STUDIES ON THE IDENTITY AND ACTIVITY OF SAPROPHYTIC BACTERIA ON STORED BARLEY

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by
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PREFACE

The work reported in this thesis concerns the identity of saprophytic bacteria on barley seed, and the possibility of their growth at moisture levels sufficiently low to enable them to interact with the xerophytic moulds which are pathogenic on stored seed. It was therefore essentially a laboratory investigation, which would have been impossible to implement without the funds, facilities, and technical assistance made available by Professor W.R. Philipson. I want to express my gratitude to him, and to acknowledge the technical assistance of the following persons in the fields indicated: Mr J. Brusse - general microbiology; Mr F. McGregor - photography; Miss S. Bullock - electron microscopy.

The 360 computer system was operated by the staff of the Mobil Computer Centre, Canterbury University, and the programme was written by Mr F.L. Ng, of the Electrical Engineering Department, to whom I am indebted for much helpful advice on computing.

A grant for equipment was received from the University Grants Committee, and a grant from the University of Canterbury Research Assistants Fund enabled me to employ Miss A. Ramsay as a personal research assistant for two weeks.

Research on this topic required some background knowledge of the technology involved in grain storage, and Mr J. Malcolm and Mr J. Smart of the Canterbury (N.Z.) Malting Company, were my mentors in this respect. I am also grateful to the Company for their co-operation in many aspects of the work, including the supply of all the seed used.

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ABSTRACT

1. The epiphytic microflora of barley seed was investigated to determine the kinds of bacteria present, and the possibility of their growth at low levels of water activity.

2. Identification of the bacteria was based on similarity indices computed for each isolate.

3. On developing grain in the field, yeasts (*Rhodotorula*) were the first microorganisms to colonize the seed in large numbers. Subsequent growth of bacteria reached a maximum one week before harvest, and then declined somewhat. At the peak of microbial development, it was estimated that 15-20% of the seed surface was occupied by microorganisms.

4. A survey of sack-stored barley showed that the largest single group of bacteria on the grain was composed of *Erwinia herbicola*. This accounted for 45% of the total isolates. Several other groups formed a heterogenous collection of gram-variable or gram-positive coryneform bacteria, which together made up a further 38% of the total. Pseudomonads, flavobacteria and cocci were of lesser importance.

5. On stored seed attacked by granary weevils (*Sitophilus granarius*) there was a decrease in the numbers of bacteria found, and, on seed which had previously been sterilized, a corresponding increase in numbers of *Aspergillus glaucus*. Aspergilli were not found on unsterilized seed but this was not considered to be the result of antagonism by the saprophytic microflora.

6. Bacteria were shown to multiply on seed kept at atmospheric humidities of 75%, 95% and 100% R.H. Although

the rate of increase was inversely proportional to the humidity, the maximum population attained was the same in each case, and once attained it was immediately followed by a drop in numbers. These results were interpreted as indicating that in a physiological sense, xerophytic bacteria do occur on barley, but that this is of no ecological significance because in the absence of liquid water the growing colony is soon poisoned by its own metabolic wastes.

CHAPTER I.

INTRODUCTION

1. FUNGI ASSOCIATED WITH CEREAL SEEDS

Prior to 1945, very little information was available on the saprophytic microflora associated with cereal seeds. The storage of large surpluses of wheat in North America since then has, however, stimulated research on this topic and on the possible role of microorganisms in deterioration of stored grain. This work has been reviewed by Semeniuk (1954), Milner and Geddes (1954), Christensen (1957) and Christensen and Kaufmann (1965). As used by these authors the term deterioration included any or all of the following phenomena: a decrease in germination percentage, discolouration of embryos or whole seeds, biochemical changes that make grain unfit for food, and heating, which usually results in a drastic reduction in quality or in complete spoilage.

Fungi were regarded as the primary agents of deterioration, and the moisture content of the seed as the most important factor determining the kinds of fungi that invade the stored seed, and the degree to which they invade. Other factors said to influence the deterioration of grains by fungi were temperature, length of storage, insect and mite infestation, mechanical damage, and age of grain.

Christensen (1957) distinguished two groups of fungi

associated with grain which he called field fungi and storage fungi. Field fungi were those which invaded seeds before harvest and formed an abundant mycoflora on freshly harvested grain. Alternaria tenuis was found to be the dominant species of this flora but a wide variety of other fungi were also common. These included species of Fusarium, Cladosporium, Helminthosporium and Pullularia. The group was said not to cause deterioration of grain in storage as these fungi were unable to grow at seed moisture contents below 25%.

Storage fungi, on the other hand, were said to develop on and within seed only after it had been placed in storage. Dickson (1962) indicated that the source of inoculum for these moulds was associated with grain silos, bins, elevators etc. As defined by Christensen (1957) the storage moulds comprise several species of Aspergillus and Penicillium which replace partially or completely the field fungal flora of stored grain. Although Pionnat (1966) showed that in his studies deterioration of stored barley was caused chiefly by growth of Penicillium species, the aspergilli are generally considered the more pathogenic, especially some in the A. glaucus group. Osmophilic members of this group have been shown to invade the seeds and gradually kill the embryos at seed moisture levels too low for the growth of other fungi (Milner, Christensen and Geddes, 1947).

Perera (1966) surveyed the fungal flora of barley stored at the Heathcote plant of the Canterbury (N.Z.) Malting Company Ltd. All barley used in the present work was also stored at Heathcote.

2. THE BACTERIAL FLORA OF CEREAL SEED

Even after Pasteur's classical experiments on the lactic acid and alcoholic fermentations in the 1860's, bacteria were sometimes regarded as living forms of enzymes. The production of diastase in germinating cereals for instance was attributed to bacteria within the tissues (Jorissen, 1885). This view was eventually discredited by work such as that of Fernbach (1888) who expressed the generally held opinion that

> "..... les tissus vegetaux normeaux constituent pour les microbes une filtre parfait, et qu'ils ne peuvent etre envahis par eux qu'a la suite de causes tout a fait accidentelles."

Hiltner (1887), Burri (1903) and Düggeli (1904), all of whom carried out detailed surveys of the numbers and kinds of bacteria present on plant surfaces, must therefore have done so in the belief that these microorganisms were of no significance to the plants.

Düggeli (ibid) demonstrated the presence of an abundant and characteristic epiphytic bacterial flora on a wide variety of seeds and seedling leaves. The kinds of bacteria

present were extremely limited, however. One yellow-pigmented organism, which Düggeli named Bacterium herbicola aureum, was dominant in almost all cases. For example it accounted for 89%to 100% of the isolates from three samples of barley seed, and 97%to 100% of those from five samples of wheat. This species has been the subject of a recent taxonomic study by Dye (1969) and is now known as Erwinia herbicola. Düggeli found only one other species that was at all common - Bacterium fluorescens (Flugge) L. & N. The sixth edition of Bergey's Manual of Determinative Bacteriology (Breed et al, 1948) lists this as a synonym of Pseudomonas fluorescens Migula.

In 1918, Morgenthaler confirmed that healthy cereal seeds show a luxuriant epiphytic flora of bacteria composed chiefly of E. herbicola, and in 1929 Woller published a detailed account of the development of the bacterial flora during the growth cycle of various plants. He extended Düggeli's observations on seeds and seedlings and showed that the epiphytic microflora was very similar on a range of crop, garden and meadow plants. He claimed that weather conditions determined the kinds of bacteria present, and that E. herbicola, although common, was not always dominant. Under some conditions he found 80%to 90% of the population on barley to consist of spore-forming bacilli. This early work in Germany culminated in a comparative study by Mack (1936) of a

large number of isolates of E. herbicola and other yellow-pigmented bacteria with which it had been confused.

One of the first papers on the microflora of stored grain to appear in the post-war period was that by James, Wilson and Stark (1946). This pointed to the existence of a large epiphytic bacterial population on all wheat passing through the Winnipeg market. This flora was dominated by two bacteria, one of which was E. herbicola and the other an unidentified Pseudomonas not related to P. fluorescens. These workers considered that the numerous bacteria and yeasts present on the grain were true commensals developing on the seed coat or in its intercellular spaces, but that the fungi present were not the result of proliferation and could not be considered epiphytic. They implied that if the seed microflora were responsible for deterioration of stored grain, then the organisms involved must be bacteria. This view was soon proved to be wrong, and once the aspergilli had been implicated as the most important agents of deterioration, interest in the bacteria declined and the earlier German work was often overlooked or ignored. Dickson (1962) for example, reviewing the microflora associated with barley kernels, refers only to

"..... the numerous undetermined white and yellow

bacteria, mostly motile rods that are both acid-tolerant and non-tolerant."

Christensen (1957) considered that bacteria were of no importance, as grain is usually stored at moisture levels too low for their growth. In their review of deterioration in stored grain, Christensen and Kaufmann (1965) do not mention bacteria.

In contrast to the work then being carried out in North America, Spicher (1956), working in Germany, claimed that with stored wheat and rye seeds there was a positive correlation between the amount of damage (besatz) and the ratio of numbers of bacteria to fungi. This is unexpected in view of claims that the bacteria are passively carried on the seed while damage of any kind increases the rate of deterioration by storage fungi (Christensen, 1957).

Spicher's work has not been repeated but recent Russian work, for example that by Chranowska (1964) on rye and by Ordin (1966) on wheat, indicates that the bacterial flora is not necessarily passive and that there may be an increase in bacterial numbers after harvest. Chranowska (ibid) claimed that this bacterial flora was antagonistic to seed-borne fungi, and both workers reported that the microflora consisted chiefly of E. herbicola.

Perera (1966) found a large and apparently active

bacterial population on barley stored at the Heathcote malting plant, Christchurch. Numbers varied from 0.6 x 10^6 to 9 x 10^6 per gram of seed, and were correlated with the condition of the seed and the method of storage. Although the general tendency was for numbers to fall during storage, they increased during one three month period when the moisture content of the seed rose from 14% to 15%. This was still well below the lowest seed moisture level at which bacteria have been reported active, but the rise in moisture content may have indicated periods of high atmospheric humidity during which the bacteria multiplied.

All isolates were gram-negative motile rods which fell into two distinct groups. The commoner type comprised 85% of the population of freshly harvested barley and 55% of the population after nine months storage; it produced yellow colonies on nutrient agar. The remaining bacteria formed white colonies and a greenish water-soluble pigment on this medium.

