1974, T. Ito LXII-2-3 (IFO 30004).

Conidia are often longer and larger than those given in the description.

[K. Tubaki and T. Ito]

44. Minimidochium setosum Sutton (Pl. 6) Hyphomycetes
Canad. J. Bot. **47**: 2095 (1969).

Sporodochia scattered on agar surface, superficial, sessile, pulvinate, hemispherical, yellowish brown to dark brown as conidia matured, 300–500 μm wide. Setae unbranched, aseptate or septate, often proliferate through collar-like fragile remains of older setae, gradually tapering towards an acute apex, dark brown to almost black, 560–800 μm long, 6–8 μm wide at base. Conidiogenous cells developed from peripheral cells of sporodochium, monophialidic, clavate or cylindrical, smooth-walled, with a minute apical collarette, sometimes septate at base, 8–11 $\times$ 3–4 μm. Conidia numerous, phialidic, developed from phialides over whole surface of sporodochium, hyaline, aseptate, curved, sub-cylindric, asymmetric, tapering towards basal scar, acute at apex, 7.2–8.5 $\times$ 1.6–2.3 μm; both ends provided with a single, simple, straight or slightly curved setula which is up to 7 μm long.

Sporodochia developed on malt agar, potato-sucrose agar, oat-meal agar and corn-meal agar on which fresh isolate is inoculated. Best result was obtained when inoculated on the sterilized leaves of *Castanopsis cuspidata* Sch. put on the agar plate.

Hab. Isolated by T. Ito from aquatic sediment of Lake Sengari, Hyogo Pref., Nov. 30, 1973, K. Tubaki 210–37–9 (IFO 30006).

The present isolate fits in many respect with the description given by Sutton except the size of the sporodochia and setae, both are markedly larger and longer than

those in the description. However, as generally known, the size of sporodochia and setae are quite changeable depend to surrounding conditions. Especially those developed in culture are usually different in size from those in nature. Conidia of the present isolate are quite same with those in the type, Herb. I.M.I. No. 103360, which was kindly offered from Dr. B.C. Sutton to whom we owe many thanks. The collar-like fragile remains of the setae are also recognized in the type specimen. The present collection is the second of the species succeeding to the type locality, Sierra Leone.

[K. Tubaki and T. Ito]

45. Pendulispora venezuelanica M.B. Ellis (Pl. 7, A-C) Hyphomycetes Mycol. Pap. CMI **82:** 41 (1961).

Colonies on the host effuse, dark brown to almost black, hairly. Conidiophores macronematous, mononematous, straight or slightly bent, subulate, with a small hyaline branch at apex, dark brown, paler towards apex, smooth-walled, 90–200 μm or more long, 6–5 μm wide at base, tapering to 3–4 μm wide at apex. Conidiogenous cells monoblastic, terminal on conidiophore, hyaline or pale brown, 4–12$\times$2–3 μm. Conidia solitary, pendulous, acrogenous, simple, caudate, subhyaline or pale brown at middle, smooth-walled, muriform, 92–115 μm long, 12–16(–18) μm wide in broadest part, 2–4 μm wide at base, 4–5 μm at apex.

Hab. On the reverse side of bark of *Fagus crenata* Bl., Mt. Fuji, Yamanashi Pref., July 8, 1973, K. Tubaki 200–3–3 (IFO. H–11659).

[K. Tubaki]

46. Periconia macrospinosa Lefevre & A.G. Johnson, apud Lefevre, Johnson & Sherwin (Pl. 8, A) Hyphomycetes Mycologia **41:** 416 (1949); Mason & Ellis, Mycol. Pap. CMI **56:** 78 (1953).

Conidiophores simple, erect, straight or curved, solitary or gregarious, dark brown, septate, smooth, 50–200 μm high, 8–14 μm thick, swollen and branched at the apex. Conidiogenous cells globose, finely echinulate, 8–10 μm pale, brown. Conidia coarsely echinulate, dark reddish brown, 20–30(–35) μm in diameter including the spines, spines conspicuous, solitary or confluent each other in groups.

Growth on malt agar very rapid, floccose, whitish, then grayish ochraceous, reverse blackish brown. Growth on potato sucrose agar as on malt agar, but rich in sporulation.

Hab. From soil, Taniyashiki, Ochi-cho, Agawa, Kochi, Oct. 10, 1972, T. Yokoyama 4720–10–16 (IFO 9649 and IFO. H–11667).

[T. Yokoyama]

47. Phialocephala humicola Jong & Davis (Pl. 7, D-E) Hyphomycetes
Mycologia **64**: 1351 (1972).

Conidiophores macronematous, arising singly or in small groups from hyphae, consisting of stipes and penicillate conidiogenous apparatus bearing a slimy mass of conidia. Stipes erect, septate, smooth, commonly 200–300 μm long, 2.5–4.0 μm wide, dark brown, paler towards apex. Primary metulae, light brown, 8–11$\times$2.5–3.5 μm; secondary metulae, 5–8$\times$2.0–3.0 μm. Conidiogenous cells monophialidic, cylindrical, hyaline, smooth-walled, 10–12$\times$1.0–2.0 μm, with inconspicuous collarettes. Conidia phialidic, 1-celled, ellipsoidal, hyaline, smooth-walled, 2.5–3.0$\times$1.5–2.0 μm, grouped in slimy masses.

Growth on malt agar, rapid, velvety, olive green, with white margin; reverse dark olive to almost black. On potato-sucrose agar, growth is rapid and better than on malt agar, with concentric surface rings; reverse dark olive to almost black.

Hab. Isolated by T. Ito from aquatic sediment of River Ichikawa, Kobe City, Hyogo Pref., May 10, 1973, T. Ito LXIII–1–11 (IFO 30007).

The present species was established recently, which was originally isolated from a soil of New Jersey. The present isolate fits in many respects with the description given by Jong & Davis. This species has been found very often also from the sediments of Lake Sengari, Hyogo Pref..

[K. Tubaki and T. Ito]

48. Pithomyces maydicus (Sacc.) M.B. Ellis (Pl. 8, B-D) Hyphomycetes
Mycol. Pap. CMI **76:** 15 (1960); Ellis, in Dematiaceous Hyphomycetes, p. 44 (1971).

Conidia holoblastic, solitary on hyaline, cylindrical, 5–10 μm long, 2–3 μm thick denticles, detached by fracture of the upper part of the denticle, a part of the denticle often remains attached to the base of the conidium, ellipsoidal, reddish brown, verrucose, mostly with 2 transverse septa, the middle cells sometimes with a longitudinal septum, slightly constricted at the septa, rounded at both ends, 12–18$\times$6–10 μm.

Growth on malt agar very rapid, cottony, white to pale yellowish brown, reverse pale brown. Growth on potato sucrose agar as on malt agar.

Hab. On the culm of *Zoysia japonica* Steud. Mt. Aso, Kumamoto, Nov. 23, 1972, collected and isolated by Y. Tahama, identified by T. Yokoyama T–26 (IFO 30015 and IFO. H–11668).

[T. Yokoyama]

49. Septosporium bulbotrichum Corda (Pl. 9, C-D) Hyphomycetes
Icon. Fung. **1**: 12 (1837); Ellis, in Dematiaceous Hyphomycetes, p. 108 (1971).

Colonies on the host effuse, dark brown to black, velvety or hairy, shiny. Setae numerous, straight, subulate, dark brown to black, thick-walled, 100–140(–160) μm long, 3–4 μm wide at base, tapering to 2–3 μm at paler apex. Conidiophores short, cylindrical, subhyaline to pale brown, 14–22 μm long, 3–4 μm wide, bearing conidia terminally. Conidia thallic, solitary, broad elliptical with a protuberant basal cell, pale brown when young, dark brown to almost black when matured, smooth-walled, muriform, 30–60 $\times$ 20–24 μm; one or more cells of conidium swell out individually which are darker even when young.

Hab. On reverse side of bark of *Fagus crenata* Bl., Mt. Fuji, Yamanashi Pref., July 8, 1973, K. Tubaki 200–3–1 (IFO. H–11639).

Although Ellis described that the swelled out cells of the conidia become transformed into short-beaked pycnidia, the formation of them is not seen in the present collection.

[K. Tubaki]

50. Solosympodiella clavata Matsushima (Pl. 10, A-C) Hyphomycetes
In Microfungi of the Solomon Islands and Papua-New Guinea, p. 55 (1971).

Conidiophores simple, erect, septate, smooth, thick-walled, dark brown, paler towards the distal end, 50–130 $\times$ 2–4 μm, irregularly swollen or sympodially elongated at the fertile apex, prominently denticulate. Conidia cylindrical with rounded apex and truncate base, narrower towards base, hyaline, smooth, 1–septate, 20–25 $\times$ 2.5–4 μm.

Growth on malt agar and potato sucrose agar rather restricted, velvety, gray to black, reverse black.

Hab. On the dead leaves of *Castanopsis cuspidata* Schottky var. *sieboldii* (Nakai) Nakai. River side of Kuira, Iriomote, Okinawa, June 2, 1973, T. Yokoyama RI–32–3 (IFO 30016 and IFO. H–11669); Maryudo Fall, Iriomote, Okinawa, June 5, 1973, T. Yokoyama RI–50–3 (IFO 30017 and IFO. H–11670); Hinaisawara Fall, Iriomote, Okinawa, June 1, 1973, T. Yokoyama RI–15–1 (IFO. H–11671).

This fungus is not uncommon in the litter of *Castanopsis*-forest throughout the Japan archipelago.

[T. Yokoyama]

51. Stachybotrys atra Corda var. **microspora** Mathur & Sankhla
(Pl. 10, D-F) Hyphomycetes

Sci. Cult. **32:** 93 (1966); Ellis, in Dematiaceous Hyphomycetes, p. 544 (1971).

Conidiophores erect, simple, hyaline to very pale yellowish umber, smooth, 60–90 μm high, 2.5–4 μm thick. Phialides pale yellowish brown, 8–11 $\times$ 2.5–3 μm. Conidia ellipsoidal, often tapering and truncate at the base, rounded at the apical end, fuliginous to blackish olivaceous, verrucose, 5–7 $\times$ 3–4.5 μm.

Growth on malt agar moderate, rather flat, sparse, white to creamy ocherous, reverse concolor. Growth on potato sucrose agar rapid, coarsely floccose to funiculose, white to silver gray, finally grayish ocherous, reverse pale umber.

Hab. On the fallen leaves of *Castanopsis cuspidata* Schottky var. *sieboldii* (Nakai) Nakai. River side of Kuira, Iriomote, Okinawa, June 2, 1973, T. Yokoyama RI-30-2 (IFO 30018 and IFO. H-11672).

[T. Yokoyama]

52. Stachybotrys cylindrospora Jensen Hyphomycetes

Bull. Cornell Univ. agric. Exp. Sta. **315:** 496 (1912); Ellis, in Dematiaceous Hyphomycetes, p. 542 (1971).

Conidiophores erect, simple, 1–2-septate, hyaline to subhyaline, pale grayish above, smooth, up to 100 μm, 3–5 μm thick, slightly swollen at the base. Phialides pale grayish, pyriform to broadly clavate, 10–14$\times$5–6 μm. Conidia cylindrical, dark gray to fuscous, with longitudinal striation, truncate at the base, rounded at the apex, 14–16$\times$3–5 μm.

Hab. On the leaves of *Ligularia tussilaginea* Makino. Miyanoura, Yakushima, Kagoshima, May 31, 1970, T. Yokoyama 4505-31-66 (IFO 9356 and IFO. H-11684).

Ichinoe (Trans. Mycol. Soc. Japan **10:** 112, 1970) reported this species from Kanagawa and Ibaragi, Japan.

[T. Yokoyama]

53. Stachybotrys dichroa Grove (Pl. 11, A-B) Hyphomycetes

J. Bot. Lond. **24:** 201 (1886).

Conidiophores erect, straight or flexuous, simple, 1–3-septate, thin-walled, hyaline, minutely granulose, 60–80 μm high, up to 120 μm and sometimes branched, 2.5–4 μm, tapering to the apex. Phialides clavate, pale olivaceous brown, 10–12$\times$3–5 μm. Conidia ellipsoidal to subcylindrical, finely verrucose, olivaceous brown to sooty brown, obliquely attenuated and truncate at the base, rounded at the apex, 8–11$\times$4–6 μm.

Hab. On the rotten leaves of *Pandanus boninensis* Warb. Chichijima, Ogasawara, Tokyo, Nov. 24, 1972, T. Yokoyama 4711-24-27 (IFO. H-11673).

[T. Yokoyama]

54. Stachybotrys nephrospora Hansf. (Pl. 12, A-D) Hyphomycetes

Proc. Linn. Soc. Lond. **1942–1943:** 44 (1943).

Conidiophores erect, stout, simple, thick-walled, hyaline, smooth, up to 200 μm high, 6–8 μm thick, sometimes minutely verrucose and pale fuliginous near the apex. Phialides clavate to ellipsoidal, subhyaline to pale gray, verrucose, 8–12$\times$4–5 μm.

Conidia reniform, dark olivaceous brown to blackish brown, distinctly verrucose, 9–11×5–7 μm.

Growth on malt agar moderate, white to pale yellow, finely floccose. Growth on potato sucrose agar very rapid, white to pale yellowish brown, reverse pale brown.

Hab. On the fallen leaves of *Castanopsis cuspidata* Schottky var. *sieboldii* (Nakai) Nakai. River side of Kuira, Iriomote, Okinawa, June 2, 1973, T. Yokoyama RI–30–5 (IFO 30019 and IFO. H–11674).

[T. Yokoyama]

55. Thozetella cristata Pirozynski & Hodges (Pl. 13, A-D) Hyphomycetes
Can. J. Bot. **51:** 168 (1973).

Sporodochia amphyphyllous, scattered, superficial, with dark brown stipe-like base, bearing cup-shaped structure consisting of aggregated conidiophores and white compact mass of conidia, conidial mass globose, subglobose to ovoidal, up to 300×200 μm, stalk 30–50 μm wide, up to 180 μm high, conidiophores branched, aggregated, hymenium-like, subhyaline to yellowish brown, terminating in paler, cylindrical phialide 2–3 μm thick. Conidia lunate to allantoid, hayline, smooth, non-septate, furnished with filiform setula 5–7 μm long, 10–15×2–3 μm. Microawns nonseptate, rough- and thick-walled and refractive at the distal half, smooth- and thin-walled and hyaline at the basal end, 30–40×2.5–3 μm.

Growth on malt agar moderate, velvety, white to olivaceous gray, reverse concolor, medium turn to yellowish brown. Growth on potato sucrose agar moderate, velvety to finely floccose, white to dark gray, then almost black, reverse black.

Hab. On the fallen leaves of *Castanopsis cuspidata* Schottky var. *sieboldii* (Nakai) Nakai. Mt. Omoto, Ishigaki, Okinawa, May 31, 1973, T. Yokoyama RI–5–5 (IFO 30020 and IFO. H–11675); Hinaisawara Fall, Iriomote, Okinawa, June 2, 1973, T. Yokoyama RI–24–2 (IFO 30021 and IFO. H–11676); On the leaves of *Quercus* sp. Taniyashiki, Ochi-cho, Agawa, Kochi, Oct. 10, 1972, T. Yokoyama 4720–33–7 (IFO 30022 and IFO. H–11677) and 4720–33–20 (IFO 30023 and IFO. H–11678).