The work described in this thesis extends this preliminary survey. A more detailed examination was made of the bacterial flora of barley stored during one season. This was carried out with two objects in view: to determine whether seed grown and stored under New Zealand conditions carried a characteristic epiphytic flora such as that described by Düggeli (1904) and later authors; and to

confirm that the bacteria present were capable of active growth under conditions of low water activity.

CHAPTER II

GENERAL METHODS OF STUDY

1. ENUMERATION AND ISOLATION OF BACTERIA AND FUNGI

Bacteria and fungi present on barley were counted by Christensen's mould count method (Bottomley, Christensen and Geddes, 1952). Basically this involves using a Waring Blendor to comminute a known weight of seed in a known volume of diluent, and preparing a dilution series from the resulting suspension. The media used in the dilution plates were as follows:

(i) malt extract agar (Difco malt extract or 'Maltexo' 2.0%, agar 1.5%)

(ii) malt-salt agar (Bottomley, Christensen and Geddes, 1952) with 10% NaCl.

(iii) nutrient agar (Difco).

Colonies were counted and strains were isolated after the dilution plates had been incubated for at least ten days at 25°C.

Enumeration

A weakness of the mould count method is that the sizes of microbial populations are normally determined relative to a weight of seed. This was found to be particularly inappropriate when determining population changes resulting from increasing moisture content of the seed. If a particular

bacterium multiplied under these conditions, the increasing weight of the seeds resulted in a marked under-estimation of numbers compared with the same results expressed on a 'per seed' basis.

Microbial growth and interactions between individuals and populations take place on surfaces, and it is the surface area available for occupancy by microbial cells which is important in relation to the number of such cells. This is true even of a complex environment such as soil where it is technically impossible to estimate the total surface area. It is not impossible to do for seeds. Sources of error are certainly present:- it is difficult to accurately measure the surface area of an asymmetrical seed; and in the case of barley, microorganisms are commonly present in the sub-surface as well as the surface layers of the pericarp, while fungal hyphae may occasionally penetrate to the endosperm (Dickson, 1962). Nevertheless, an estimate of the numbers of bacteria per square millimetre of seed surface is a more meaningful figure than the number of bacteria per gram of seed. Even 'number per seed' is a better term than 'number per gram'.

The relationship between the terms is best illustrated by an example. One sample of stored barley with an average surface area of 57.68 ± 5.46 sq. mm. was found to have a bacterial population of 2.4×10^5 per gram. This is equivalent

to 9.5×10^4 bacteria per seed, or 1,647 per sq. mm. of surface. This calculation can be carried a step further by assuming that the area of seed actually occupied by bacteria is $9.5 \times 10^4 \times 2$ sq. μm (most of the bacteria were rods measuring approximately 1.0×2.0 μm). This is equivalent to only 0.3% of the total surface area of 5.8×10^7 sq. μm, so that what appeared to be a large population of bacteria in fact occupies an insignificant proportion of the area available to it.

As the appropriate measurements were not made in all cases, the colony counts were generally converted to 'no. per seed' in order to obtain uniformity of presentation. Where relevant, however, the 'no. per sq. mm.' was calculated and the percentage of the seed surface occupied was estimated.

The surface area per seed for any sample was calculated by using a micrometer caliper gauge to measure the length, and the greatest and least diameter at four points along the length, of 50 randomly selected seeds. The length x mean circumference was taken as an estimate of surface area.

Isolation

After counting, all bacterial colonies on one or more plates were 'picked' and streaked twice before culturing the inoculum for the characterization tests. This was done when the culture was 18 - 48 hours old, depending on the rate of growth of the isolate.

Except where indicated in the text, fungi were not isolated in pure culture but were identified directly from the dilution plates. The number of bacteria isolated from a particular sample varied from 30 - 100. It is clear that the fewer the isolates examined, the greater the chance of missing significantly large groups in the population. At the 95% level of probability, 100 isolates from a population will include at least one member from a group comprising 3.2% of this population. If only 20 isolates are seen, the size of 'detectable' group rises to 14.0% of the population. However, time and laboratory facilities are limited and the larger the number of isolates examined, the fewer the tests that can be carried out upon them. The result of this is that isolates differing in characters not tested for are grouped together i.e. the number of groups detected is inversely proportional to the number of characters tested for.

In a study of mixed bacterial types, such as is found on most naturally occurring substrates, a compromise has to be found between the conflicting demands of accuracy of identification of the isolates and accuracy of description of the population from which they were isolated. In the investigation of the changing microflora of the ripening seed, it was arbitrarily decided that a group of bacteria comprising 5% of the total population was likely to be

ecologically significant and too large to be overlooked. A minimum of 60 isolates was therefore made from any population examined, this being a large enough sample to detect groups comprising 4.8% of the population. In the studies of stored seed, where the microflora was assumed to be relatively static, and where only the major components were required to be identified, smaller samples of 30 - 35 isolates were taken. These were capable of detecting groups larger than 9.5% to 8.2% of the total at the 95% level of probability.

2. TESTS USED TO CHARACTERIZE BACTERIA

All isolates were not examined by the full range of tests described below; this is explicit in the text.

In general, cultures were grown in 1 oz. McCartney bottles ("Universal Containers") and the term "tube" as used below refers to such a container.

Unless otherwise stated, all incubation was at 25°C.

Morphological Characters

Cell Morphology

Wet mounts prepared from young cultures (12 - 30 hours) in nutrient broth were examined by phase contrast, and the same cultures re-examined when old (3 - 7 days).

Motility

Motility was recorded during the phase contrast examination of nutrient broth wet mounts.

Symplasmata

The formation of symplasmata (Graham and Hodgkiss, 1966) by some Erwinia isolates was also noted during the phase contrast examinations.

Gram Reaction

Air dried smears of 20 - 48 hour cultures (depending on rate of growth) were fixed and stained as detailed by Skerman (1967). If the result was doubtful, the test was repeated, when possible, using both younger and older cultures.

Flagellation

Flagellation of 24 hour cultures was determined using a Hitachi HS -7 electron microscope. A cell suspension of 10^9/ml in filtered distilled water was negatively stained with potassium phosphotungstate (Horne, 1965). The stained cells were mounted on carbon coated nitrocellulose grids and examined at a magnification of x9,000 - x12,000 diameters. (see Figure 1).

Acid Fastness

The Ziehl-Neelson method for staining acid-fast bacteria was used to stain smears of 20 - 48 hour cultures from nutrient agar (Harrigan and McCance, 1966).

Cultural Characters

Colour and Slime Formation

This was routinely observed on the glucose, yeast extract, $CaCO_3$ medium (GYCA) of Dye (1962). In some cases nutrient

agar + 5% glycerol was also used. Mucoid growth and pigment production were recorded after 2 - 4 days growth.

Production of Fluorescent Pigments

Production of diffusible fluorescent pigments was observed on medium B of King et al. (1954), but using tryptone (Difco) instead of proteose peptone. Plates were streaked and recorded after 2 - 4 days.

Tolerance of Sodium Chloride

This was determined in tubes of nutrient broth containing 0, 6, 8, and 10% NaCl. The tubes were examined for turbidity over a period of ten days.

Utilization of Inorganic Nitrogen

The ability to grow without organic nitrogen was tested in the synthetic medium of Ayres et al. (Dye, 1964). Tubes were examined for turbidity over a period of ten days.

Sensitivity to Polymyxin B

Sensitivity discs containing polymyxin B 300 units (Biolab) were placed on plates of nutrient agar seeded with the isolates under test. The presence of a zone of inhibition was taken to indicate sensitivity to this antibiotic.

Utilization of Carbon Compounds

Mode of Utilization of Glucose

This was determined by Hugh and Leifson's (1953) method as modified by Park and Holding (1966). Most of the work was done using 1 oz. McCartney bottles containing 15 ml. of medium. However, a considerable saving in media and incubator space was effected by using ¼ oz. ('bijou') McCartney bottles and only 5 ml. of medium. This was found to be quite satisfactory provided the test was read twice a day. A final recording of slow or inactive strains was made after four days.

Production of Acid from Carbon Sources

To begin with, this was observed in tubes of Phenol Red broth (Difco) containing 0.5% glucose, lactose, sucrose, salicin, inositol, rhamnose, mannitol, and adonitol. The pK' value of Phenol Red (7.8) was found to be too high to clearly differentiate the isolates however, and most of the work was done using the inorganic medium of Dowson (1957) with bromthymol blue (pK' 7.1) as an indicator. Sterilization was by autoclaving at 121°C for one minute. Cultures were examined for growth and acid production over a period of 30 days.

Degradation of Cellulose

Inoculated tubes containing filter paper strips in the cellulose mineral salts medium of Harrigan and McCance (1966) were examined for growth and degradation of the

filter paper over a period of 30 days.

Methyl Red and Voges-Proskauer Tests

The medium and the methods used were those described by Harrigan and McCance (1966), Barritt's modification of the V.P. test being chosen.

Utilization of Ethanol

The yeast extract - ethanol agar described by Carr (1968) was used, the tubes being examined periodically for 30 days for the acid production characteristic of acetic acid bacteria.

Biochemical Tests

Catalase Test

A loopful of growth from a nutrient agar slant was emulsified in a drop of 10 volume H_2O_2 on a clean glass slide and examined for the production of gas bubbles.

Oxidase Test

Kovac's method, as modified by Steel (1961) was used to determine oxidase production.

Gelatin Hydrolysis

Stab inoculations were made into nutrient gelatin (Difco) and the tubes incubated at room temperature. Growth and liquefaction of the medium were recorded periodically for 30 days.

Growth in Milk

This was observed in purple milk (Difco), sterilized by steaming on three successive days. Cultures were observed

periodically for 14 days and the isolates classed as

(i) proteolytic
(ii) acid producing
or (iii) having no effect on milk.

Reduction of Nitrate

This was tested by growing the bacteria in nitrate broth (Difco) for four days and testing for the presence of nitrite with sulphanilic acid and dimethyl - a - naphthylamine. Zinc dust was added to cultures giving a negative reaction to ensure that this was not due to reduction of nitrate beyond the nitrite stage.

H_2S Production

Stab inoculations were made into lead acetate agar (Difco) and the tubes examined periodically for 14 days. The method was not considered satisfactory in that several results were equivocal. As this test seemed unimportant in classifying the isolates however, it was discontinued and no other method was used.

Hydrolysis of Starch

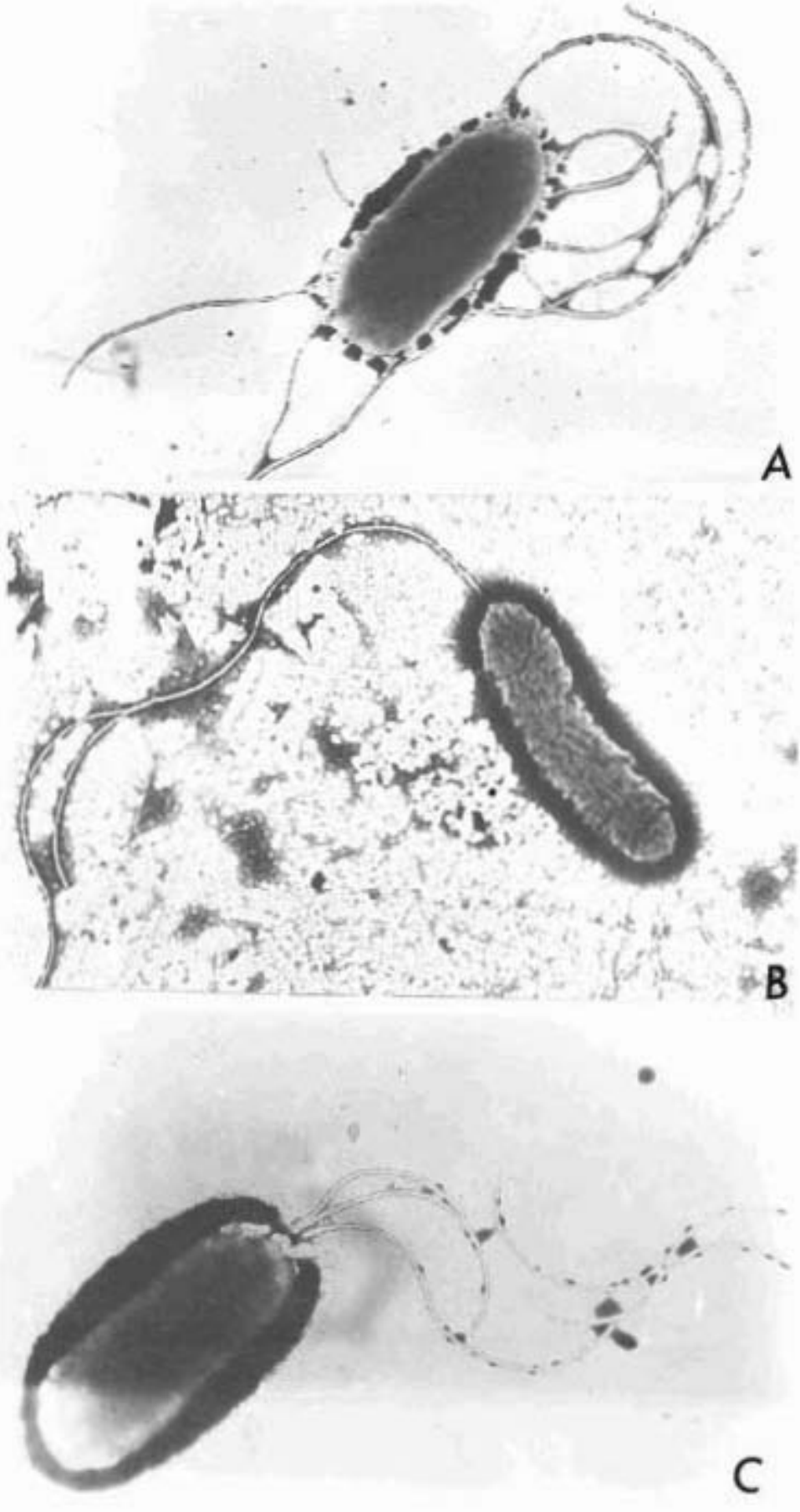
A medium containing (w/v); peptone (Bacto) 0.5%, beef extract (Oxoid) 0.3%, soluble starch (Difco) 0.2% and agar 1.5%, pH 7.0, was sterilized at 115°C for 10 minutes. Plates were inoculated by streaking, and hydrolysis detected after four days growth by flooding the surface of the agar with Lugol's iodine solution.

Figure 1 (facing). ELECTRON MICROGRAPHS OF ISOLATES WITH

A:- PERITRICHOUS FLAGELLA

B:- A SINGLE POLAR FLAGELLUM

C:- THREE POLAR FLAGELLA


A
B
C

3. USE OF THE COMPUTER TO GROUP BACTERIAL ISOLATES

As the number of tests used to characterize the bacterial isolates was limited by the large number of isolates being studied, it was hoped to use a simple determinative scheme such as that of Park and Holding (1966) to place the bacteria into genera or generic groups. As preliminary work had shown that yellow coliforms were common, a minimal series of tests to clearly differentiate Erwinia herbicola from other bacteria was devised. It then became apparent that although E. herbicola was ubiquitous on barley seed, it was not always dominant, and the tests used did not clearly separate the other bacteria commonly present. This was particularly true of the large number of isolates tentatively identified as 'coryneform'. These did not form recognizable groups but rather a spectrum of strains from those which approached Flavobacterium (as defined by Hendrie et al., 1968) to those which were clearly Arthrobacter.

In an attempt to obtain the maximum information from the data available it was decided to use the methods of numerical taxonomy to group the isolates, and then to further characterize selected isolates from within the groups.

The data from each experiment were therefore analysed by computing association coefficients for each isolate, using the formula of Sokal and Mitchener (1958) viz.

$$S = \frac{m}{m + u}$$

where m= number of matched characters (positive or negative) and u= number of characters positive in one strain and negative in the other.

Clustering of isolates was then carried out by the single linkage method of Sneath (1957). In order to avoid the premature linking of quite dissimilar groups, however, it was necessary to recalculate the mean similarity values within groups and between groups after each computational cycle. This was done by computing $\triangle S$, the mean of the triangular matrix, as recommended by Sokal and Sneath (1963).

All computations were made on an IBM 360/44 system using a programme especially written by Mr F.L. Ng. This programme is deposited in the Botany Department of the University of Canterbury.

It should be stressed that the use of a computer to group isolates in this way does not necessarily constitute numerical taxonomy. In the present study, there was no intention of constructing a taxonomic classification of the isolates. Indeed, with the limited number of characters available for comparison (20 - 25 in different experiments) this could not have been done. Although there has been no unequivocal answer to the question of how many characters should be considered in a numerical taxonomic study, the suggestion of Mitchener and Sokal (1957) that the minimum be

not less than 60 has been widely accepted (Sokal and Sneath, 1963; Davis and Newton, 1969; Tsukamura, 1969), although Rovira and Brisbane (1967) used only 37 tests in a study of rhizosphere bacteria. Mitchener and Sokal's figure is based on the premise that association coefficients are based on a proportion of matched characters out of a total of all possible matches, and that the reliability of the estimates of similarity increases as the sample size (number of characters) increases.

In the present study, the basic computational techniques of numerical taxonomy were used when an edge-punched card system failed to make most use of the cumbersome data that accumulate when over 1,500 isolates are characterized. Any other form of classification necessarily involved the use of a dichotomous key, and although such a scheme was suitable for some groups of isolates such as the pseudomonads and coliforms, the end result was that over half the isolates were left in an amorphous and extremely variable 'coryneform' group which could not be further divided on accepted taxonomic grounds with the data available.

The only logical way of sub-dividing this group was to set up a matrix of all the characters of all the isolates, and calculate their association coefficients. With under 30 character states to compare, this might not result in taxonomically valid units (this was obvious in some cases)

but it did produce clusters of isolates which were recognizable in different experiments and which could be given a meaningful description within the limitations of the tests used to characterize them. Moreover, the computer analysis did not result in the splitting of any recognizable taxonomic unit; pseudomonads were grouped with pseudomonads and arthrobacters with arthrobacters.

CHAPTER III

ESTABLISHMENT OF THE BARLEY SEED MICROFLORA

1. EXPERIMENTAL DESIGN AND METHODS

The bacteria and other microorganisms present on seed as it comes into store are thought to be derived from populations which have been active on the maturing grain in the field (Semeniuk, 1954). This has been investigated only in the case of fungi, however, and in one of the few studies made, Hyde and Galleymore (1951) concluded that the sub-epidermal mycelium in wheat seed may arise either from spores and hyphae present on the outside of the developing grains or from a systemic mycelium similar to that described for seeds of Lolium.

The present study was undertaken primarily to determine whether or not the characteristic and restricted microflora of harvested barley was derived from a more varied microflora on the immature seed. The establishment and development of microorganisms on the seed was therefore followed in a plot of Kenia barley sown in the University Botanic Garden. The plot measured 11 ft. x 21 ft. and was hand sown in spring (September) in 8 in. double rows 18 in. apart. It was in an open grassed area about 100 yards from a field of wheat. No fertilisers were applied.

The numbers and types of microorganisms on the seeds were determined at five stages of maturity (as described by Bergal and Clemencet, 1962). These were:

1. awns emergence (stage B) on 18 December
2. straight ear (stage E) on 3 January
3. arcuate ear (stage A) on 16 January
4. late milk stage (stage J) on 30 January
5. field ripe (stage R) on 7 February

When most of the crop had ripened to the desired stage of maturity, 25 heads were collected at random and immediately processed in the laboratory. Individual seeds were pulled from the heads, taking care to maintain asepsis. The number of seeds required to give 5 g fresh weight was determined, and these were comminuted in a Waring Blendor. Duplicate mould count dilution series were then set up, using nutrient agar and malt extract agar.

Another 5 g sample was used to determine the moisture content of the seed.

As the mould count method gives no indication of the number of contaminated seeds in a sample, nor the distribution of microorganisms on these seeds, spore prints were made of seeds from the first three stages of ripening. This was done by pressing 100-150 seeds onto the surface of nutrient agar in large (22cm. diam.) petri dishes. All the seeds in one dish were from the same head. The position of the seeds was marked and after two hours they were removed and discarded. The flag leaves from the same plants were also 'printed' in the same dishes. This was done to see if the phyllosphere

microflora was the source of inoculum for that which developed on the seed.