[T. Yokoyama]

56. Torulomyces lagena Delitsch (Pl. 14, A) Hyphomycetes
System. Schimmelpilze (1943); Barron, Mycologia **59:** 716 (1967); Tokumasu, Bull. Sugadaira Biol. Lab. Tokyo Kyoiku Univ. **6**: 46 (1974).
Syn. *Monocillium humicola* Barron, Can. J. Bot. **39**: 1573 (1961).

Conidiophores macronematous, mononematous, erect from slender vegetative hyphae, hyaline, smooth-walled, 10–17 μm long, up to 20 μm, 0.5–1.5 μm wide at basal stalk; swelling to ovoidal or ellipsoidal vesicle near apex delimited by a basal septum,

which measuring 4–5 μm long and 2.5–3.0 μm wide; tapering to short cylindrical neck, 1–2 μm wide with a collarette. Conidia phialidic, small, globose, pale olive brown, at first smooth but becoming minutely roughened, 1.5–2.0 μm in diam., catenate in a chian with narrow connectives.

Growth on malt agar moderate, restrict, velvety or somewhat powdery, radially wrinckled at surface, with concentric rings, pale gray-green; reverse hyaline to pale brown green.

Hab. Isolated by T. Ito from aquatic sediment of Lake Sengari. Hyogo Pref., May 10, 1974, T. Ito LXI–6–3 (IFO 30008).

The present species was also isolated from aquatic sediment of Ichikawa River, Kobe City, and all isolates fit in many respects with the description given by Barron.

(K. Tubaki and T. Ito]

57. Ciliospora gelatinosa Zimmermann

(Pl. 15, A-D; Pl. 16, A-C) Coelomycetes

Centralbl. f. Bakter. Abt. 2, **8:** 217 (1902); Petch, Trans. Brit. mycol. Soc. **26:** 65 (1943).

Pycnidia immersed at first, then erumpent, solitary or gregarious, globose to irregular, cushion-shaped, up to 1 mm wide, 0.5–1 mm high, white, gelatinous, viscid at maturity. Conidiophores narrow, branched, aggregated, lining the inner wall of the cavity of the pycnidia. Conidia oblong to cylindrical, obtusely rounded at the ends, slightly curved, hyaline, smooth, continuous, furnished at or near each ends with 4–5 hyaline, filiform appendages up to 20 μm long, most of appendages bent near the attachment site.

Growth on malt agar very rapid, white, transluscent, zonate, aerial mycelium very poor. Growth on potato sucrose agar as on malt agar.

Hab. On the fallen leaves of *Quercus* sp. Taniyashiki, Ochi-cho, Agawa, Kochi, Oct. 10, 1972, T. Yokoyama 4720–33–12 (IFO 30024 and IFO. H–11679); On the leaves of *Castanopsis cuspidata* Schottky var. *sieboldii* (Nakai) Nakai. Mt. Omoto, Ishigaki, Okinawa, May 31, 1973, T. Yokoyama RI–6–6 (IFO 30025 and IFO. H–11680); On the fallen leaves of broad-leaved tree. Chichijima, Ogasawara, Tokyo, Nov. 24, 1972, T. Yokoyama 4711–24–22 (IFO 30026 and IFO. H–11681); On the rotten leaves of unidentified tree. Miyanoura, Yakushima, Kagoshima, June 1, 1970, T. Yokoyama 4506–1–12 (IFO 30027 and IFO. H–11682).

[T. Yokoyama]

58. Lasiodiplodia theobromae (Pat.) Griffon & Maubl.

(Pl. 16, D-E) Coelomycetes

Bull. Soc. mycol. Fr. **25:** 1 (1909).

Pycnidia spherical, with an elongated beak, black, up to 300 μm wide. Conidio-

phores cylindrical to clavate, hyaline, 8–15 μm thick. Conidia ellipsoidal to fusoid, dark brown, with several subhyaline longitudinal striations, 1-septate, 22–28×12–15 μm.

Growth on malt agar very rapid, aerial mycelium rich, pale blackish brown to mouse gray, reverse blackish brown. Growth on potato sucrose agar more vigorously, white to grayish brown, reverse blackish brown.

Hab. On the fruit of *Pandanus boninensis* Warb. Chichijima, Ogasawara, Tokyo, Nov. 27, 1972, T. Yokoyama 4712–27–1 (IFO–30028 and IFO. H–11683).

[T. Yokoyama]

59. Pestalotia distincta Guba (Pl. 11, C-D) Coelomycetes

In Monograph of Monochaetia and Pestalotia, p. 107 (1961); Yokoyama, Mycologia, in the press.

Conidia 5–celled, broadly ellipsoid or fusoid somewhat inaequilateral or tapering towards the basal end, 19–28×6–8 μm, apical cells hyaline, conical to obconic, rarely hemispherical, 3–5×4–6 μm, setulae 3–9 (mostly 5) in number, hyaline, straight or curved, adorning the periphery of the basal end of the apical cells, rarely also the distal end of the apical cells, 6–10×1–1.2 μm, 3-intermediate cells concolorous, pale brown to umber, dark brown or fuliginous in the wall and septa, thick walled, constricted at the septa, 15–18 μm long, basal cells hyaline, attenuated into short appendages 3–5 μm long, appendages hyaline, straight or slightly curved, 1 μm thick.

Growth on malt agar moderate, compactly floccose, white to pale ocherous brown. Growth on potato sucrose agar rapid, floccose, white to dingy yellow, then pale grayish ocher to olivaceous brown.

Hab. On the leaves of *Castanopsis cuspidata* Schottky. Esuzaki, Wakayama, Apr. 18, 1969, T. Yokoyama 4404–18–33 (IFO 9981 and IFO. H–11650) and 4404–18–36 (IFO 9982 and IFO. H–11651); Miyanoura, Yakushima, Kagoshima, June 1, 1970, T. Yokoyama 4506–1–21 (IFO 9983 and IFO. H–11652).

[T. Yokoyama]

60. Micronectriella cucumeris (Klebahn) Booth (Pl. 14, B-D) Hypocreales

In The genus Fusarium, p. 39 (1971).

St. conid. *Fusarium tabacinum* (v. Beyma) Gams, Persoonia **5**: 179 (1968).

Perithecia develop in abundance on corn-meal agar, scattered, globose to ovoid, with beaked ostiolar papilla, brown at base, paler above, (110–)210–290×90–110 μm. Asci clavate with a short stalk and a round, slightly thickened apex which is not blued by iodine, containing eight ascospores, 40–55×6–8 μm. Ascospores 1–septate, elliptical, slightly constricted at a septum, hyaline, 10–12×3–4 μm. Conidial state is of the *Fusarium*-type. Conidiophores monophialidic, develop from aerial mycelium, single or branched, hyaline, 16–22 μm long, 2–3 μm wide at base. Conidia phia-

lidic, cylindrical, straight or slightly curved with a rounded apex, with or without a central median septum, (5–)7–12×2.5–4.0 μm.

Perithecia develop on most agar media, but malt agar, corn-meal agar and potato-carrot agar (CMI) are best for the production. Perithecia also develop in abundance on the sterilized straw piece put on the plain agar.

Hab. Isolated by T. Ito from aquatic sediment of Lake Sengari, Hyogo Pref., Jan. 10, 1973, T. Ito XXXV-1-3 (IFO 30005).

This species was transferred to *Plectosphaerella cucumeris* (Lindfors) Gams (1972). The present isolate fits in all respects with a typical strain of *M. cucumeris* kindly offered from Dr. C. Booth to whom we owe many thanks.

[K. Tubaki and T. Ito]

61. Triangularia bambusae (v. Beyma) Boedijn (Pl. 17) Sphaeriales
Ann. Mycol. Notitiam Sci. Mycol. Univ. **32**: 302 (1934); Cain, Can. J. Bot. **34**: 693 (1956).
Syn. *Trigonia bambusae* v. Beyma, Zent. Bakt. Parasit. **89**: 237 (1933).

Perithecia on agar media scattered, superficial or sometimes immersed, brownish black, pyriform or lacrymoid, 280–450×200–320 μm, with short and cylindrical neck, covered with a few brown, septate, flexuous hairs measuring up to 150 μm long and 2–4 μm wide. Peridium membranaceous, areolate with darker and thicker areas. Asci eight-spored, cylindrical with a distinct thickened ring in apex which is negative by iodine, 120–160×10–17 μm; discharges ascospores forcibly. Paraphyses filiform, lying pararell. Ascospores uniseriate, triangular, brownish black, with hyaline lower cells attached along shorter side of upper darker cells; darker cell consists of two sides measuring 10–14 μm and 16–20 μm long respectively and of base measuring 10–12 μm long. Conidiogenous cells phialidic, acropleurogenous, cylindrical, pale brown and paler apex, forming a small collarette at apex, 8–16×2.5–3.0 μm. Conidia globose to subglobose, smooth walled, hyaline, 2–3 μm in diam.

Growth on agar media rapid and spreading on whole surface of agar; perithecia develop in most abundance on oat-meal agar; medium was changed to a distinct pinkish color.

Hab. Isolated from aquatic sediment of Hatsuka River, Tokura, Hyogo Pref., Nov. 30, 1973, isolated by T. Ito, K. Tubaki 210-39-3 (IFO 30009).

The present species was originally found by v. Beyma on bamboo in Java and the present collection is apparently the second from nature.

[K. Tubaki]

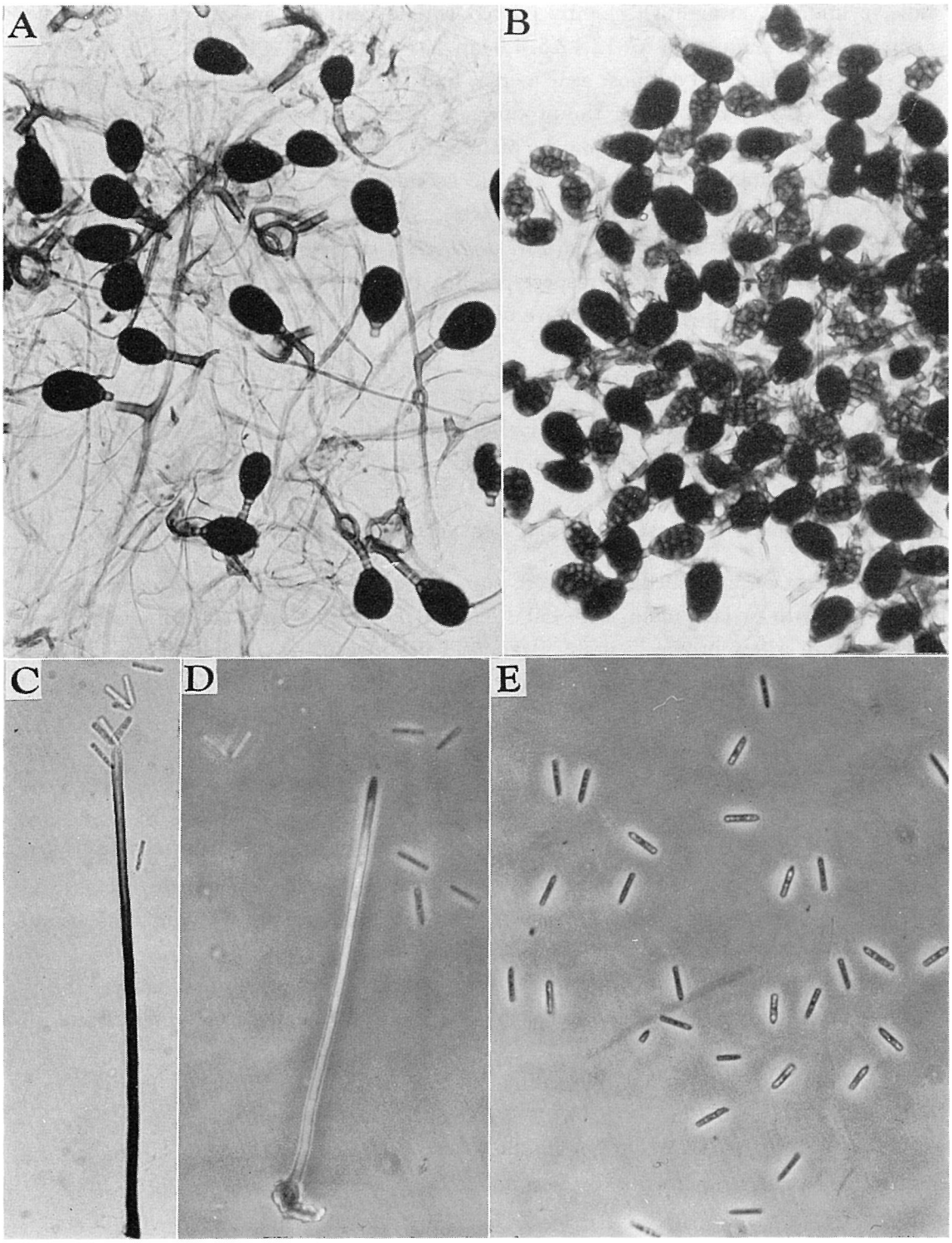

Plate 1. A-B. *Acrodictys erecta*. A. Mature conidia (×250), B. Young conidia (×250), C-E. *Blastophorum truncatum*. C. Conidiophore and conidia (×250), D. Ditto (×250), E. Conidia (×250).

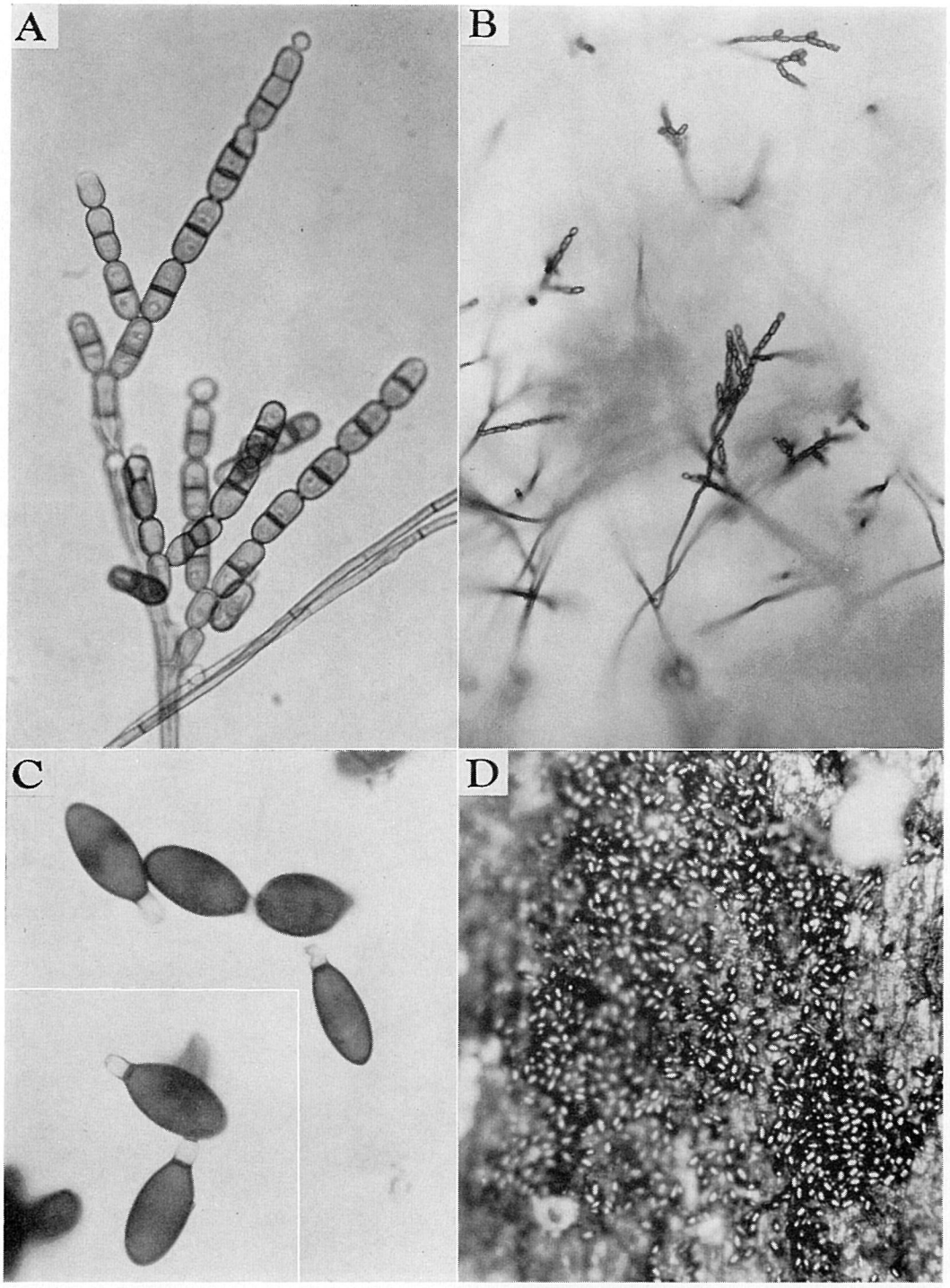

Plate 2. A-B. *Bispora betulina*. A. Conidial structures (×400), B. Ditto (×100). C-D. *Brachydesmiella biseptata*. C. Conidia (×400), D. Habit (×65).

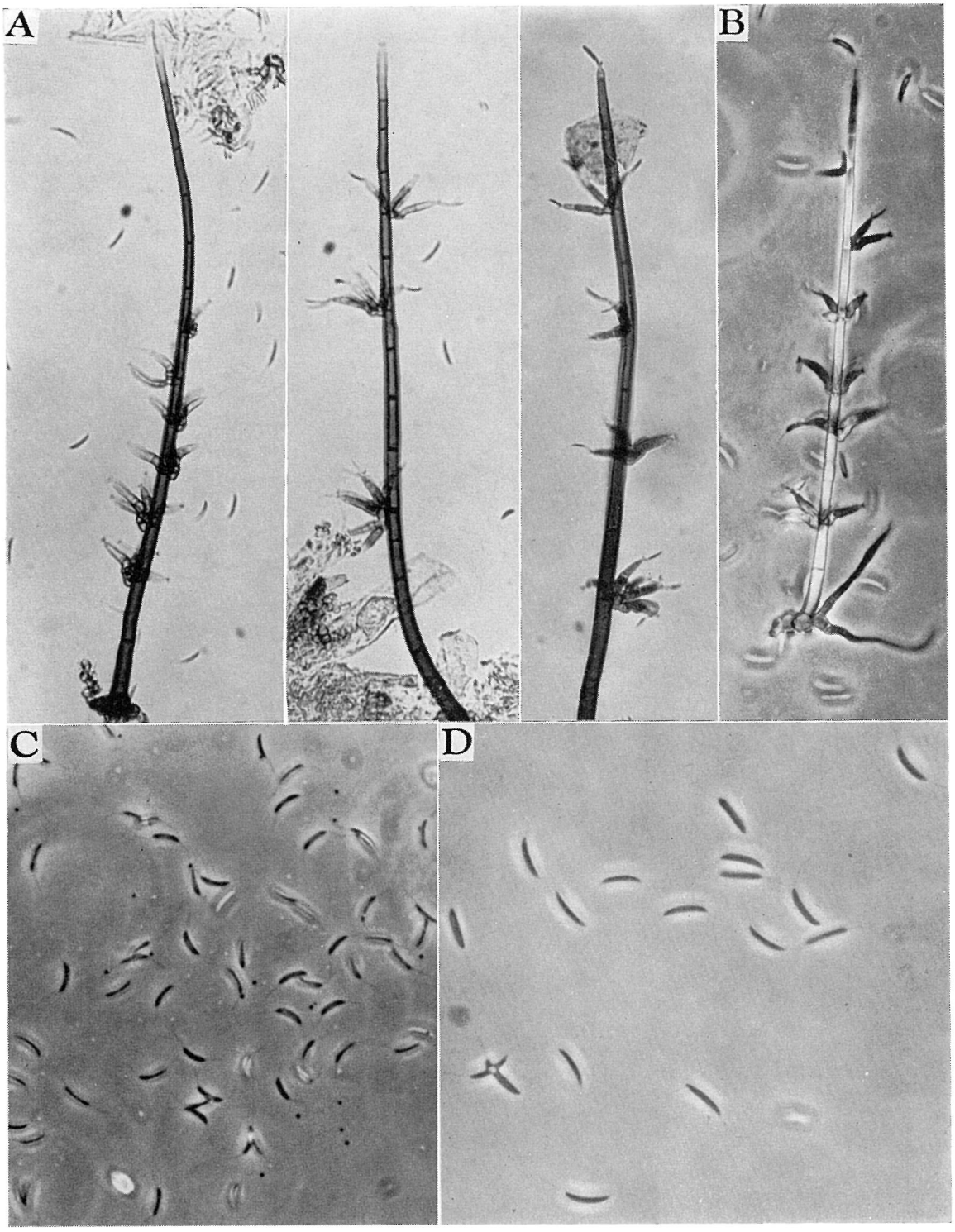

Plate 3. *Codinaea gonytrichodes*. A. Conidiophores (×250), B. Ditto (×250), C. Conidia (×250), D. Ditto (×400).

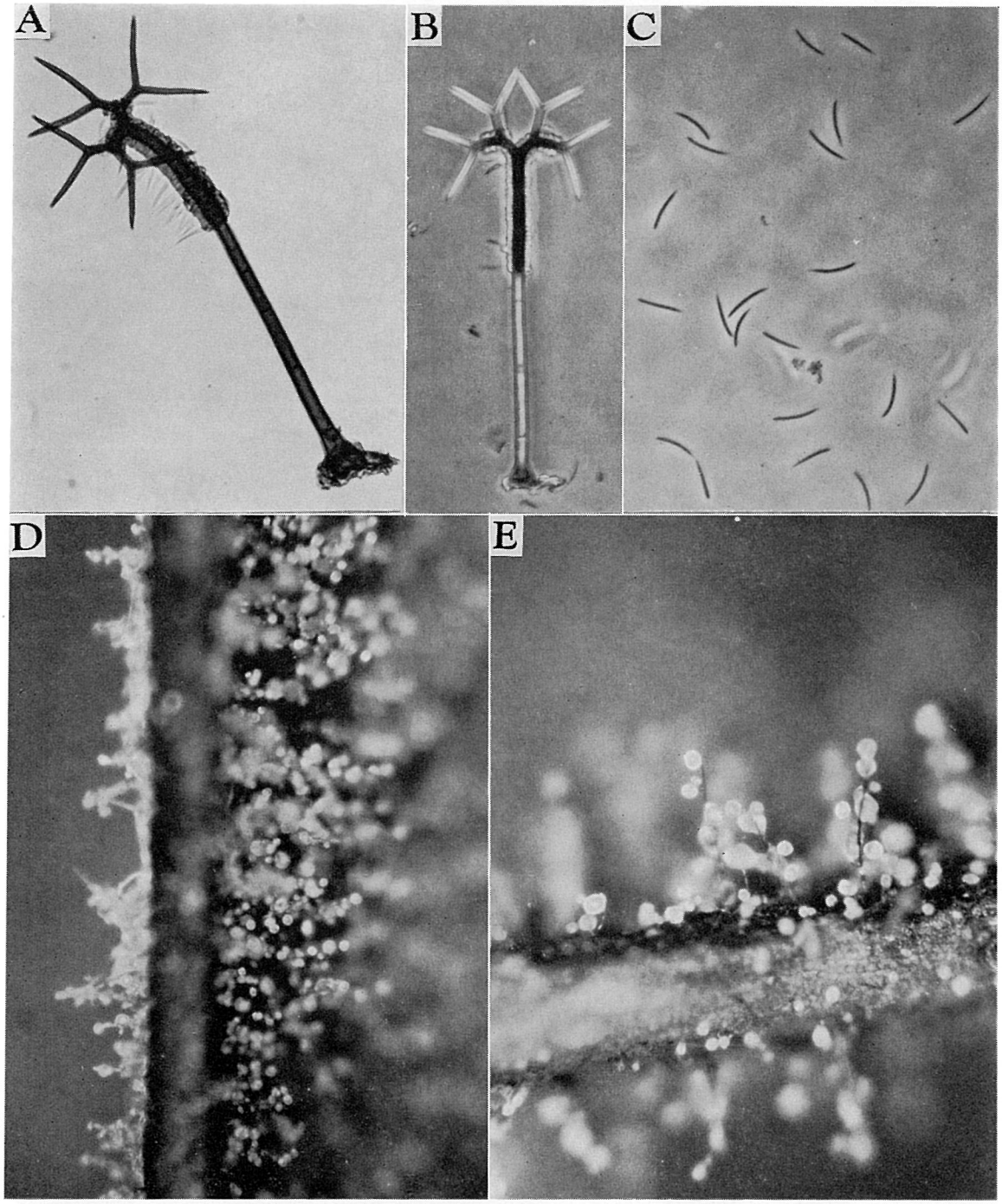

Plate 4. A-C. *Cryptophiale guadalcanalense*. A. Conidiophore (×250), B. Ditto (×250), C. Conidia (×250). D-E. *Codinaea gonytrichodes*. D. Habit (×38), E. Ditto (×65).

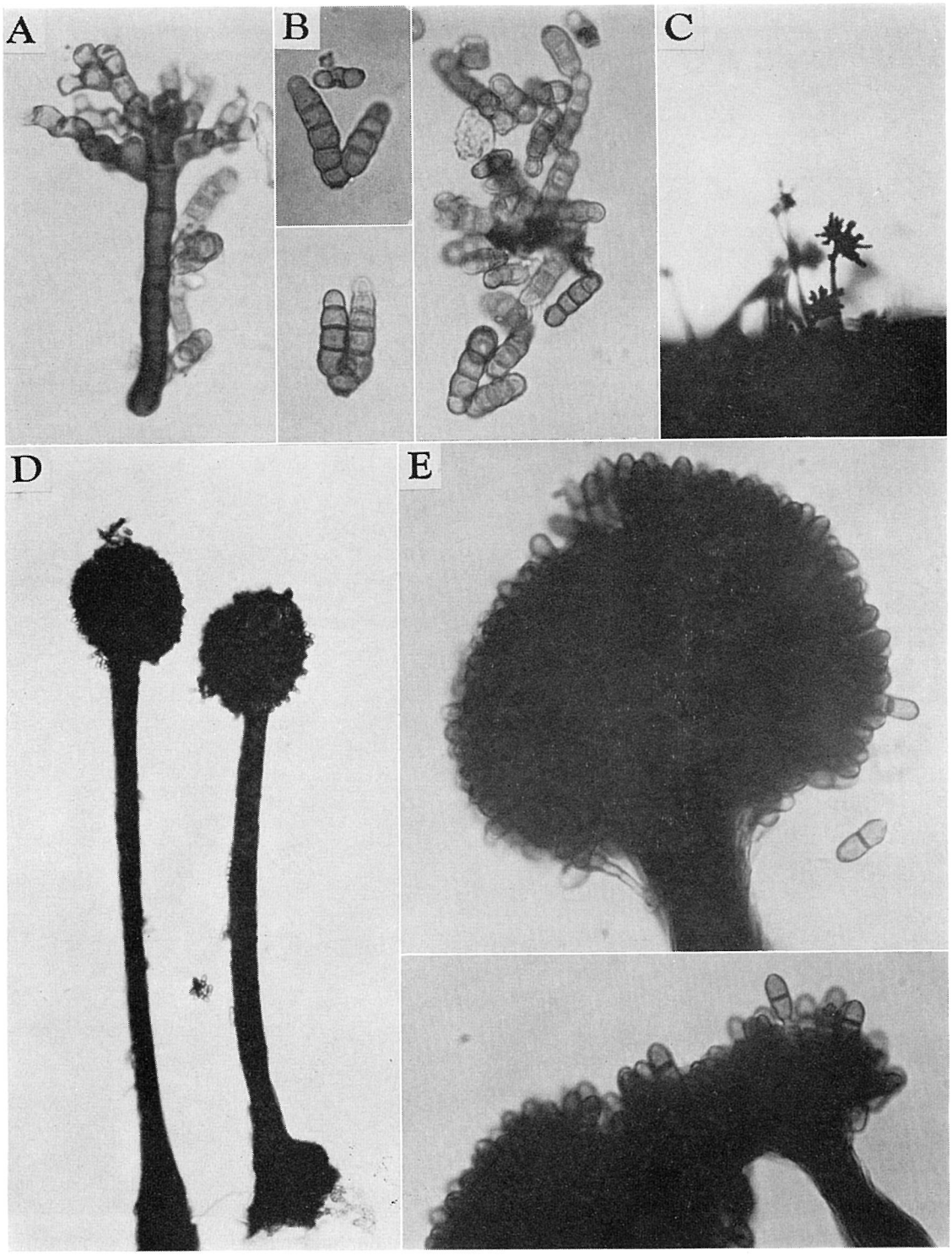

Plate 5. A-C. *Dendryphion comosum.* A, Conidiophores and conidiogenous cells (×400); B. Conidia, (×400), C. Habit (×65). D-E. *Didymobotryum rigidum.* D. Synnemata (×1100), E. Conidial heads and conidia (×400).