2. SEED MOISTURE DETERMINATIONS

The moisture content of the seed during the period of ripening, and the rainfall during this period at the University Botanic Garden, are shown in Figure 2. The weather had been sunny and dry for several weeks before this period, and the barley ripened 2 - 3 weeks earlier than is normal for a September sown crop in Canterbury.

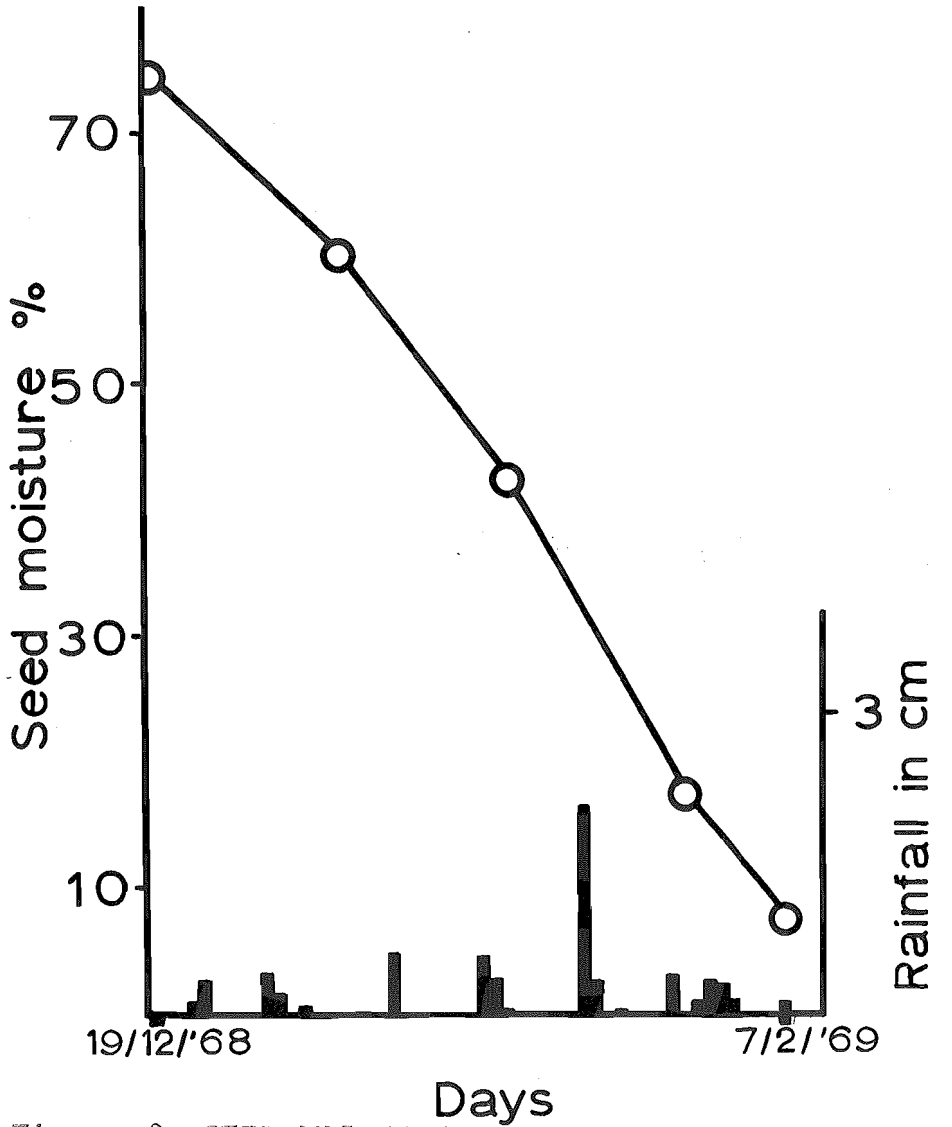


Figure 2. SEED MOISTURE CONTENT AND RAINFALL DURING MATURATION OF THE SEED

3. IDENTIFICATION OF THE BACTERIA

Numbers were so low at the "awns emergence" stage that all 39 colonies developing at the lowest dilution were isolated. In all other cases, 70 - 90 bacteria were isolated and tested for the following characters.

(i) cell morphology and motility
(ii) gram reaction
(iii) pigmentation and slime formation
(iv) mode of utilization of glucose
(v) production of acid from lactose
sucrose
salicin
inositol
rhamnose
(vi) catalase production
(vii) effect on milk
(viii) gelatin hydrolysis
(ix) reduction of nitrate.

The results of these tests, which are shown in Table VIII, were used to group all the isolates by computer analysis. A dendrogram constructed from the similarity indices of the 300 isolates is shown in Figure 3. The similarity index (S.I.) of any linkage is the mean of the association coefficients of the linking isolates x 100 to give the percentage of matching characters. The best division of the isolates was obtained by clustering at the 70-phenon line i.e. by choosing groups of isolates with at least 70% of their tested characters in common. There were nine such groups, and further tests were carried out on selected

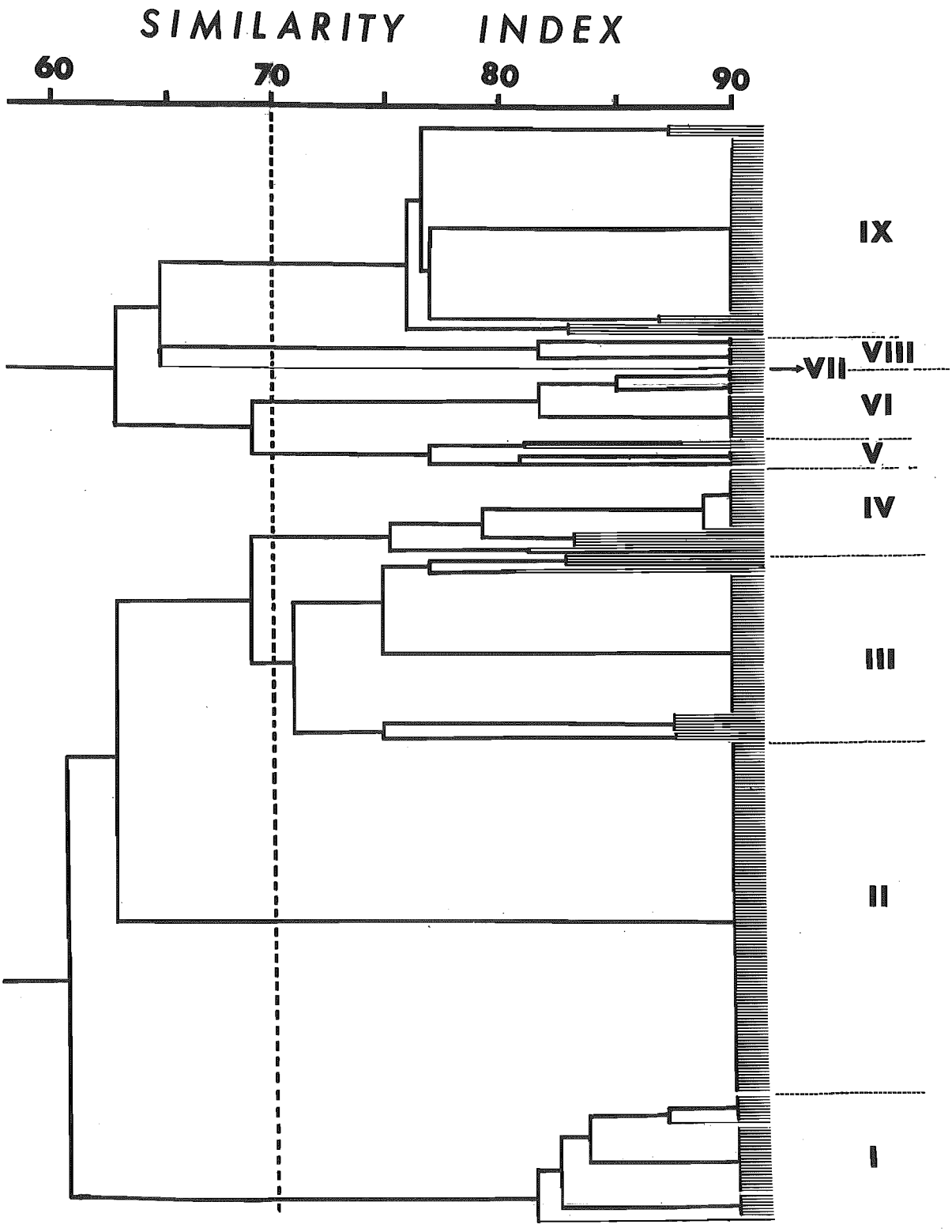


Figure 3. DENDROGRAM SHOWING RELATIONSHIPS OF BACTERIA ISOLATED FROM RIPENING BARLEY

isolates from within these groups as described below.

Group I (pseudomonads)

This group of 42 isolates was quite distinct, and on the basis of the computer analysis it was also moderately homogenous. As some of the isolates appeared to be pseudomonads on the basis of the routine tests, additional evidence was obtained by determining the flagellation of 20 random isolates, and testing these for pigment production in King's medium B, sensitivity to polymyxin B, and oxidase reaction.

All isolates were gram-negative, motile, oxidative, catalase-positive short rods which did not reduce nitrates. Most peptonized milk (34/42 tested), did not produce acid from lactose (39/42), were polarly flagellated (16/20) and liquefied nutrient gelatin (40/42), sometimes rapidly (19 isolates gave positive results within 24 hours). The other tests, some of which have been used by other workers to characterize Pseudomonas, gave variable results e.g. only 10/20 isolates tested produced a water soluble pigment in King's medium B, only 13/20 were sensitive to polymyxin B, and only 7/20 gave a positive reaction to the oxidase test.

The term pseudomonad, as applied to this group, should therefore be interpreted in the widest possible sense to mean a group of isolates including strains of Pseudomonas and other strains more similar to Pseudomonas than to the

other groups described.

Group II (*Erwinia herbicola*)

As discussed later, the 112 members of this homogenous group (S.I. 93%) appeared to be close to *Erwinia herbicola*. Additional evidence was obtained by choosing 30 isolates at random and determining their flagellation, NaCl tolerance, growth in Ayre's medium, starch hydrolysis and reaction in the oxidase test.

All isolates were gram-negative, motile, fermentative, catalase-positive short rods forming chains of cells in young nutrient broth cultures. Flagellation was peritrichous (30/30), the number of flagella varying from 1 - 8. Colonies on GYCA were yellow, varying from pale yellow to bright yellow, and from 1 - 6 mm. in diameter. Nitrates were reduced to nitrites, growth was positive on Ayre's medium and in nutrient broth containing 8% NaCl; H_2S was not produced and starch was not hydrolysed. All 30 isolates tested were oxidase negative.