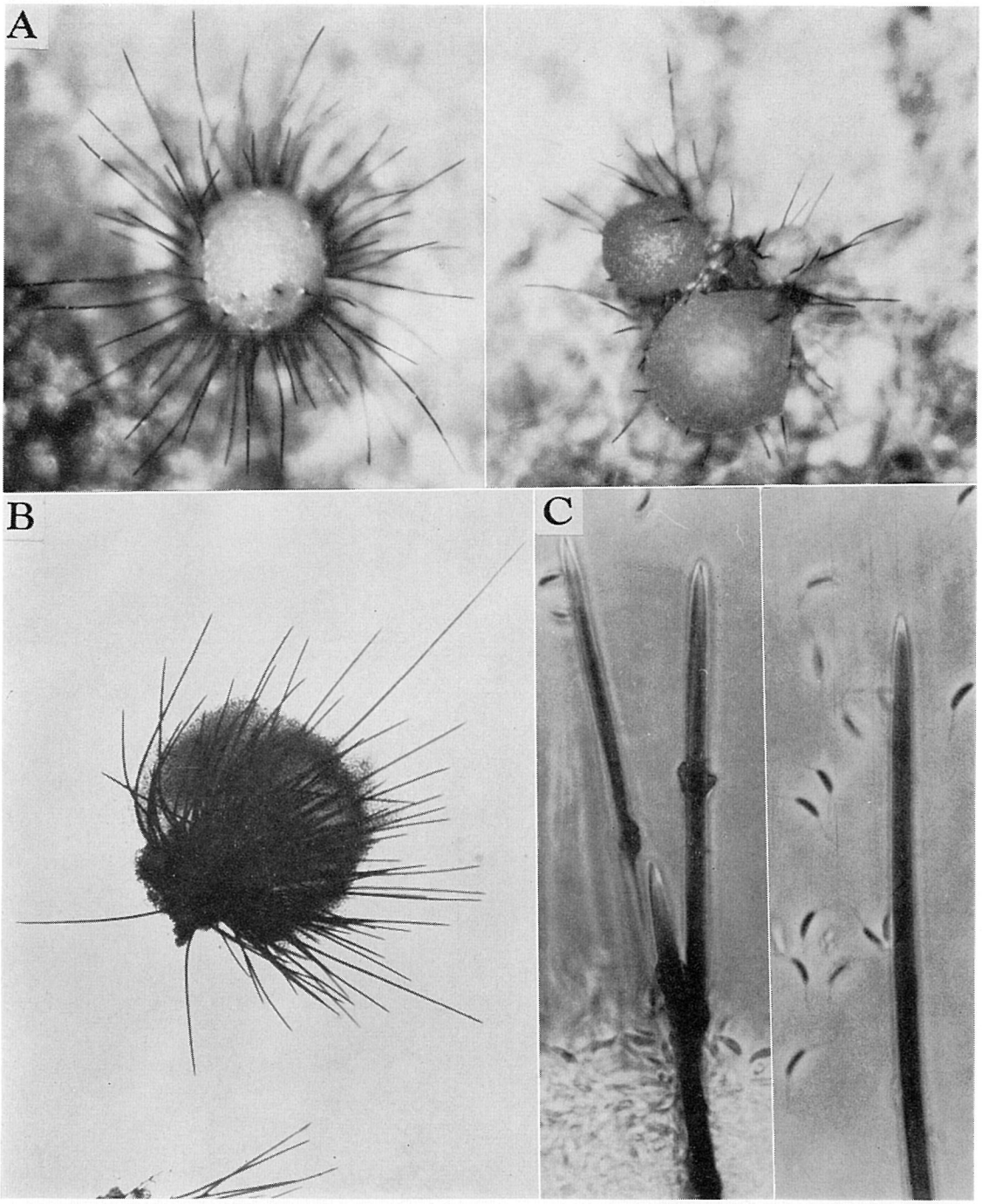

Plate 6. *Minimidochium setosum.* A. Sporodochia on sterilized leaves (×38), B. Sporodochium (×40), C. Phialides and conidia (×400).

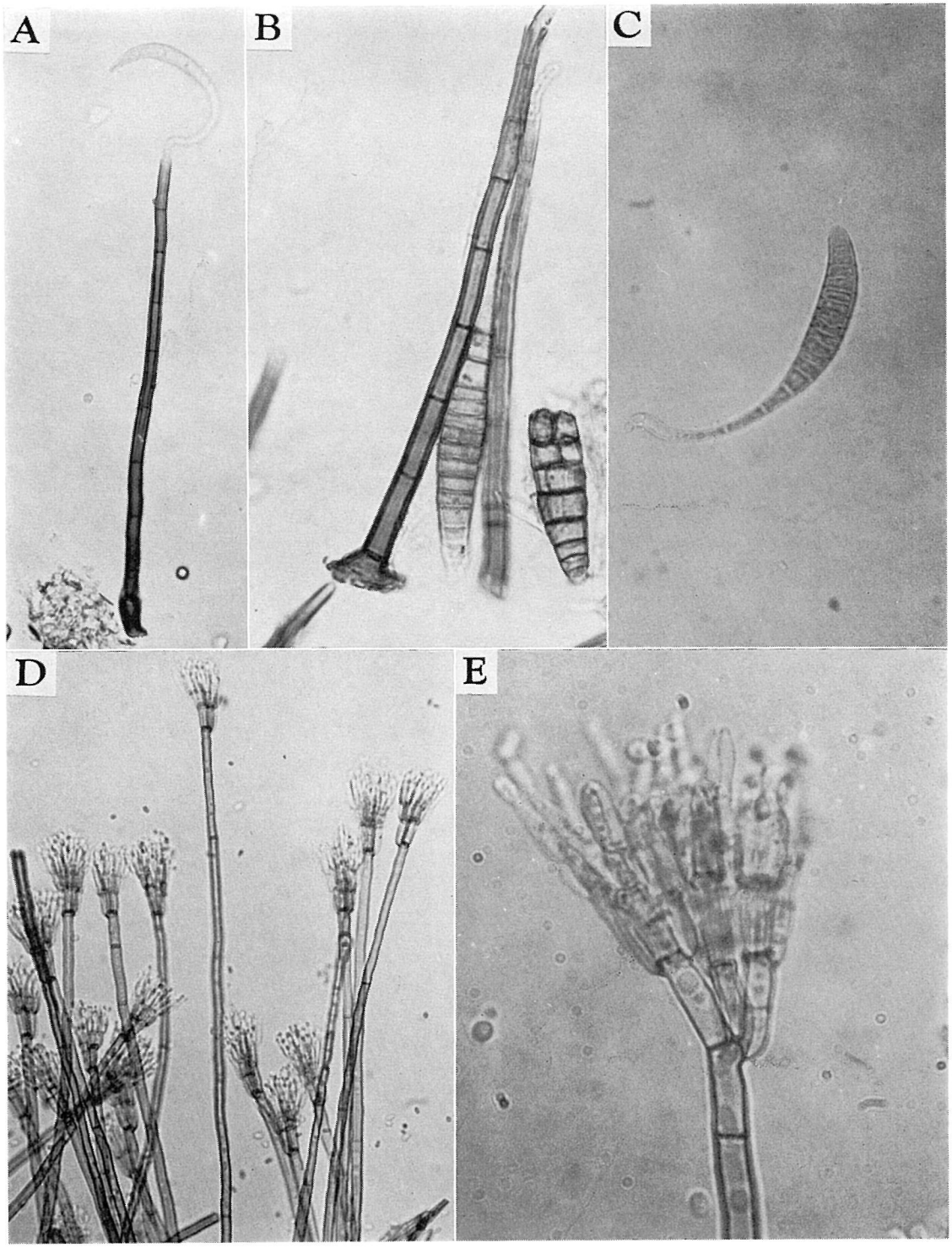

Plate 7. A-C. *Pendulispora venezuelanica*. A-B. Conidiophores and conidia (×250 & ×400). C. Conidium (×400). D-E. *Phialocephala humicola* (×250 & ×900).

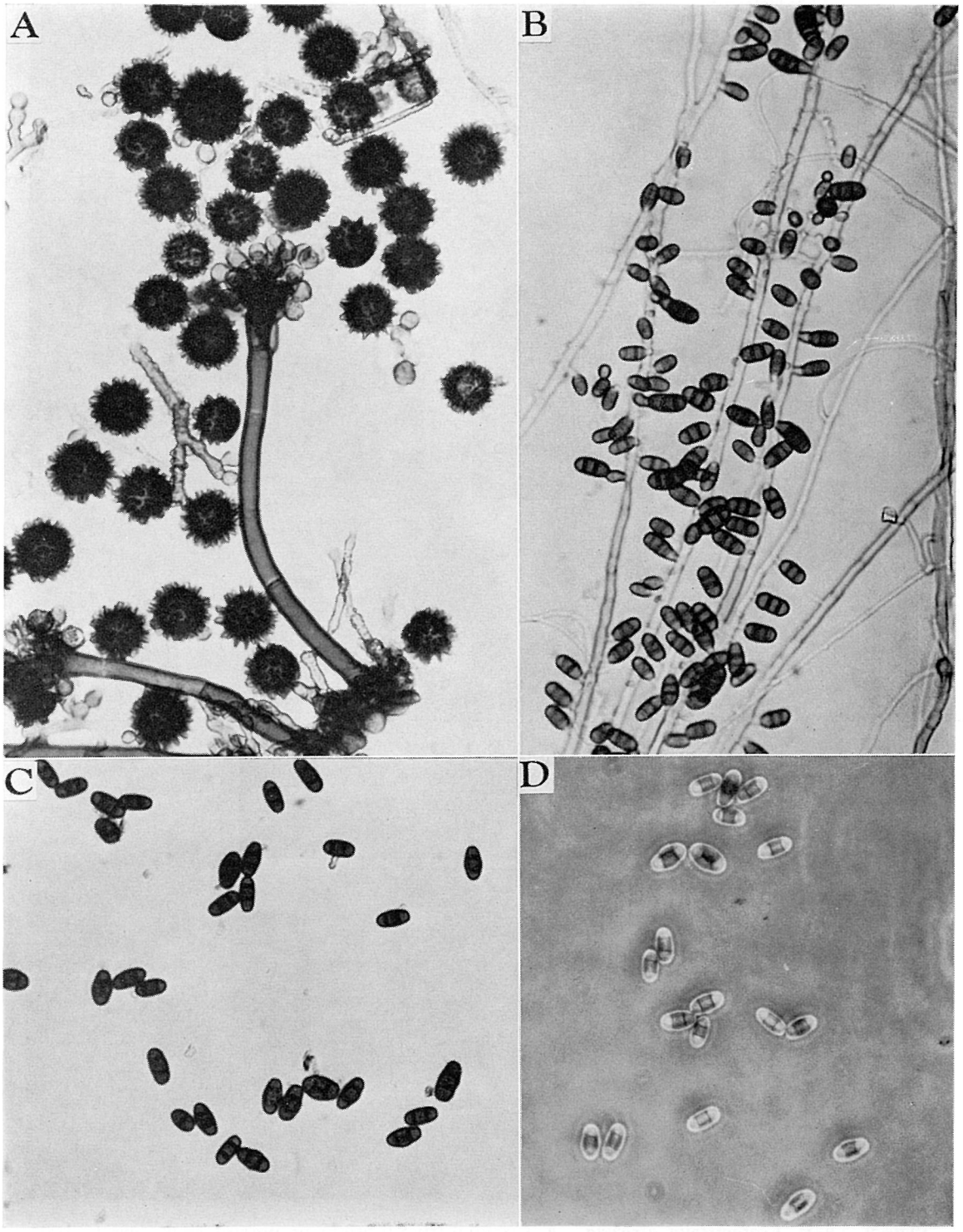

Plate 8. A. *Periconia macrospinosa*. Conidiophores and conidia (×250), B-D. *Pithomyces maydicus*. B. Conidiogenous cells and conidia (×250), C. Conidia (×250), D. Ditto (×250).

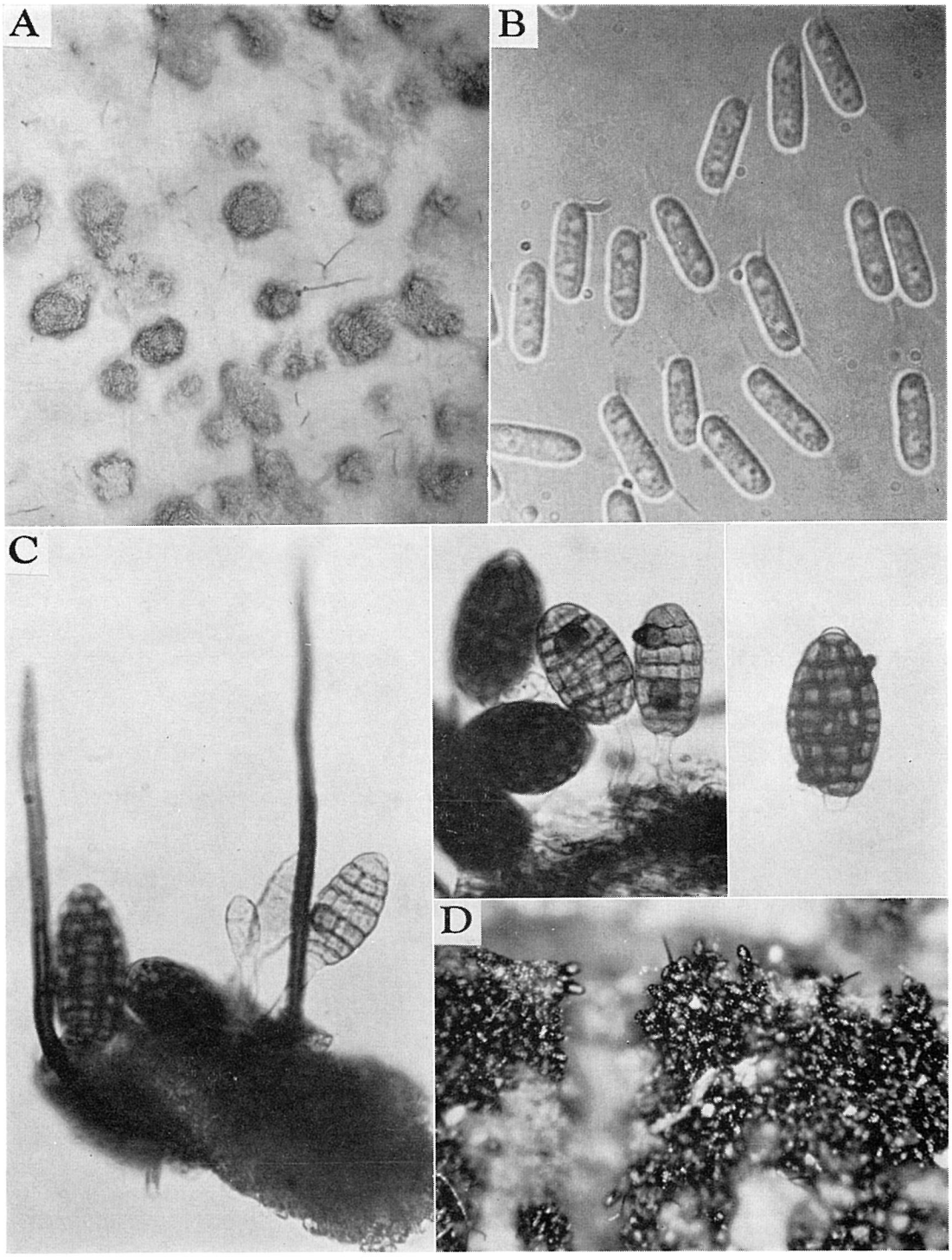

Plate 9. A-B. *Leptodiscella africana*. A. Conidial fructifications on potato-carrot agar (×100) B. Conidia (×900). C-D. *Septosporium bulbotrichum*. C. Setae and conidia (×400), D. Habit (×65).

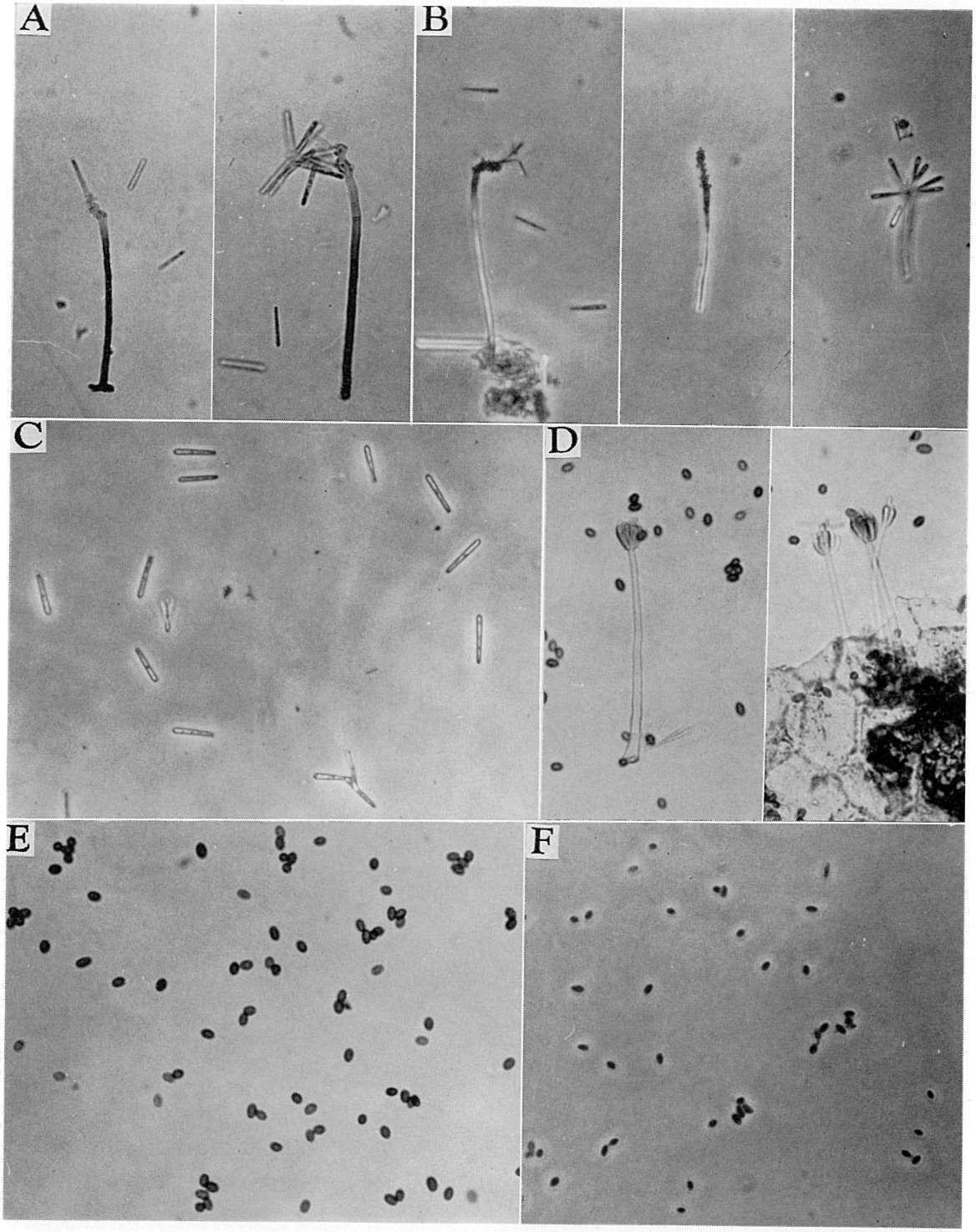

Plate 10. A-C. *Solosympodiella clavata*. A. Conidiophores (×250), B. Ditto (×250), C. Conidia (×400), D-F. *Stachybotrys atra* var. *microspora*. D. Conidiophores (×250), E. Conidia (×250), F. Ditto (×250).

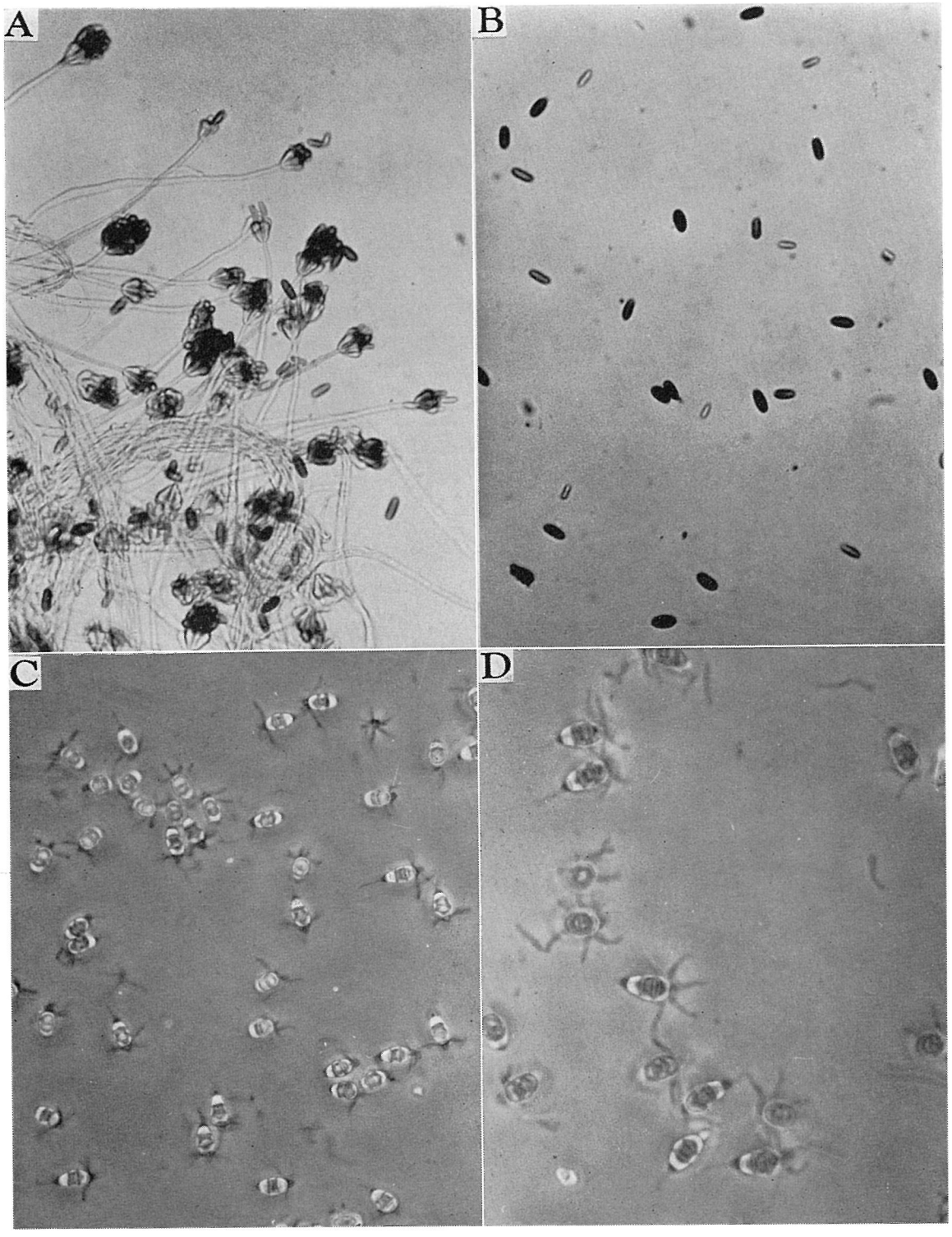

Plate 11. A-B. *Stachybotrys dichroa*. A. Conidiophores (×250), B. Conidia (×250), C-D. *Pestalotia distincta*. C. Conidia (×250), D. Ditto (×400).

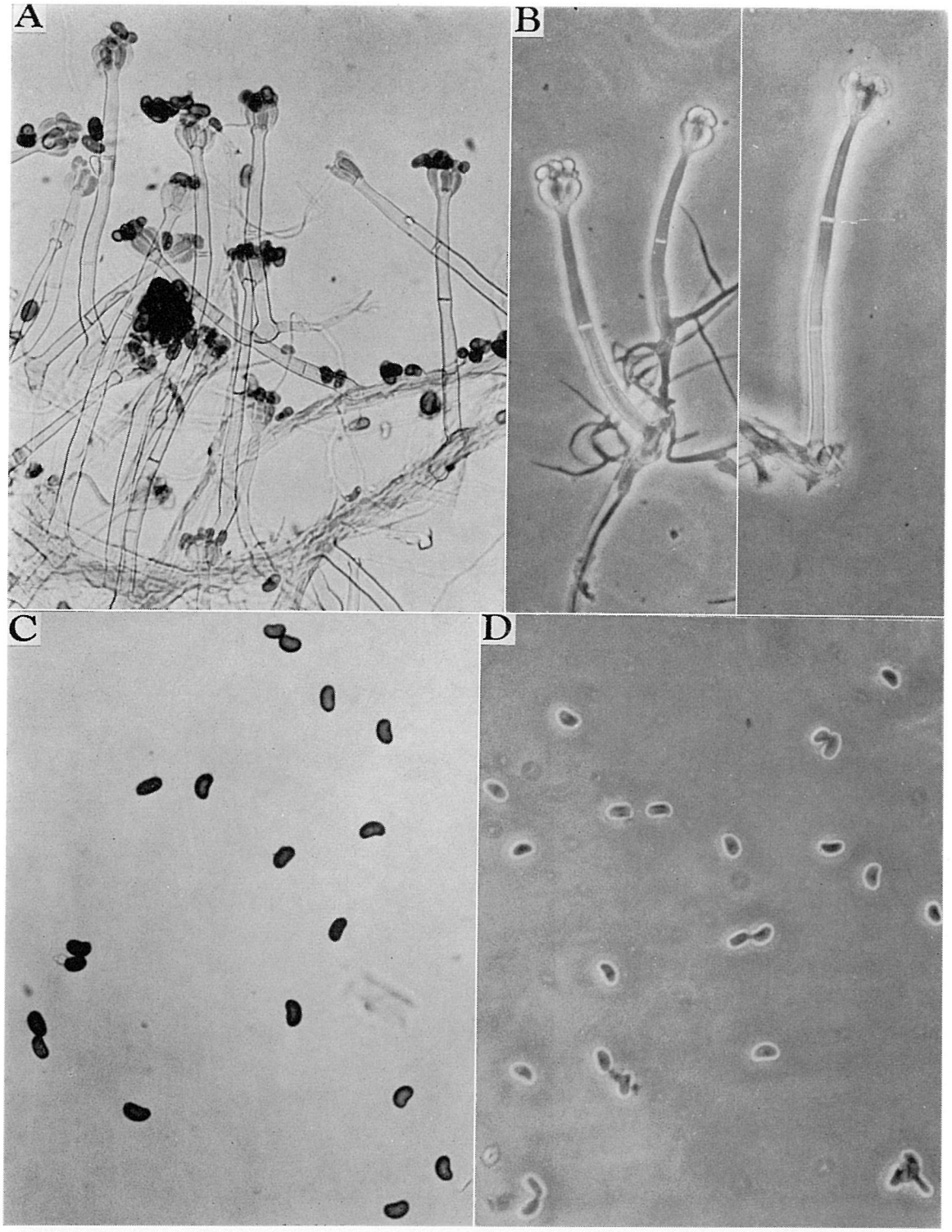

Plate 12. *Stachybotrys nephrospora*. A. Conidiophores (×250), B. Ditto (×250), C. Conidia (×250), D. Ditto (×250).

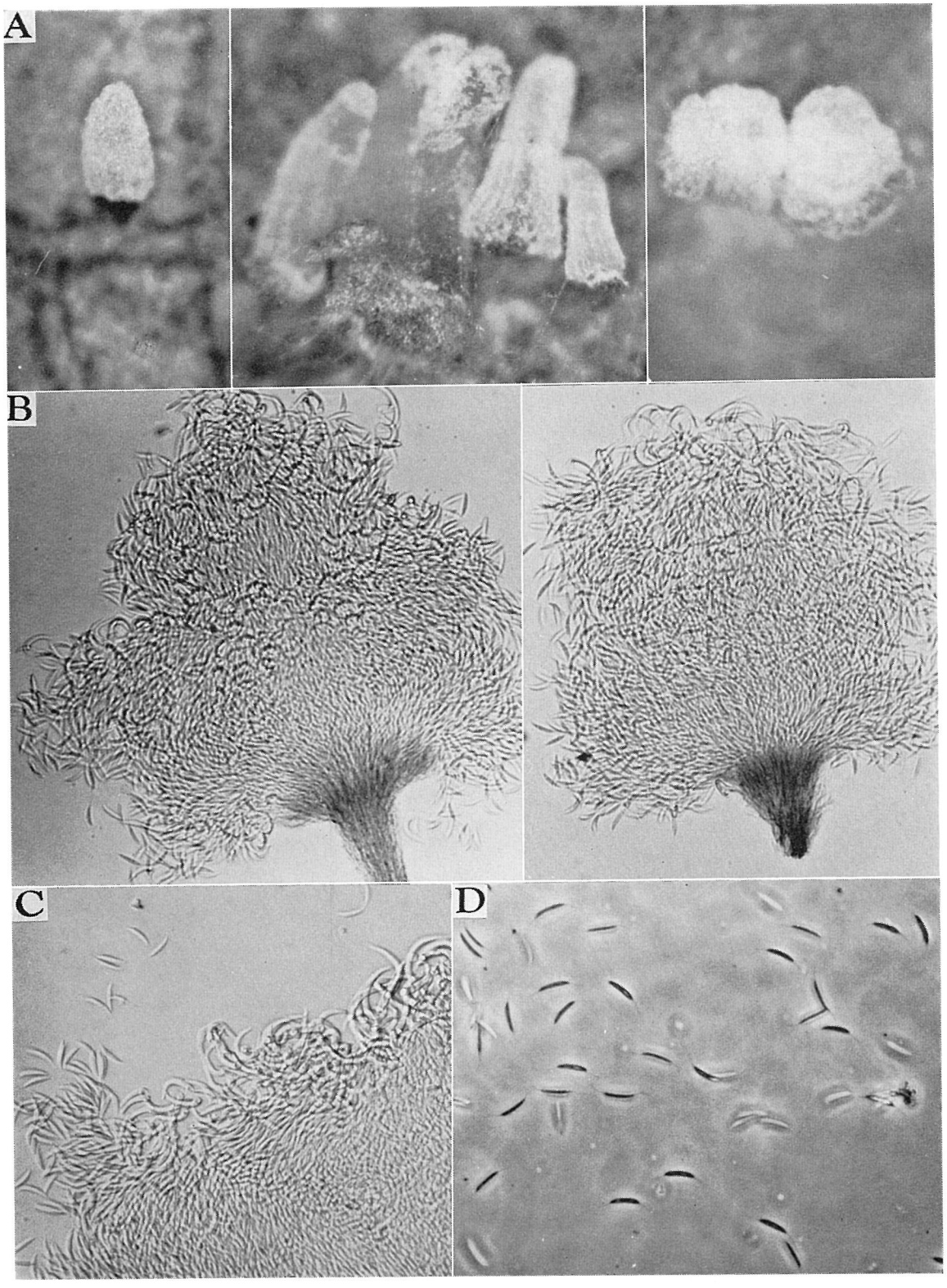

Plate 13. *Thozetella cristata*. A. Habit (×38), B. Sporodochial structures and conidial mass (×100), C. Conidia and microawns (×250), D. Conidia (×250).