Most isolates produced acid from sucrose (100/112), salicin (90/112), inositol (88/112) and rhamnose (91/112), but not from lactose (20/112). Acid was also commonly produced in milk (104/112). Most liquefied gelatin slowly (100/112) taking 7-21 days to produce any noticeable effect: the remainder produced no liquefaction in 30 days.

Mucoid growth was recorded for 33 of the 112 isolates

and 13/30 grew in nutrient broth + 10% NaCl. Symplasmata were noted in 21/112 isolates but the 'biconvex bodies' described by Graham and Hodgkiss (1967) were not seen.

Group III (flavobacteria)

This group of 56 isolates comprised 47 strains of yellow-pigmented gram-negative rods which produced no acid in the Hugh and Leifson test, and are therefore most conveniently placed in Flavobacterium. The remaining isolates were five non-pigmented strains identified as Alcaligenes by the scheme of Park and Holding (1966), and four gram-negative but coryneform isolates producing pink colonies on GYCA. No additional tests were carried out on members of this group.

All isolates were gram-negative, catalase-positive, motile short rods producing no acid in Hugh and Leifson's medium with glucose. Two of the yellow isolates produced chains of cells in young nutrient broth cultures. No mucoid or spreading growth was seen, the colonies being circular, entire and butyrous. Reaction in milk, nitrate broth, and acid production from carbohydrates was variable, but most isolates produced acid from lactose (40/56) and sucrose (42/56), and most isolates liquefied gelatin (48/56).

Group IV (coryneform)

The 27 isolates in this group were catalase-positive motile short rods which were gram-positive, although most of them (23/27) were only weakly so. They did not exhibit the 'snapping' division typical of corynebacteria. One isolate was oxidative, the others giving no reaction in Hugh and Leifson's medium with glucose. All isolates produced acid from lactose, sucrose, salicin and rhamnose; and most (23/27) produced acid from inositol, and gave an acid reaction in milk. Gelatin was liquefied slowly (no reaction in 7 days) by most isolates (23/27).

One strain produced cream coloured colonies on GYCA; the remainder were yellow. Mucoid growth was not seen.

Seven of the 27 strains were tested and found to be not acid fast.

Group V (coryneform)

This was a small group of 9 gram-positive, catalase-positive, fermentative, motile rods. Pigmentation was varied, two being unpigmented, two giving cream coloured colonies, two yellow and three orange. Two isolates exhibited 'snapping' cell division.

No acid was produced from carbohydrates except that the only isolate tested in mannitol gave a positive reaction in that medium. Gelatin was hydrolysed by 4/9 isolates, but starch was not hydrolysed by the two isolates tested. These

two were also shown not to be acid fast.

Group VI (cocci)

All 21 cocci isolated fell into this group, which was readily sub-divided into 8 catalase-positive Micrococcus strains, and 13 catalase-negative isolates which may have been lactic acid bacteria. The latter were found only at the 'awns emergence' stage of maturity. They were gram positive, slowly fermentative in Hugh and Leifson's medium, and occurred as single cells or in short chains. Colonies on GYCA were small ($<$ 0.5 mm) and unpigmented or orange. The Micrococcus isolates were similar morphologically and in pigmentation. They produced larger surface colonies however and did not utilize glucose.

Group VII

This single isolate was a gram-variable, catalase-negative, non-motile, fermentative short rod. Cells occurred singly or in rafts with no evidence of 'snapping' division. Acid was produced from lactose and sucrose but not from salicin, inositol or rhamnose. Gelatin was not liquefied, but nitrates were reduced and there was a slightly acid reaction in milk. Colonies on GYCA were pink and mucoid.

Group VIII (coryneform)

This group of nine isolates was comprised of gram-

positive, oxidative, catalase-positive, non-motile coccoid rods. They produced acid from sucrose but not from lactose, salicin, inositol or rhamnose. Gelatin was not hydrolysed and nitrates not reduced. An alkaline reaction was produced in milk by five isolates, the remainder having no effect.

Colonies were orange on GYCA, and 7/9 were mucoid. Two strains grown on nutrient glycerol agar were pink in colour, and these strains were not acid fast.

Group IX (coryneform)

All isolates in this group were gram-positive (34/68) or gram-variable (34/68), catalase-positive rods which did not utilize glucose in Hugh and Leifson's medium. One third (23/68) were pleomorphic in that young cultures showed an appreciable variation in cell size and a tendency to filament formation, but only six were identified as Arthrobacter. These formed cocci in older cultures, and cystites (as described by Stevenson, 1963) were seen in two cases. The only streptomycete isolated was included in this group by the computer analysis. The marked palisade formation and V's typical of Corynebacterium were noted in 14/68 cultures.

Most isolates were non-motile (56/68); none produced acid from lactose or salicin, and all did so from sucrose and inositol. Of 15 isolates tested in mannitol and

adonitol, 13 produced acid in both media. Most isolates were proteolytic in milk (58/68) and slowly liquefied gelatin (49/68), but few reduced nitrate (11/68).

Of 15 isolates tested further, none were acid fast; none hydrolysed starch or degraded cellulose; all were oxidase negative and MR and V-P negative, and most (13/15) were not sensitive to polymyxin B.

Colony form and pigmentation varied considerably. On GYCA non-pigmented forms were commonest (28/68); others were described as cream (2), orange (18), orange-brown (3), pink (4) and yellow (13). Mucoid growth was noted in nine cases.

4. THE MICROFLORA OF RIPENING GRAIN

(i) 'B' Stage. The mould count method showed a population of 14.3 bacteria and 3.3 fungi per seed. All fungi were identified as Cladosporium sp. The bacteria were classified as

	No. per seed
Group III (flavobacteria)	1.5
Group V (coryneform)	1.5
Group VI (cocci)	6.2
Group IX (coryneform)	5.1

Results obtained by the spore print method are summarized in Table I.

TABLE I. Number of Seeds Infected with Microorganisms

'B' Stage Sample

Total No. Seeds	Sterile Seeds	No. with 1-3 bacteria	No. with 4-10 bacteria	No. with >10 bacteria	No. infected by fungi
118	90	16	2	5*	6**
(24 heads)	76.3%	13.6%	1.7%	4.2%	5.1%

* 4 were from same head

** 3 were from same head

The flag leaves examined all had a sparse population of mixed bacterial types (as judged by colony appearance) including Bacillus mycoides.

(ii) 'E' Stage. The dominant component of the microflora at this stage consisted of pink yeasts tentatively identified as Rhodotorula sp. on morphological criteria. They were not seen to produce ascospores, and the cell and colony appearance agreed with the description of R. glutinis (Fres.) Harrison given by Lodder and van Rij (1967). The number of these yeasts, as judged by the mould count on malt agar, was 1.1×10^4 per seed.

The bacteria present numbered 194 per seed and were classified as follows:

	No. per seed
Group II (E. herbicola)	64
Group III (flavobacteria)	4
Group VIII (coryneform)	1
Group IX (coryneform)	3

Cladosporium was the only filamentous fungus seen in the mould count plates, and its count had increased to 2,378 per seed.

The spore print plates showed that all 171 seeds examined were infected by both Rhodotorula and Cladosporium, and that at least 120 also carried bacteria; yellow and unpigmented types were equally common. The microflora of the flag leaves was dominated by the same organisms. In two cases, the leaves were left on the surface of the agar during the five days incubation. In both cases, Alternaria tenuis, Stemphylium sp. and Chaetomium sp. were identified in addition to the organisms noted above.

(iii) 'A' Stage. The Rhodotorula count was now lower than that for bacteria, the relative populations being estimated as 2.1×10^5 and 3.3×10^5 per seed respectively. The bacteria were classified as

	No. per seed
Group I (pseudomonads)	22,000
Group III (flavobacteria)	136,500
Group IV (coryneform)	77,500
Group V (coryneform)	18,500
Group VIII (coryneform)	3,500
Group IX (coryneform)	74,000

The mould count of Cladosporium had increased to 15,500 per seed.

Only 50 seeds from ten heads were examined by the spore

print method at this stage, a like number being left on the agar plates during incubation. The flag leaves had dried off by this time and were not examined.

The same types of microorganisms were found by both methods viz. Rhodotorula, Cladosporium and bacteria.

(iv) 'J' Stage. The number of Rhodotorula cells found had dropped to 1.7×10^5 per seed, while bacterial numbers reached a peak of 2.7×10^6 per seed. The bacteria present were:

	No. per seed
Group I (pseudomonads)	129,500
Group II (E. herbicola)	1,210,000
Group III (flavobacteria)	389,000
Group IV (coryneform)	86,500
Group VI (cocci)	43,000
Group IX (coryneform)	864,000

A reliable estimate of the numbers and types of moulds could not be made because of the very large numbers of bacteria present at suitable dilutions.

(v) 'R' Stage. The mould count gave an estimated Rhodotorula population of 6.3×10^4 per seed, and a total bacterial count of 5.7×10^5 per seed. The bacteria present were:

	No. per seed
Group I (pseudomonads)	234,000
Group II (E. herbicola)	158,500
Group III (flavobacteria)	8,500

	No. per seed
Group VI (cocci)	8,500
Group VII (coryneform)	8,500
Group VIII (coryneform)	58,500
Group IX (coryneform)	92,000

The mould count of Cladosporium, which was still the only filamentous fungus detectable by this method, was 30,000 per seed.

Fluctuations in numbers of the main microbial types found by the mould count are illustrated in Fig. 4.

5. DISCUSSION

Until recently, there was much confusion regarding the identity of the gram-negative, yellow-pigmented, fermentative rods commonly found associated with plants. This was resolved when Dye (1964) showed that these bacteria were peritrichously flagellated and best considered as Erwinia herbicola. The current (7th) edition of Bergey's Manual (Breed et al., 1957) restricts the genus Erwinia to plant pathogens, but further studies of E. herbicola by Graham and Hodgkiss (1967) and Komagata et al. (1968) confirmed Dye's identification. Recently, Dye (1969) has proposed a 'herbicola' group of organisms to embrace E. herbicola var. herbicola (the common yellow saprophyte) and three other closely related bacteria.

The bacteria in Group II which were given additional tests can be identified as members of this 'herbicola' group.

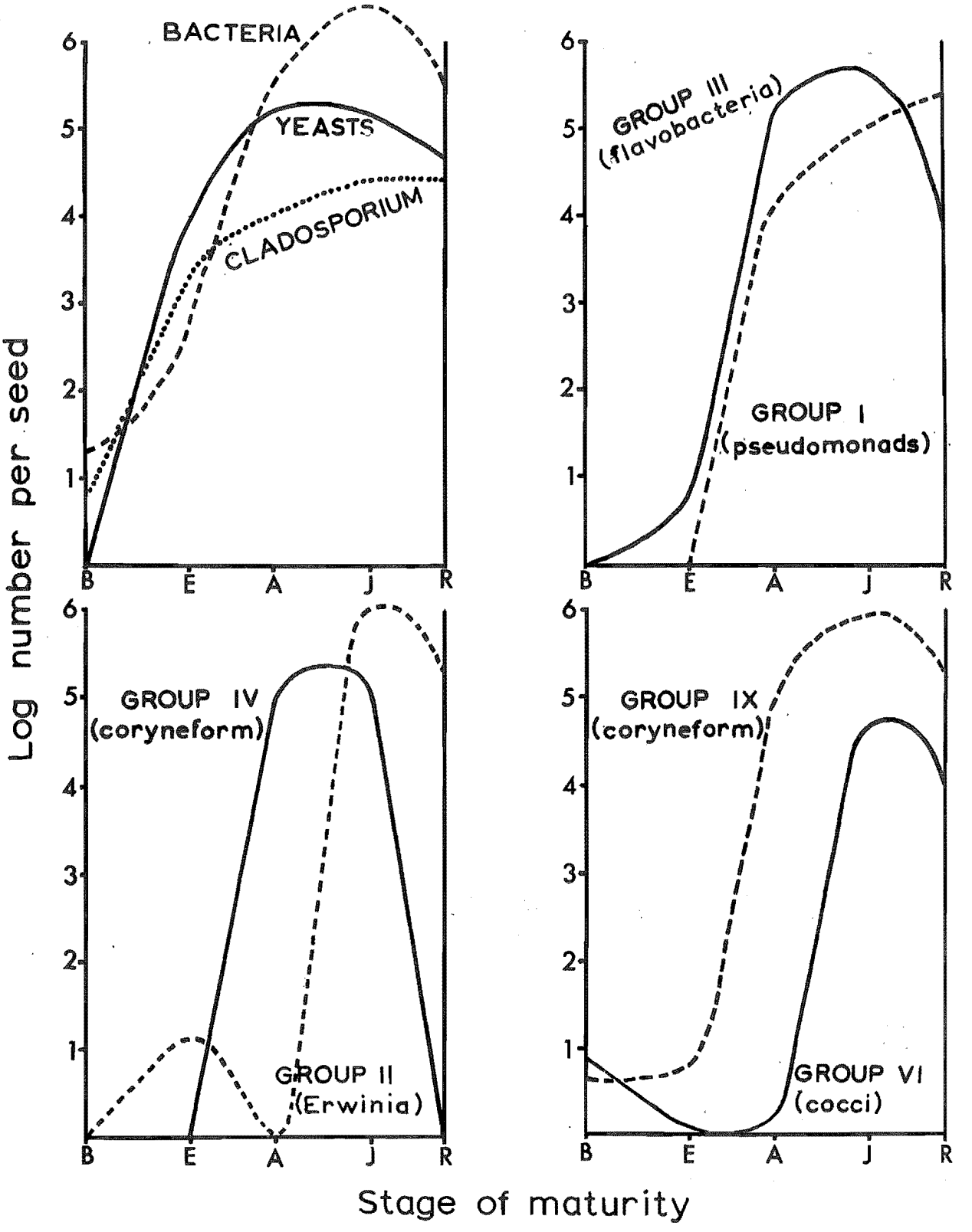


Figure 4. MICROBIAL POPULATIONS ON RIPENING BARLEY

As gram-negative, peritrichously flagellated, fermentative rods they are claased in the Enterobacteriaceae, and all the other characters ascribed to the Group II bacteria agree with those recorded for the E. herbicola group by one or other of the workers mentioned above. There is no other recognized group in which they could be placed.

Some slight doubt remains about the identity of the isolates which were not examined for flagellation. Occasional yellow-pigmented strains of Vibrio or Aeromonas are found which can only be positively separated from enterobacteria be determining the type of flagellation (Bain and Shewan, 1968). Because of the close similarity of all the strains in Group II, it is unlikely that Vibrio or Aeromonas were included in this group, but their absence is not proven by the data available.

The bacteria in Group IX constituted the third most numerous group on the ripe grain. With the exception of the single streptomycete isolate, they would key out as coryneforms in a scheme such as that devised by Harrigan and McCance (1966), and on the basis of the tests carried out cannot be described otherwise. Yet it must be recognized that most of them did not show the 'snapping division' which Robinson (1968) described as the one feature common to organisms of the Corynebacteriaceae, and the group as a whole was obviously heterogenous.

Corynebacteria are characteristic of the autochthonous

soil microflora, and to this extent these results show a less specialized epiphytic microflora than that reported by other workers. Wallace and Lochhead (1951), after examining bacteria from seeds of six different crop plants (including wheat and oats) stressed the virtual absence of gram-positive bacteria, and Verona (1963) is even more specific:

> "... sur la graine il est rare de rencontrer des especes... pleomorphiques du type Corynebacterium."

Studies of phyllosphere bacteria from ryegrass by Stout (1960) and from barley by Diem (1967) also suggest a preponderance of gram-negative types on plant surfaces. Coryneform bacteria were however described as dominant on herbage cut for silage by Gibson et al. (1958).

Pelhate (1968), discussing interactions of storage moulds on wheat, states that a "premier occupant" tends to maintain its supremacy because its extracellular enzymes render the substrate unsuitable for other organisms. This may well be true of seed in store, but in the field the physical and chemical nature of the substrate is constantly changing as a result of the ripening process, and it is almost axiomatic that conditions favouring the supremacy of a particular organism will not persist for long. It is therefore not surprising that a succession of different microorganisms occurred. At the 'B' or 'awns emergence' stage of development, the immature barley seeds were not

quite sterile. As judged by the mould count method they carried a population of 14.3 bacteria and 3.3 fungi (Cladosporium) per seed. The spore print method clearly showed that this was not a sparse, evenly distributed population but that nearly all the bacteria were present on only 5.9% of the seed, and all the fungi on 5.1%.

Reference to Figure 4 shows that yeasts were the first colonizer of the immature seed and that they dominated the microflora until the late milk (J) stage. If the average size of a Rhodotorula cell is taken as 20 sq. μm then this organism occupied over 7% of the total surface area of the seed at the peak of its development. Large populations of this same yeast were found by Di Menna (1959) on rye grass/clover foliage in New Zealand.

As the yeast growth stopped, there was a second wave of colonization, this time by bacteria, and especially E. herbicola. This resulted in even greater occupancy of the seed surface, the bacteria alone accounting for 9.4% of the total area, and the microflora as a whole occupying at least 15% and possibly 20%, depending on how one interprets the Cladosporium counts. This population explosion took place during a fortnight in which the seed dried out from a moisture content of 42.6% to one of 17.5%. The only heavy rainfall recorded occurred during this period, however, and this may have resulted in longer periods when free water was available for bacterial movement. It is also possible

that the seed is particularly suitable for microbial growth at this stage of maturation. Hyde and Galleymore (1951) noted maximum development of field fungi on wheat at the stage when the moisture content of the grain was falling rapidly, and Ponchet (1966) stated that "the yellowing stage is the starting signal for the rapid colonization of the pericarp and of the phenomena of competition that accompany it."

The final stage of the succession took place during the final week of ripening, when the seed moisture fell to the very low level of 7.9%. During this time the pseudomonads were the only significant group to increase in number. Populations of the other groups decreased to such an extent that the pseudomonads were in fact the most numerous group on the ripe grain in spite of the fact that they were not detected on it until three weeks previously.

The microflora of the mature grain consisted mainly of pseudomonads, E. herbicola, corynebacteria of Group IX, Rhodotorula and Cladosporium. As the immature seed at emergence from the ear was almost sterile, and as microbial growth had presumably stopped by the time of the final sampling, the organisms listed above must have been present in large numbers because

a) conditions at some stage during ripening had been suitable for their multiplication to high levels;

and b) they were capable of surviving when conditions became unsuitable.

Other organisms isolated during the ripening process failed to meet one or other of these requirements. Cocci, for instance, found conditions suitable for multiplication between the 'A' and 'J' stages of seed maturation, but they did not increase as rapidly as did the Erwinia group during this period and never attained a dominant position in the ecosystem. The corynebacteria of Group IV on the other hand, did form a substantial part of the total population in the earlier stages of maturation, but had completely disappeared by the time the grain ripened. This was presumably because they were unable to remain viable under the environmental conditions prevailing on the seed after it reached the 'J' stage.

It is difficult to estimate the significance of Cladosporium to the development of the seed microflora. The mould count method estimates the number of fungal spores (Bottomley, Christensen and Geddes, 1952) and this is not necessarily related to activity. Nevertheless, the enormous increase in the count of this fungus between stages 'E' and 'A' must have been correlated with extensive mycelial growth and colonization of the seeds. This conclusion is supported by the fact that practically every seed plated on agar or 'printed' on agar was infected.

Fokkema (1968), studying the growth of Cladosporium herbarum on rye leaves, concluded that the pollen grains of the host plant were an important nutrient source for this

fungus, resulting in rapid increases in population shortly after flowering. He also noted that the mycelium of the fungus could be readily washed from the leaves, indicating that it was entirely superficial. Flowering of barley occurs one to four days after emergence of the ear (Bergal and Clemencet, 1962) i.e. shortly before the second sampling was done in this experiment, and although no direct confirmatory evidence was obtained, Fokkema's hypothesis would explain the predominance of Cladosporium in the early stages of ripening. It would also explain why Alternaria tenuis was not detected by the mould count method in spite of the fact that A. tenuis, and not Cladosporium, is the characteristic 'field fungus' found in stored cereals (Christensen, 1957; Perera, 1966). The heavily sporulating but superficial Cladosporium would make the detection of Alternaria difficult both in 'spore prints' and dilution plates. If the former fungus was growing on pollen rather than on the seed coat, however, it would not be so apparent on stored seed.