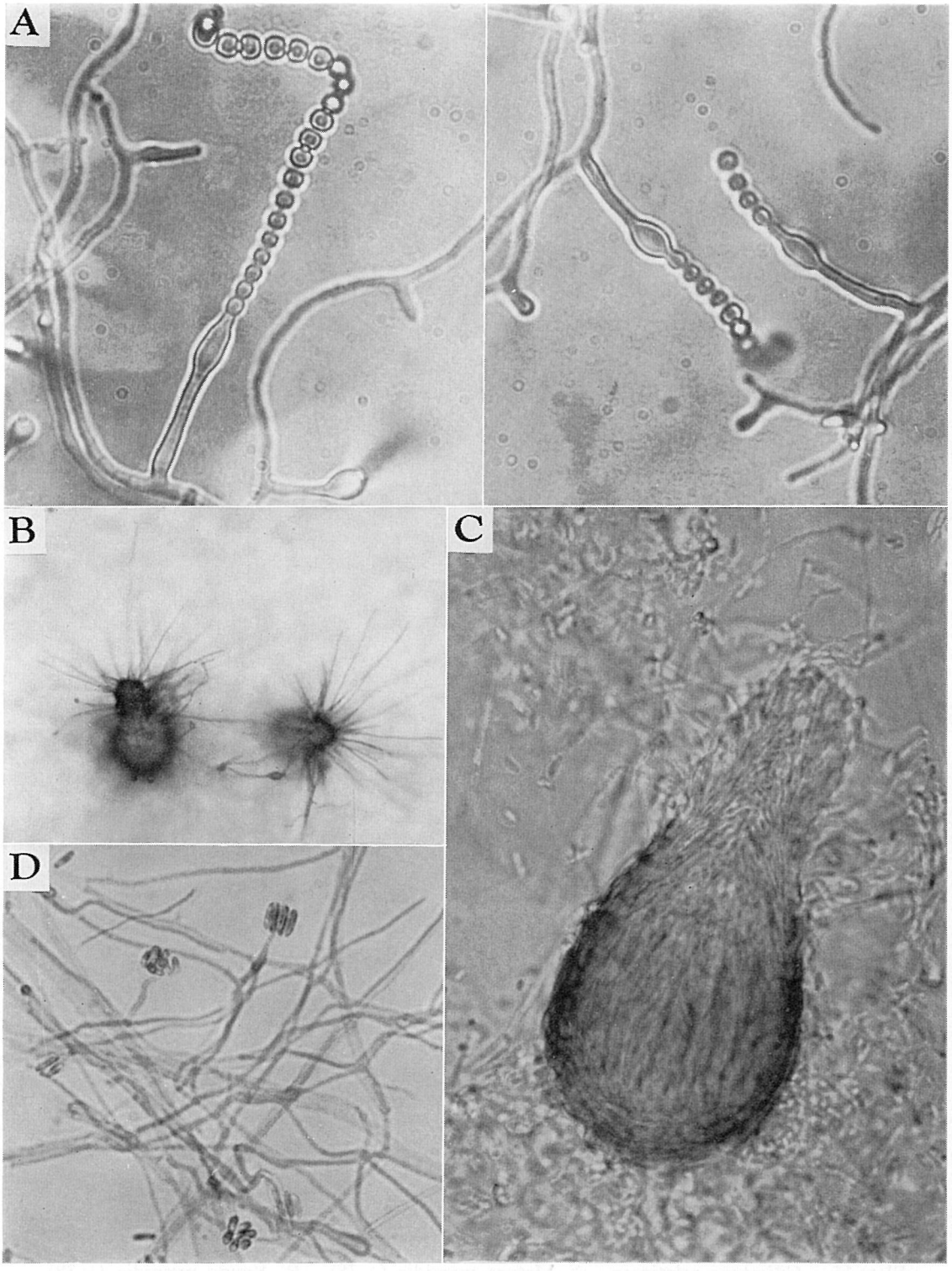

Plate 14. A. *Torulomyces lagena*. Conidiophores and conidia (×900). B-D. *Micronectriella cucumeris*. B. Perithecia on corn-meal agar (×38), C. Perithecium (×100), D. Conidiophores and conidia (×400).

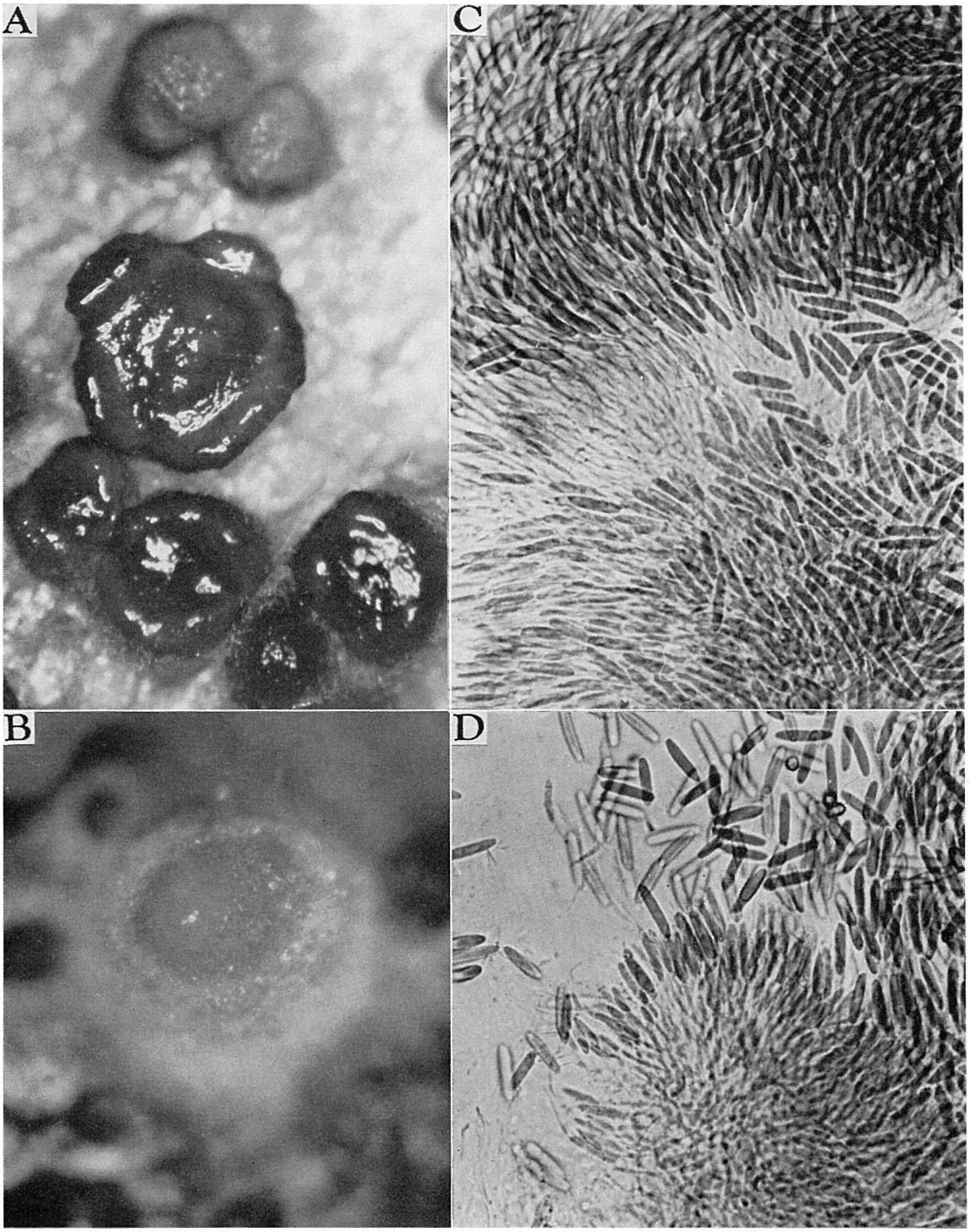

Plate 15. *Ciliospora gelatinosa*. A. Mature pycnidia (×38), B. Young pycnidia (×38), C. Inner structure of pycnidial cavity, (×250), D. Conidiophores and conidia (×250).

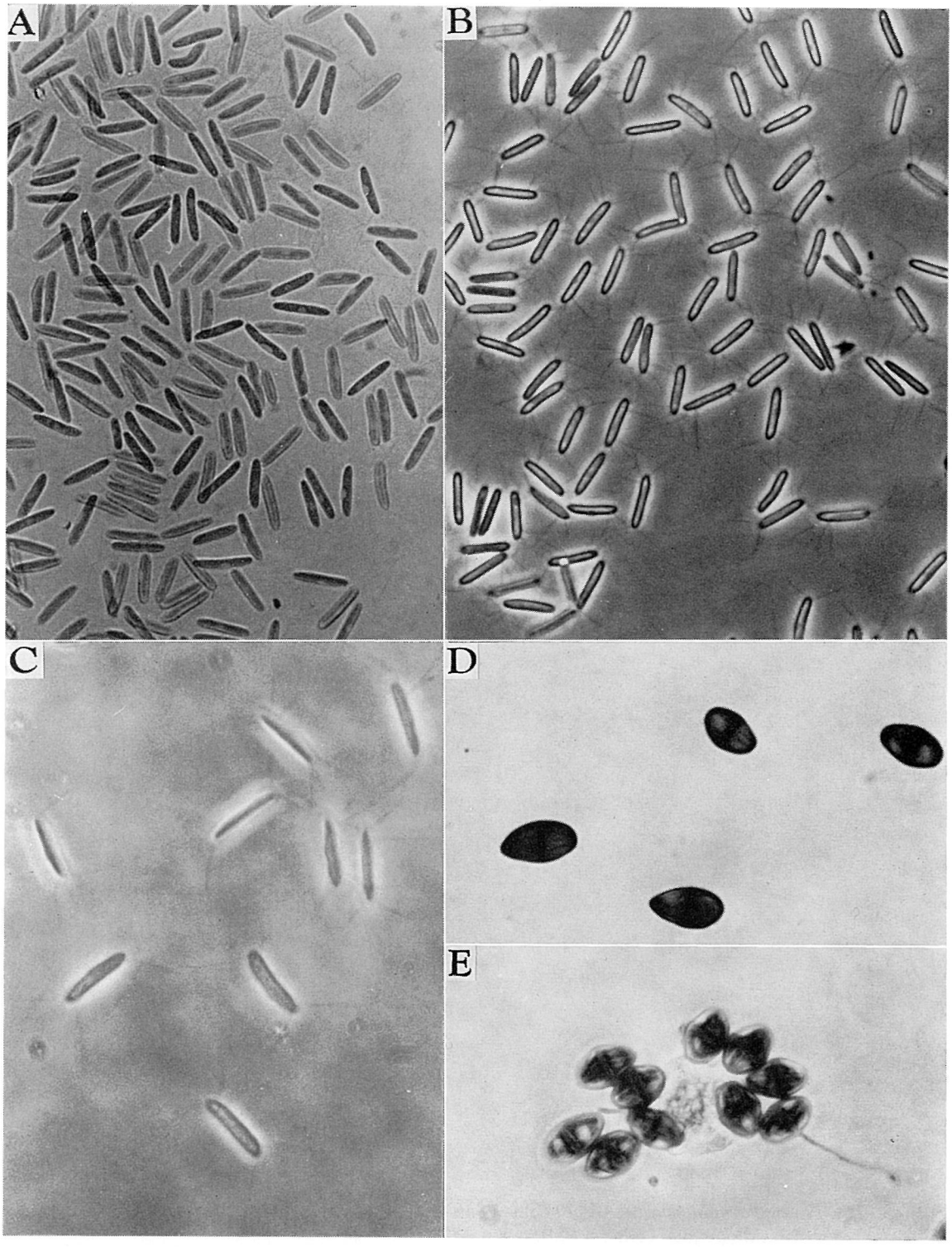

Plate 16. A-C. *Ciliospora gelatinosa*. A. Conidia (×250), B. Ditto (×250), C. Ditto (×400), D-E. *Lasiodiplodia theobromae*. D. Conidia (×250), E. Ditto (×250).