As mentioned previously, the aims of this work were twofold: to confirm earlier work describing a characteristic bacterial flora on barley seed; and to determine to what extent the bacteria were capable of multiplying on seed in store, and thus of playing a role in the microbial invasion and deterioration of the grain. The work so far described appears to confirm the existence of numerous

Pg 47 missing

bacteria adapted to growth on the ripening seed and capable of surviving on the ripe grain. This work was, however, carried out on one small plot of barley, and this limits the value of the results obtained. If a survey of stored grain showed the same kinds of organisms to be commonly present, one would have greater confidence in the results obtained from this study of the development of the seed microflora. Such a survey forms the subject of the next chapter.

CHAPTER IV

THE MICROFLORA OF STORED BARLEY

1. EXPERIMENTAL DESIGNS AND METHODS

The numbers and types of bacteria on stored barley were assessed by testing samples of grain from sacks stored in the yard of the Canterbury Malting Company at Heathcote. Samples of seed were transferred to new polyethylene bags by means of a sack sampler, and these were immediately taken to the laboratory. Mould count dilution series were prepared as before, using nutrient agar.

A total of 23 different sacks was sampled in this way; one in February containing freshly harvested seed, 12 in July, and 10 in November after nine months storage. The origin of the grain could not be identified in every case but seed of both the commonly grown varieties (Kenia and Research) was tested and two of the samples were known to have come from as far afield as Blenheim and North Otago.

In the case of the February sample, 70 bacteria were isolated from the dilution plates, and 30 - 35 from each of the July and November samples. The tests used to group the isolates in the latter two series were the same as those noted previously (p. 27) except that acid production was tested in different carbohydrates. Inositol and rhamnose were not used, but mannitol was substituted in the July series. In February, isolates were routinely tested for

H_2S production, starch hydrolysis, tolerance to NaCl, and growth in Ayre's medium, in addition to the tests listed on p. 27.

2. IDENTIFICATION OF BACTERIA

A. FEBRUARY

Table IX (Appendix) shows the results of the tests used to characterize these isolates. They were readily identified without computer analysis, more than 78% being Erwinia herbicola (Group II), and all but two of the remainder Pseudomonas sp. (Group I).

B. JULY

The results of the tests were used to group the 343 isolates by computer analysis, and a dendrogram illustrating their relationships is shown in Figure 5. Nine groups were again selected, this time by clustering at the 69 - phenon line. Table X shows the characteristics of these groups as judged by the routine tests.

Additional tests carried out on selected isolates gave the following results.

Additional Tests (Number of positive results)

Group I (10 isolates)		Group II (20 isolates)	
polar flagellation:	9	peritrichous flagellation:	20
water soluble pigment:	7	Group IIj (1 isolate)	
acid from ethanol:	0	acid from ethanol:	0
		Group X (3 isolates)	
		acid from ethanol:	0

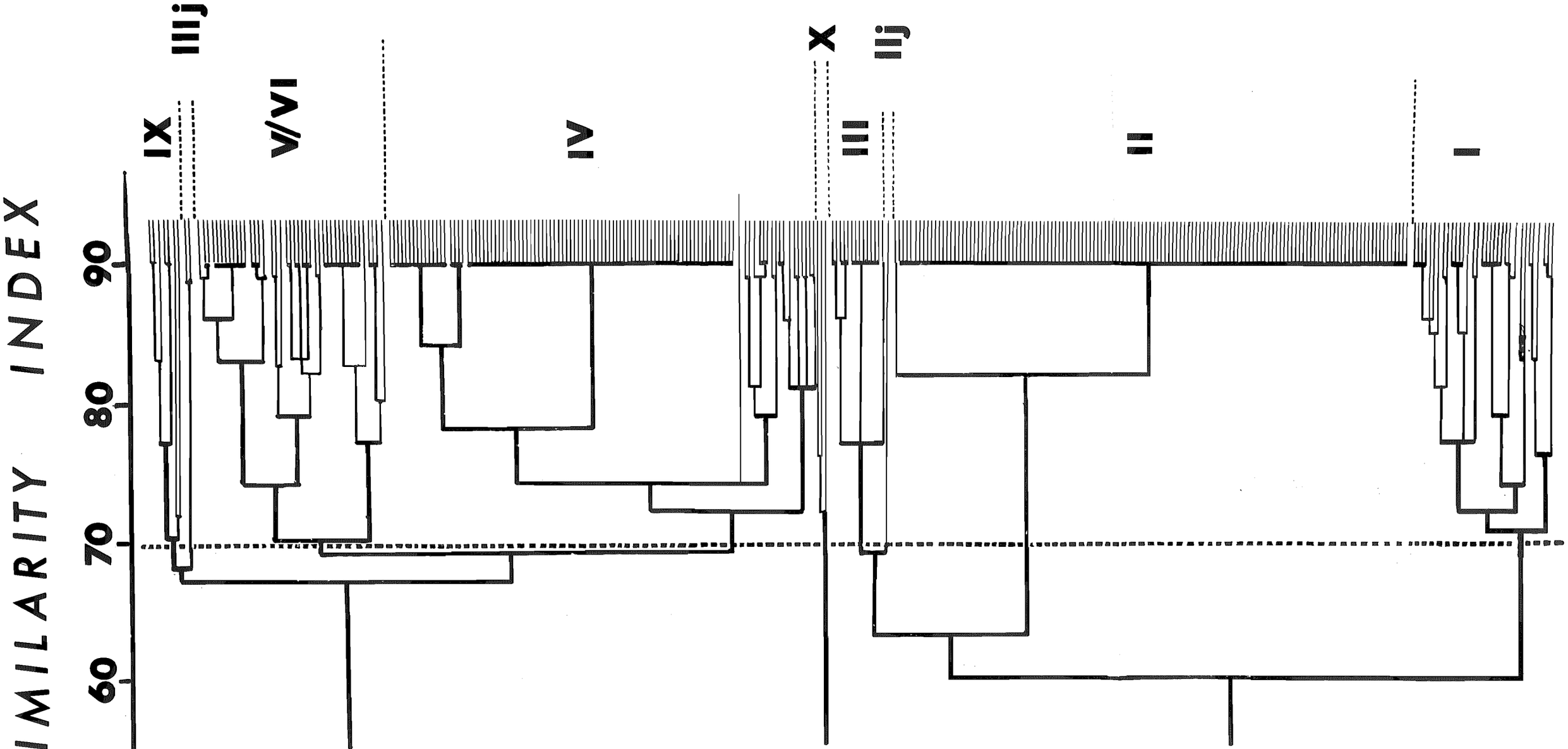


Figure 5. DENDROGRAM SHOWING RELATIONSHIPS OF BACTERIA ISOLATED FROM STORE: JULY

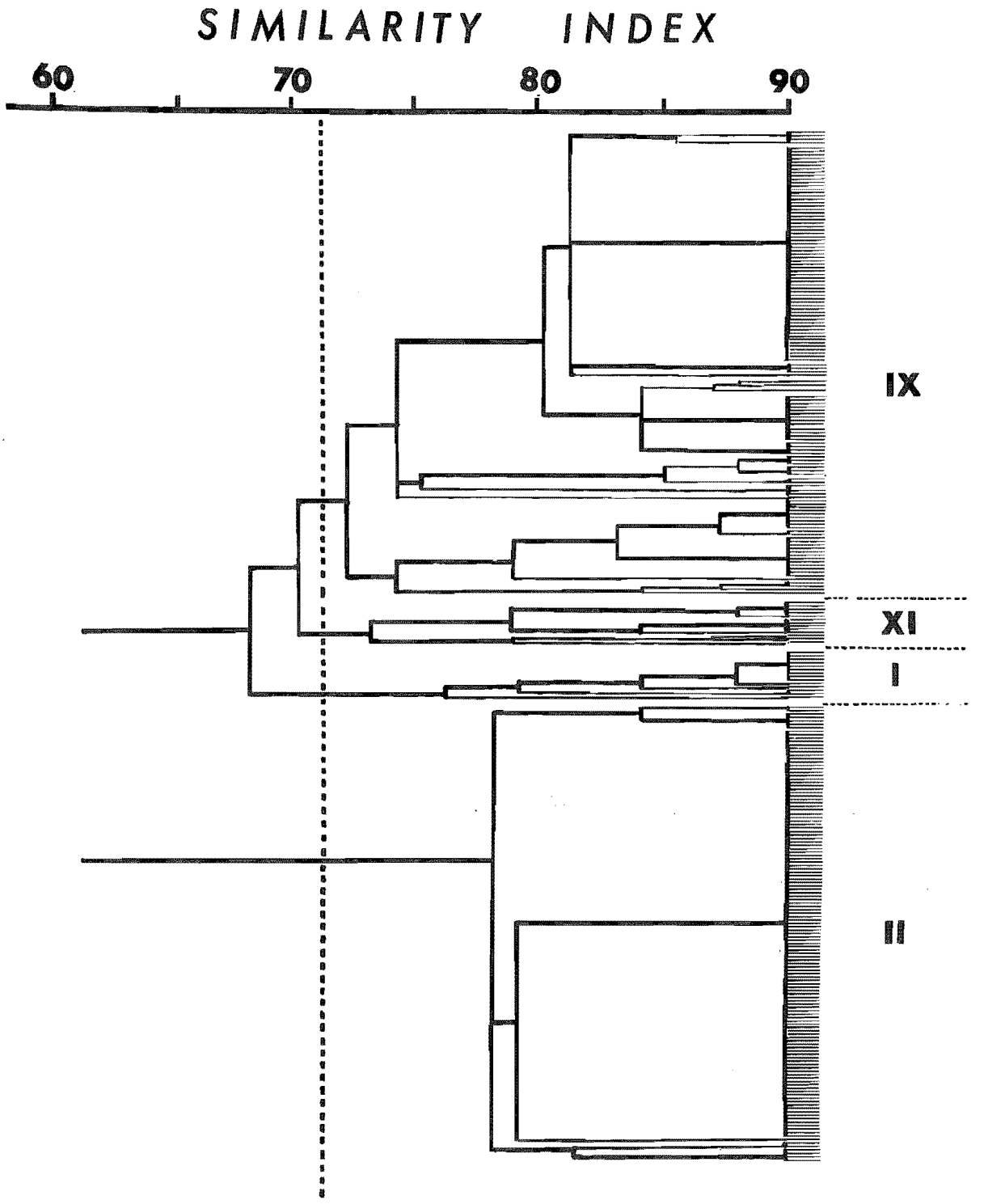


Figure 6. DENDROGRAM SHOWING RELATIONSHIPS OF BACTERIA ISOLATED FROM STORE: NOVEMBER

Table X shows that although Groups I, II, III and IV were similar to the previously described groups with the same numbers, this was not true of the remaining groups. The small groups designated IIj, IIIj and X were 'new' in the sense that the isolates were unlike any found on grain from the field plot, while V, VI and IX were equated with the previous groups so numbered in spite of major differences which are summarized below.