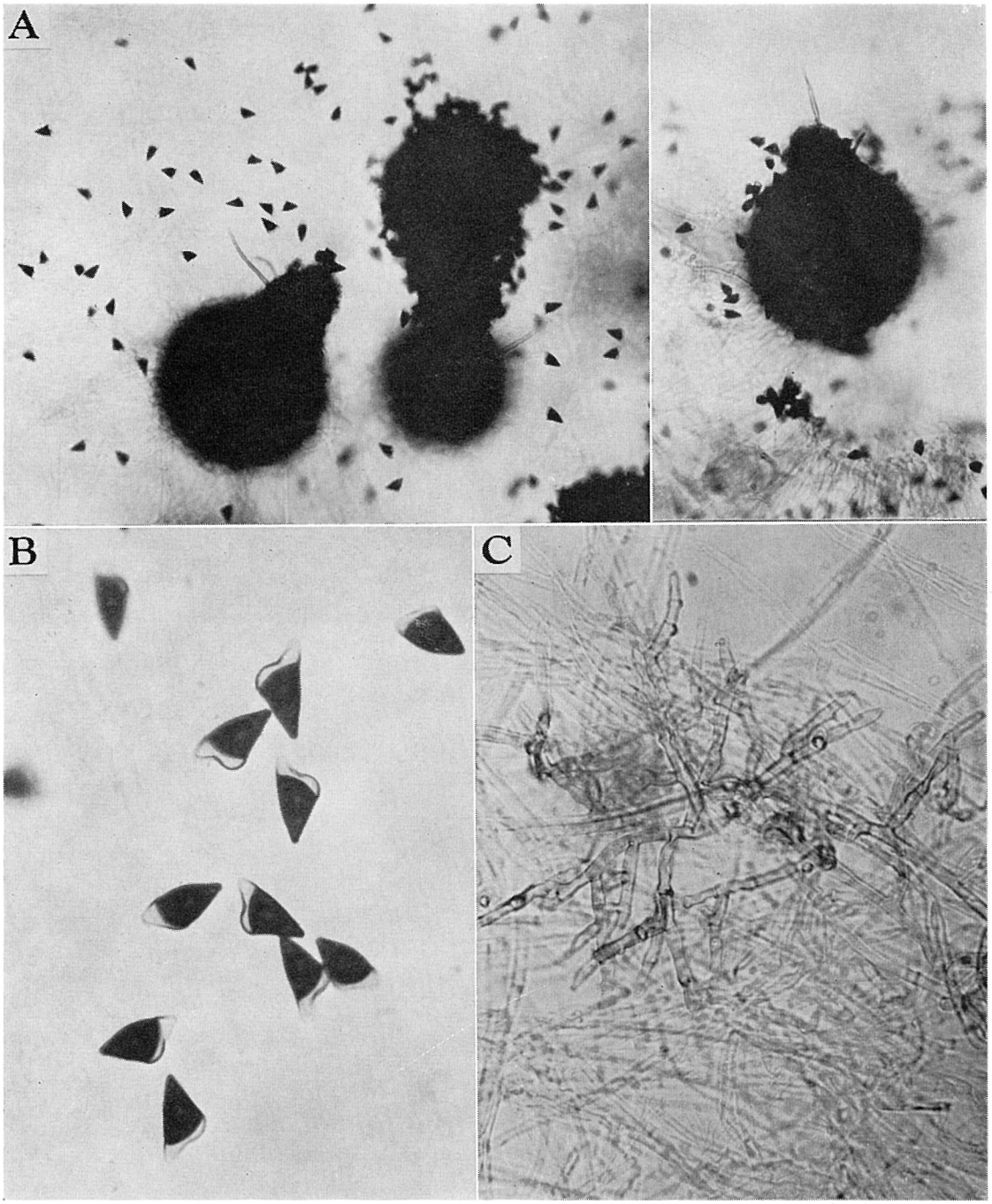

Plate 17. *Triangularia bambusae*. A. Perithecia on oat-meal agar (×100), B. Ascospores (×400), C. Conidiogenous cells (×400).

IFO Res. Comm. 7,
143–145, 1975 (*March*)

DESCRIPTIVE CATALOGUE OF IFO CULTURE COLLECTION OF BACTERIA 2.

In the routine work to update the strains and data in the IFO bacterial collection, a few strains were reidentified as different species from formerly named species and some were found to be aberrant from the standard properties of the species. The descriptions of some other strains which had been identified as the known species and used in various researches have not been published yet or insufficiently described in published papers. The object of this catalogue is to provide the descriptions of these bacterial strains.

4 and **5.** *Enterobacter cloacae* (Jordan) Hormaeche and Edwards 1960
IFO 3320 and 12935

6. *Enterobacter aerogenes* Hormaeche and Edwards 1960
IFO 12012

7. *Enterobacter liquefaciens* (Grimes and Hennerty) Ewing 1963
IFO 12979

8 to **15.** *Klebsiella pneumoniae* (Schroeter) Trevisan 1887
IFO 3317, 3318, 3319, 3321, 12009, 12019, 12059, and 12932

As the genus *Aerobacter* had been rejected by the judicial commision of ICNB (opinion 46), 12 strains which have been maintained as *Aerobacter* sp. were reidentified. All these strains are gram-negative rods, $0.5–0.8 \times 1.0–2.0\,\mu$m, occurring singly and grow readily in ordinary media aerobically and anaerobically. Physiological properties of these strains are described in Table 1.

From these results, the strains IFO 3320 and 12935 were reidentified as *Enterobacter cloacae* (Jordan) Hormaeche and Edwards; IFO 12010 as *Enterobacter aerogenes* Hormaeche and Edwards; IFO 12979 as *Enterobacter liquefaciens* (Grimes and Hennerty) Ewing; and IFO 3317, 3318, 3319, 3321, 12009, 12019, 12059 and 12932 as *Klebsiella pneumoniae* (Schroeter) Trevisan.

Note: Six strains of *K. pneumoniae*, IFO 3317, 3318, 3319, 3321, 12019, and 12059 gave weakly positive result in semisolid agar medium test for the motility, though the flagella staining of their cells and the hanging drop method did not demonstrated the motility. Furthermore, 2 strains of them, IFO 3318 and 3319, gave the positive reaction in ornithine decarboxylation test at 25 C. There remains a suspicion that these six strains are intermediate organisms between *K. pneumoniae* and *E. aerogenes*.

[I. Banno and T. Sakane]

Table 1. Physiological properties.

	Strain No.											
	IFO 3320	IFO 12935	IFO 12010	IFO 12979	IFO 3317	IFO 3318	IFO 3319	IFO 3321	IFO 12009	IFO 12019	IFO 12059	IFO 12932
Motility	+	+	−	+	−*	−*	−*	−*	−	−*	−*	−
Indole production	−	−	−	−	+	−	−	−	−	−	−	−
MR test	−	−	W	+	+	W	W	−	+	−	−	W
VP test	+	+	+	+	+	+	+	+	+	+	+	+
Nitrate reduction	+	+	+	+	+	+	+	+	+	+	+	+
H_2S production	+	+	+	+	+	+	+	+	+	+	+	+
Urease	+	+	−	−	+	+	+	+	+	+	+	+
Gelatin liquefaction	+	+	+	+	−	−	−	−	−	−	−	−
Lysine decarboxylation	−	−	+	+	+	+	+	+	+	+	+	+
Arginine dihydrolysis	+	+	−	−	−	−	−	−	−	−	−	−
Ornithine decarboxylation	+	+	+	+	−	−**	−**	−	−	−	−	−
Phenylalanine deamination	−	−	−	−	−	−	−	−	−	−	−	−
Citrate utilization	+	+	+	+	+	+	+	+	+	+	+	+
Malonate utilization	−	+	+	−	+	+	+	+	+	−	+	+
Gas from glucose	+	+	+	+	+	+	+	+	+	+	+	+
Acid from												
glucose	+	+	+	+	+	+	+	+	+	+	+	+
lactose	+	+	+	−	+	+	+	+	+	+	+	+
sucrose	+	+	+	+	+	+	+	+	+	+	+	+
mannitol	+	+	+	+	+	+	+	+	+	+	+	+
dulcitol	−	−	−	−	−	−	−	−	−	−	−	−
glycerol	−	−	+	+	+	+	+	+	+	+	+	+
adonitol	−	−	+	−	NT	−	−	+	−	NT	+	−
inositol	−	−	+	+	+	+	+	+	+	+	+	+
Growth at 24 C	+	+	+	+	+	+	+	+	+	+	+	+
" 37 C	+	+	+	W	+	+	+	+	+	+	+	+

W: weakly positive, NT: not tested,
* weak motility in soft agar medium,
** positive at 25 C.

16. *Arthrobacter globiformis* (Conn) Conn and Dimmick 1947
IFO 3062

This strain was brought by Tauchi, Tokai Regional Fisheries Research Laboratory, under the name of *Micrococcus subflavus* Flügge in 1950.

Young cells (10–16 hr) are gram-variable rods, 0.6–0.9×1.0–2.5 μm on nutrient agar. In older cultures (2 days) the cells become gram-negative coccoid, 0.5–0.7 μm in diameter.

Non-motile.

Chromogenic (lemon yellow).

Litmus milk: Clearing (3 days) without coagulation; cream-colored sediment.

Catalase positive.

Oxidase negative.

Does not survive heating at 63 C for 30 min in skim milk.

Cellulose is not decomposed.

Starch is hydrolyzed.

Gelatin is liquefied.

Nitrites are weakly produced in nitrate broth.

Indole not produced.

Acetylmethylcarbinol not produced.

Urease produced.

Hydrogen sulfide produced.

Vitamins and amino acids are not necessary for growth.

Betain, glycine, *p*-hydroxybenzoate, and tyramine are utilized as sole carbon sources.

Ammonium sulfate, potassium nitrate, sodium urate, allantoin, and urea are utilized as sole nitrogen sources.

Slightly acid but no gas from glucose, sucrose, and raffinose.

Temperature relations: Optimum, between 25 C and 30 C; no growth at 37 C.

Special application: Bioassay for chloramphenicol.

Comment: According to *Bergey's Mannual of Determinative Bacteriology*, 7th edition, this strain is identical with *Arthrobacter aurescens* (Clark) Phillips which is considered synonym of *A. globiformis* (Conn) Conn and Dimmick in the 8th edition of *The Mannual.*

[K. Imai]

ABSTRACTS 1973-1974

Hideo KIKKAWA* and Teiji IIJIMA

A Screening Test Method for Metabolic Disease Patients Using Nutrition-Requiring Mutants of *Escherichia coli*

Aspects of Cellular and Molecular Physiology, p. 209–216
(K. HAMAGUCHI ed.) University of Tokyo Press (1972)

Inherited metabolic diseases in man, characterized by an accumulation of metabolites, can be detected by an auxanographic method, *i.e.*, the Nutrition Mutant Method. This method utilizes filter paper discs which are impregnated with samples or standard solutions or metabolites and placed on a basal medium containing a nutritional mutant. Rapid screening for patients is possible by observation of the growth zone around the paper discs, as by Guthrie's inhibition assay method.

When a mixed indicator plate is used, the efficiency of detecting metabolic diseases may be increased in cases where syntrophism is excluded.

* Department of Genetics, Medical School, Osaka University, Osaka

Teiji IIJIMA and Takeshi SAKANE

A Method for Preservation of Bacteria and Bacteriophages by Drying *in Vacuo***

Cryobiology **10**: 379–385 (1973)

An efficient and practical method was established to preserve bacterial strains and bacteriophages. The method is characterized by drying without freezing and by use of a cotton wool plug (nonabsorbent) to prevent contamination. Drying conditions

** From Proceedings of Japan-U.S.A. Conference on Freezing and Freeze-Drying, held October 15–20, 1972, at Cacapon State Park, Berkeley Springs, West Virginia, sponsored by the Japan Society for the Promotion of Science, the National Science Foundation, and the American Type Culture Collection.

were examined by measuring temperature, vacuum, and residual moisture of the samples. From the measurement, it was found that the cotton wool plug acts as a buffer and a desiccant. Thus, the specimens reached optimal conditions during storage. Another point of advantage is that the temperature of the specimen during the drying procedure was 2–5C; therefore, the evaporation of the water is rapid and the time of completion is shorter than that during lyophilization.

Ko IMAI, Teiji IIJIMA, and Takezi HASEGAWA

Transport of Tricarboxylic Acids in *Salmonella typhimurium*

J. Bacteriol. **114**: 961–965 (1973)

Salmonella typhimurium possesses at least three inducible transport systems for the tricarboxylic acids (citric, isocitric, *cis*-aconitic, and tricarballylic). The first system was induced by citrate, isocitrate, or *cis*-aconitate, and transported citric acid and isocitric acid. The second system was also induced by the same acids as in the first system and transported *cis*-aconitic acid. This system required Mg^{2+} ions and was stable at pH 8.4 but unstable at pH 7.0. The metal ion was replaced with Sr^{2+} or Ca^{2+} ions but not with Ba^{2+} ions. The third system was induced by tricarballylate and transported citric acid, *cis*-aconitic acid, and tricarballylic acid.

Keisuke TUBAKI

Some Aspects of Geographical Distribution of Leaf Litter Fungi in Japan

Forsch. Gebiet Pflanzenkrankh. **8**: 61–69 (1973)
(Shokubutsu Byogai Kenkyu)

After the comparative study on microflora on the embedded sterilized leaves and on the naturally decayed leaves in the litter of the forests in Ootsu, Shiga Pref., Tanegashima Island and Yakushima Island, it became clear that there is a difference in the leaf-microflora between the central and the southern parts of Japan. Ninety-four genera are listed and their frequency of occurrence is shown. Among them, four genera predominate in the central Japan, while thirteen genera predominate in these southern islands.

Keisuke TUBAKI

Aquatic Sediment as a Habitat of *Emericellopsis*, with a Description of an Undescribed Species of *Cephalosporium*

Mycologia **65**: 938–941 (1973)

A new species of Hyphomycetes, *Cephalosporium polyaleurum* Tubaki, was described, which are not uncommon in the coastal muds and is characteristic in the smaller amount of conidial production and in the formation of numerous aleuriospores. In addition, three species of *Emericellopsis* were shown to be distributed also in muds.

Keisuke TUBAKI and Tadayoshi ITO

Fungi Inhabiting in Brackish Water

Rept. Tottori Mycol. Inst. (Japan) **10**: 523–539 (1973)

From brackish water lakes in Japan, Hinuma, Lake Shinji and Mikatagoko, twenty-four species of marine and terrestrial fungi have been found on test wood-blocks submerged in water. This paper is concerned with taxonomical studies on them and with vegetative growth and spore germination of selected species. In addition, in order to establish the tolerance levels of those fungi isolated from the brackish water a study of their physiology has been undertaken. Most of the fungi isolated respond to an increase in salinity and maxima generally occur between 30 to 70% seawater.

Tatsuo YOKOYAMA and Keisuke TUBAKI

Successive Fungal Flora on Sterilized Leaves in the Litter of Forest IV

Rept. Tottori Mycol. Inst. (Japan) **10**: 597–618 (1973)

Successive fungal colonization on the embedded sterilized leaves of *Castanopsis cuspidata* and *Quercus phillyraeoides* in the leaf-litter of the forest was investigated in a given condition in the artificially controlled growth cabinet. It was found that the potential fungal flora and the difference of the process of seral succession of the fungi in a given leaf-litter have some effect on the subsequent successive colonization of the

fungi on the newly incorporated sterilized leaves. This fact makes addition to our previous knowledge that the respective leaf-litter formed in the different environmental condition may have each own fungal flora and pattern of seral succession. Based on the present experimental evidence, we delimit four overall successive stages of fungi: lag stage, developmental or ascending stage, climax stage and descending stage, respectively.

Tatsuo YOKOYAMA and Keisuke TUBAKI

Some Hyphomycetes from Papua and New Guinea

Bull. Natn. Sci. Mus. Tokyo **16**: 655–660 (1973)

Fifteen species of litter and soil fungi are briefly described as new to Papua and New Guinea. These are part of the collections which have been collected and isolated during the second Japanese Mycological Expedition to New Guinea held in November, 1971 to January, 1972. All belong to Deuteromycotina and species are: *Circinotrichum falcatisporum* Pirozynski, *Cladorrhinum foecundissimum* Sacc. & Marchal, *Conioscypha lignicola* Höhnel, *Cryptophiale guadalcanalense* Matsushima, *Doratomyces microsporus* (Sacc.) Morton & Smith, *Gyrothrix circinata* (Berkeley & Curtis) Hughes, *G. microsperma* (Höhnel) Pirozynski, *Helicosporium nematosporum* Linder, *Lacellinopsis sacchari* Subramanian, *Periconia echinochloae* (Batista) M. B. Ellis, *P. macrospinosa* Lefebvre & Johnson, *Stachybotris parvispora* Hughes, *Tetraploa aristata* Berk. & Br., *T. ellisii* Cooke, *Trichocladium pyriforme* Dixon.

Kôiti NAKAZAWA

Production of "Crystals" by Streptomycetes

Trans. mycol. Soc. Japan **15**: 155–157 (1974)

Observations were made on the production of crystals of various species of Streptomycetes. Several cultures were examined from the point of view of their ability to produce crystals and observed that various species of streptomyces as follows produced crystals in the medium: *S. fradiae*, *S. antibioticus*, *S. olivaceus*, *S. aureus*, *S. albus S. flaveolus*, *S. griseolus*, *S. flavus*, *S. lavendulae*, *S. nitrosporeus S. parvus*, *S. colombiensis*, *A. cretaceus*, *S. graminofaciens*, *S. griseosporeus* and *S. albogriseus*.

It is considered that the ability of producing crystals in the medium is widely distributed among the species of Streptomycetes.

Takeshi SAKANE, Kozaburo MIKATA and Isao BANNO

Preservation of Yeasts by Drying in Vacuo

J. Japan. Soc. Res. Freez. Dry. **20**: 29–35 (1974)

A drying in vacuo without freezing was applied to preservation of yeasts. A selection of yeast strains belonging to 14 genera was examined for ability to survive during the drying-process and storage of the dried specimens. The cells of the yeast were dried in suspending media containing various ingredients in ampules with a cotton plug under a vacuum condition. The ampule sealed in vacuo after the drying was subjected to an accelerated storage test at an elevated temperature (37 C) to estimate the long term viability of the dried cells.

Many of the strains tested survived well after drying and during storage. The best suspending medium was 1/10 M phosphate buffer containing sodium glutamate (5%), lactose (5%), and polyvinylpyrolidone (6%), PH 7.0. From microscopical examination of the cells after rehydration of the dried specimens it was suggested that destruction of cell wall and organella may be one of the causes to decrease the viability of cells when an inadequate suspending medium was used.

[in Japanese]

Keisuke TUBAKI and Yuichi WADA

Materials for the Fungus Flora of Japan (15)

Trans. mycol. Soc. Japan **15**: 186–188 (1974)

Kernia pachypleura Malloch & Cain, an ascomycete, was described as a first record from Japan. This species was originally found on the dung in Uganda and this is the second record. The present isolate was made on the submerged balsa wood panel immersed for two months in Lake Kitagata, Fukui Pref., and the present aquatic habitat is so significant.

Tatsuo YOKOYAMA

Hyalohelicomina, a new genus of Hyphomycetes

Trans. mycol. Soc. Japan **15**: 158–160 (1974)

A new Hyphomycete genus, *Hyalohelicomina* Yokoyama, was proposed for *Helicomina deutziae* Yokoyama which has been reported previously as parasitic on the living leaves of *Deutzia crenata* Sieb. & Zucc. It can be distinguished from all other genera in the plant parasitic Helicosporae by using the provided key.

Tatsuo YOKOYAMA and Keisuke TUBAKI

Materials for the fungus flora of Japan (16)

Trans. mycol. Soc. Japan **15**: 189–195 (1974)

Cristulariella pyramidalis Waterman & Marshall was found to cause zonate leafspot disease with premature defoliation on some wild creeping plants in Japan. Five species were added as new hosts, i.e., *Hydrangea petiolaris* Sieb. & Zucc., *Paederia chinensis* Hance, *Vitis vinifera* L. cultivar. Neo Muscatel, *Cocculus trilobus* DC. and *Quamoclit angulata* Bojer. *Botrytis moricola* Hino (=*Sclerotinia moricola* Hino) on mulberry in Japan and *Sclerotium cinnamomi* Sawada on camphor-tree both in Japan and Formosa are treated here as conspecific with *C. pyramidalis*.

MISCELLANEOUS SCIENTIFIC PAPERS

Isao BANMO. 1974. Taxonomy of bacteria. *In* T. Hasegawa (ed.) New Methods for Classification of Microorganisms. p. 52–105. Kodansha Scientific, Ltd., Tokyo. [In Japanese]

Keisuke TUBAKI. 1974. Taxonomy of fungi. *In* T. Hasegawa (ed.) New Methods for Classification of Microorganisms. p. 106–138. Kodansha Scientific, Ltd., Tokyo. [In Japanese]

Keisuke TUBAKI and Tatsuo YOKOYAMA. 1971. The fungal flora developing on sterilized leaves placed in the litter of Japanese forests. *In* T. F. Preece and C. H. Dickinson (ed.) Ecology of Leaf Surface Microorganisms, p. 457–461. Academic Press, New York.

Keisuke TUBAKI. 1974. How to identify Fungi Imperfecti. I–III. Shokubutsu-Boeki **28**: 249–255, 293–298, 369–373. [in Japanese]

Tatsuo YOKOYAMA. 1974. Taxonomic aspect of *Cristulariella* spp. Shokubutsu-Boeki **28**: 346–348. [in Japanese]

PRESENTATION OF PAPERS AT SCIENTIFIC MEETINGS 1973-1974

Author(s)	Title	Scientific Meeting
T. YOKOYAMA	White mold of *Deutzia* spp. caused by *Hyalohelicomina deutziae* (Yokoyama) Yokoyama gen. et comb. nov.	Phytopathological Society of Japan. Meeting in Kyoto (March, 1973)
K. IMAI, T. IIJIMA & T. HASEGAWA	Transport of the tricarboxylic acids in *Salmonella typhimurium*. II.	Agricultural Chemical Society of Japan. Meeting in Tokyo (April, 1973)
K. TUBAKI	On ascospores of *Trichocoma paradoxa* and *Talaromyces luteus*.	Mycological Society of Japan. Meeting in Tokyo (April, 1973)
T. YOKOYAMA	Perfect state of *Actinopelte castanopsidis* Yokoyama & Tubaki.	Mycological Society of Japan. Meeting in Tokyo (April, 1973)
K. IMAI & T. IIJIMA	Transport of the tricarboxylic acids in *Salmonella typhimurium*. III.	Agricultural Chemical Society of Japan. Meeting in Kyoto (May, 1973)
T. IIJIMA, I. NOGAMI* & M. YONEDA*	Derivation of guanosine- and inosine-producing mutants from *Bacillus*.	The 15th Symposium of Japanese Society of Breeding. Meeting in Morioka (October, 1973)
Y. SAKAMOTO & T. IIJIMA	Migration of bacterial colonies belonging to genus Bacillus. (I).	Biophysical Society of Japan, Meeting in Tokyo (October, 1973)
O. WAKAE*[2], K. MATSUURA*[2], T. ENDO*[2], K. FUJIMORI*[2] & T. YOKOYAMA	Studies on control effect of sheath blight of rice plant by validamycin IX. Effect on microflora of rice plant (1).	Phytopathological Society of Japan. Meeting Okayama (November, 1973)
T. YOKOYAMA	Zonate leafspot of some wild creeping plants caused by *Cristulariella pyramidalis*.	Phytopathological Society of Japan. Meeting in Okayama (November, 1973)
K. IMAI & T. IIJIMA	Transport of the C_4-dicarboxylic acids in *Salmonella typhimurium*.	Agricultural Chemical Society of Japan. Meeting in Kyoto (February, 1974)
K. IMAI, T. SAKANE & I. BANNO	Utilization of organic acids by the species belonging to Enterobacteriaceae.	Agricultural Chemical Society of Japan. Meeting in Tokyo (April, 1974)

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Author(s)	Title	Scientific Meeting
I. NAKANISHI[*1], K. KIMURA[*1], T. SUZUKI[*1], M. ISHIKAWA[*1], I. BANNO, T. SAKANE & T. HARADA[*2]	Studies on bacterial gel-Forming β-1,3-glucans (cardlan-type polysaccharides). (V) Staining of microbial colonies.	Agricultural and Chemical Society of Japan. Meeting in Tokyo (April, 1974)
N. NISHIHARA[*3] & T. YOKOYAMA	*Kabatiella sorghi* Nishihara & Yokoyama, a new leaf blight fungus on *Sorghum* spp.	Phytopathological Society of Japan. Meeting in Tokyo (April, 1974)
T. YOKOYAMA	The genus *Cristulariella,* taxonomic position and relationship to the genus *Botrytis* and allied genera.	Phytopathological Society of Japan. Meeting in Tokyo (April, 1974)
T. SAKANE, K. MIKATA & I. BANNO	Preservation of yeasts by drying in vacuo.	Japanese Society for Research of Freezing and Drying. Meeting in Osaka (May, 1974)
K. TUBAKI	On Japanese *Hypomyces.*	Mycological Society of Japan. Meeting in Sapporo (May, 1974)
T. YOKOYAMA & N. NISHIHARA[*3]	Cultural aspects of *Kabatiella sorghi.*	Mycological Society of Japan. Meeting in Sapporo (May, 1974)
I. BANNO & T. HASEGAWA	*Saccharomyces* Yeasts Isolated in Japan. (1) A Numerical analysis of *Saccharomyces cerevisiae* group isolated from natural sources.	The 4th International Symposium on Yeasts, Vienna (July, 1974)
T. IIJIMA & K. IMAI	Genetic analysis of *tct* gene (tricarboxylic acid transport) in *Salmonella typhimurium*	Genetics Society of Japan. Meeting in Sendai (September, 1974)
K. NAKAZAWA	Production of "crystals" by streptomycetes.	Post-congress (IAMS), Symposium on Actinomycetes, Kyoto (September, 1974)
K. TUBAKI	*Hypomyces*-species and their conidial states in Japan.	Post-Congress (IAMS) Mycological Meeting, Tottori (September, 1974)
T. YOKOYAMA & K. TUBAKI	Leaf litter fungi associated with ever-green oak forests in Japan.	1st Intersectional Congress of IAMS, Tokyo (September, 1974)
S. KŌMOTO[*4], N. NISHIHARA[*3] & T. YOKOYAMA	A new disease of sudan-grass caused by two species of *Curvularia.*	Phytopathological Society of Japan. Meeting in Tokushima (October, 1974)

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[*2] Institute of Scientific and Industrial Research, Osaka University.
[*3] National Grassland Research Institute.
[*4] Chugoku National Agricultural Experiment Station.

Author(s)	Title	Scientific Meeting
Y. SAKAMOTO, T. IIJIMA S. IYOBE* & S. MITSUHASHI*	Typing of *Pseudomonas aeruginosa* by phage resistance and lysogeny.	International Symposium on Bacterial Resistance, Tokyo (October, 1974)

* Department of Microbiology, School of Medicine, Gunma University.

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CORRECTIONS

In the issue of IFO Research Communication No. 6, the following corrections should be made.

Page	Line	Type	should read
13	Fig. 6	drying (F)	drying (E)
25	9	luxriant	luxuriant
	23	summarise	summarize
	36	invadors	invaders
26	24	invadors	invaders
30	37	sucessin	succession
45	7	priod	period
83	10	13.	24.
84	1	14.	25.
	25	15.	26.
	36	Gosho	Gose
85	2	appoered	appeared
	7	16.	27.
	31	17.	28.
86	26	18.	29.
87	12	19.	30.
	31	20.	31.
88	12	21.	32.
89	5	22.	33.
	10	swallen	swollen
	14	swallen	swollen
	30	23.	34.
90	8	bottum mad	bottom mud
99	19	Name of the journal	J. Sericult. Sci. Japan **40**, 8–12 (1971)
100	17	Techniol.	Technol.
	23	but not	but

Additional corrections should be made in Table 1 on page 20, Table 3 on page 24 and Table 1 on page 31. The correct tables are reprinted below.

Table 1. on page 20

Table 1. List of fungi developed on the embedded leaves in the three communities examined.

Zygomycotina		
	Mucor	*Mortierella*
Ascomycotina		
	Ceratocystis	*Toxotrichum*
	*Leptosphaeria**	***Nectria***
	*Mollisia**	
Basidiomycotina		
	Lepista	***Thanateporus***
	Marasmius	
Deuteromycotina		
	*Actinopelte**	*Monacrosporium*
	Beltrania	***Neottiosporella***
	Botrytis	*Oidiodendron*
	Calcarisporium	*Paecilomyces*
	Candelabrum	*Penicillium*
	Catenularia	*Pestalotia*
	Cephalosporium	*Polyscytalum*
	Chalara	*Pseudobotrytis*
	Cladosporium	*Ramularia**
	*Clonostachys**	*Rhinocladiella*
	Codinaea	***Scolecobasidium***
	Crinula	*Scolecosporium*
	Cryptophiale	*Sporothrix**
	Fusarium	*Stilbum*
	Fusidium	*Subulispora*
	*Gliocladium**	*Sympodiella*
	Helicosporium	*Trichoderma*
	Idriella	*Verticillium*
	Illosporium	
Myxomycotina		
	Stemonitis	

Table 3. on page 24

Table 3. List of fungi which seasonal occurrence incliminates towards winter or summer.

Organisms	Occurrence on the leaves	
	winter October-March (1969–1970)	summer May-January (1970–1971)

Table 1. on page 31

Table 1. List of genera of fungi on the embedded leaves in the five communities examined.

Zygomycotina			
Gongronella	F	*Mucor*	D E F G
Mortierella	D E F G H		
Ascomycotina			
Calonectria	D E F G	*Nectria*	E F H
Ceratocystis	E G	*Toxotrichum*	D E G
Leptosphaeria	D F		
Deuteromycotina			
Acrothecium*	E	***Menisporopsis***	D E F G
Beltrania	D E F G	*Monacrosporium*	D E F G H
Blastophorum	F	***Mirandina***	H
Candelabrum	D G	*Neottiosporella*	D E F G H
Catenularia	D E G	*Oidiodendron*	D E F G H
Cephalosporium	D G H	*Paecilomyces*	D E F G H
Chalara	D E F G H	*Penicillium*	D E F G H
Chaetopsina	D E F G H	*Pestalotia*	D E F G H
*Chloridium**	F	***Phialocephala****	H
Circinotrichum	D E	*Phoma**	E
Cladosporium	D E F G H	***Phyllosticta****	E
Clonostachys	D G H	*Polyscytalum*	D E F H
Codinaea	D E F G H	*Pseudobotrytis*	H
Crinula	E F	*Ramularia**	D F
Cryptophiale	D G H	*Rhinocladiella**	D
Cylindrocladium	D E F G H	*Scolecobasidium*	D E F G H
Dactylaria	D F	*Scolecosporium*	G
Diheterospora	D E F	***Selenophoma***	G
Discosia*	D E	***Selenosporella***	F G
Ellisiopsis	D E F G H	***Septonema***	G
Endophragmia	D	***Solosympodiella***	E G H
Fusarium	E	*Sporothrix*	G H
Fusidium	D E H	*Stilbum*	E
Gliocladium	D E H	*Subulispora*	D E G H
Gonatobotrys	E F	*Sympodiella*	D E
Gonytrichum	D F G	*Thysanophora*	E
Gyrothrix	D E F G H	*Trichoderma*	D E F G H
Helicosporium	D G	*Verticillium*	D E F G H
Idriella	D E F G	***Zanclospora***	E F
Mahabatella	F	***Zygosporium***	F
Basidiomycotina			
Marasmius	G		
Mycena	E		
Thanateporus	D E F		
Myxomycota	D		

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