V: Four of the 43 isolates were the same as those already described but the group as a whole was much more variable e.g. oxidative and fermentative strains were present as well as those not utilizing glucose in Hugh and Leifson's medium. Moreover, the July group linked with the four cocci isolated at a relatively high S.I.

VI: The cocci were all Micrococcus and linked to gram-positive rods in Group V as noted above.

IX: All seven isolates in this group were identified as Arthrobacter or Nocardia. These would have been included in the previous group IX, but the latter was a larger and more heterogenous group.

C. NOVEMBER

The 295 isolates were grouped by computer analysis as before and a dendrogram illustrating their relationships

is shown in Fig. 6. By clustering at the 71 - phenon line, four groups were obtained, three of them similar to previous groups I, II and IX, and the fourth a group of coryneforms unlike those already described. The characteristics of these groups are shown in Table XI (Appendix).

The new group (XI) differed from other coryneform groups in that all 13 isolates showed 'palisade' formation and the snapping division typical of Corynebacterium. It also differed in that it contained approximately equal numbers of oxidative, fermentative and inactive isolates as judged by the mode of utilization of glucose in Hugh and Leifson's medium.

3. THE MICROFLORA OF SACK-STORED BARLEY

The composition of the bacterial flora of the 23 sacks examined is summarized in Figures 7 and 8. Counts of Rhodotorula are included in Figure 8 as this yeast was abundant in some sacks in July. It was not recorded in November. A duplicate series of dilution plates was poured with malt-salt agar in November, and counts made of Aspergillus and Penicillium species. The results are shown together with the bacterial counts in Figure 7.

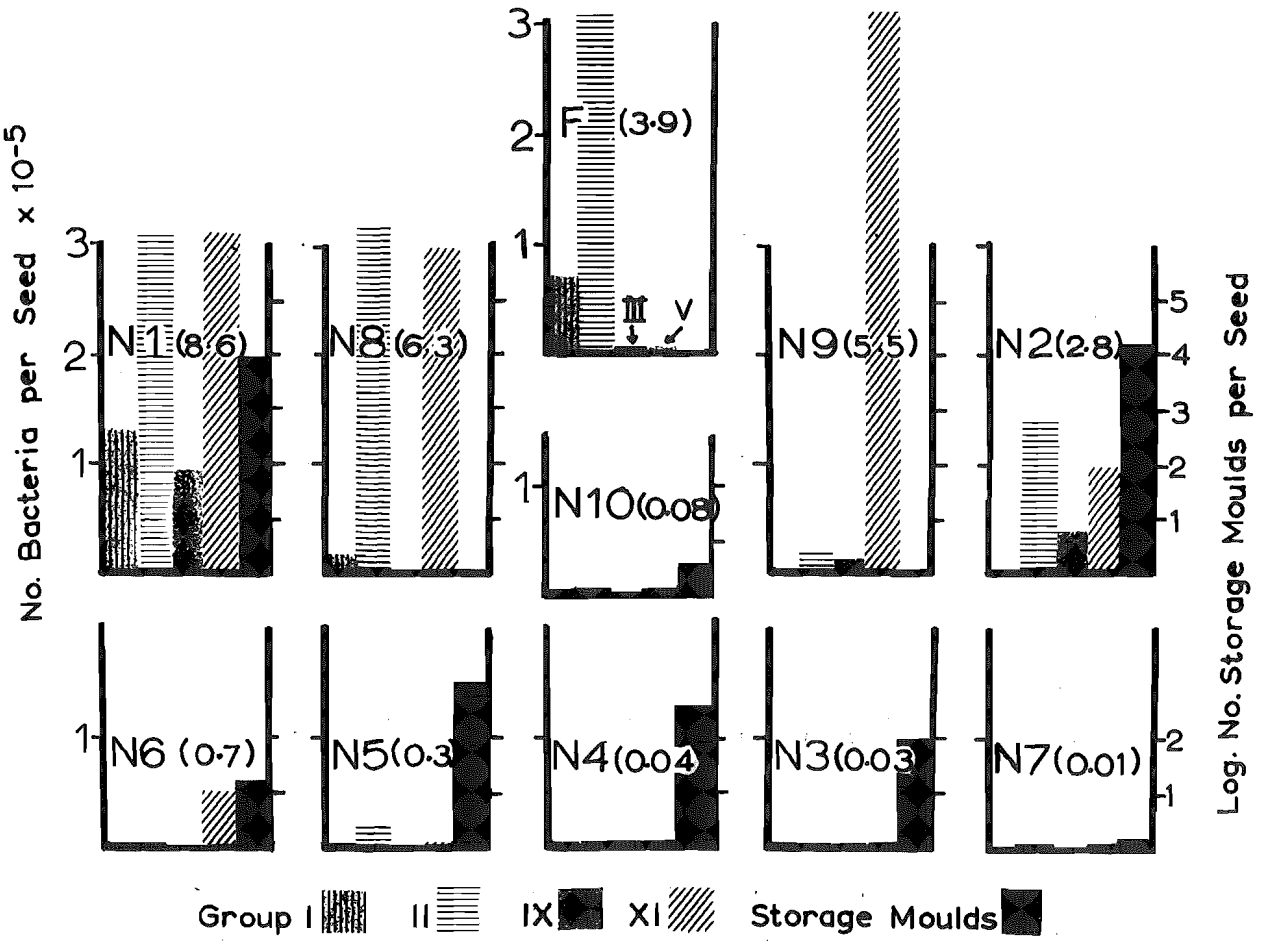


Figure 7. MICROBIAL POPULATIONS ON SACK-STORED BARLEY: FEBRUARY AND NOVEMBER

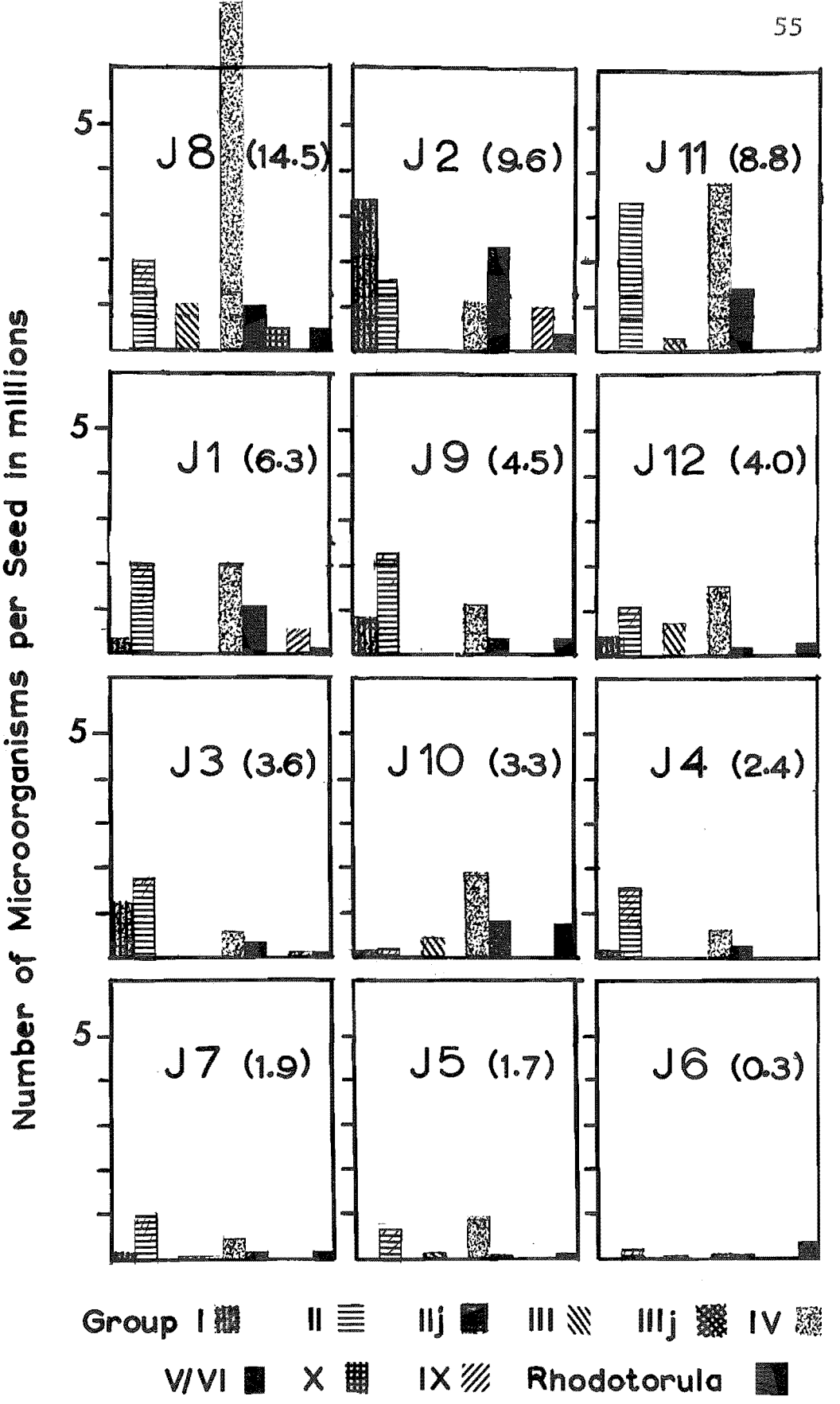


Figure 8. MICROBIAL POPULATIONS ON SACK-STORED BARLEY: JULY

The sack that was sampled in February was stored in the laboratory and re-sampled in July and November in order to determine the change in total numbers of bacteria with time. Counts were made as before, and the results are shown in Figure 9. There was a logarithmic decline in numbers from 390,000 to 97,000 per seed.

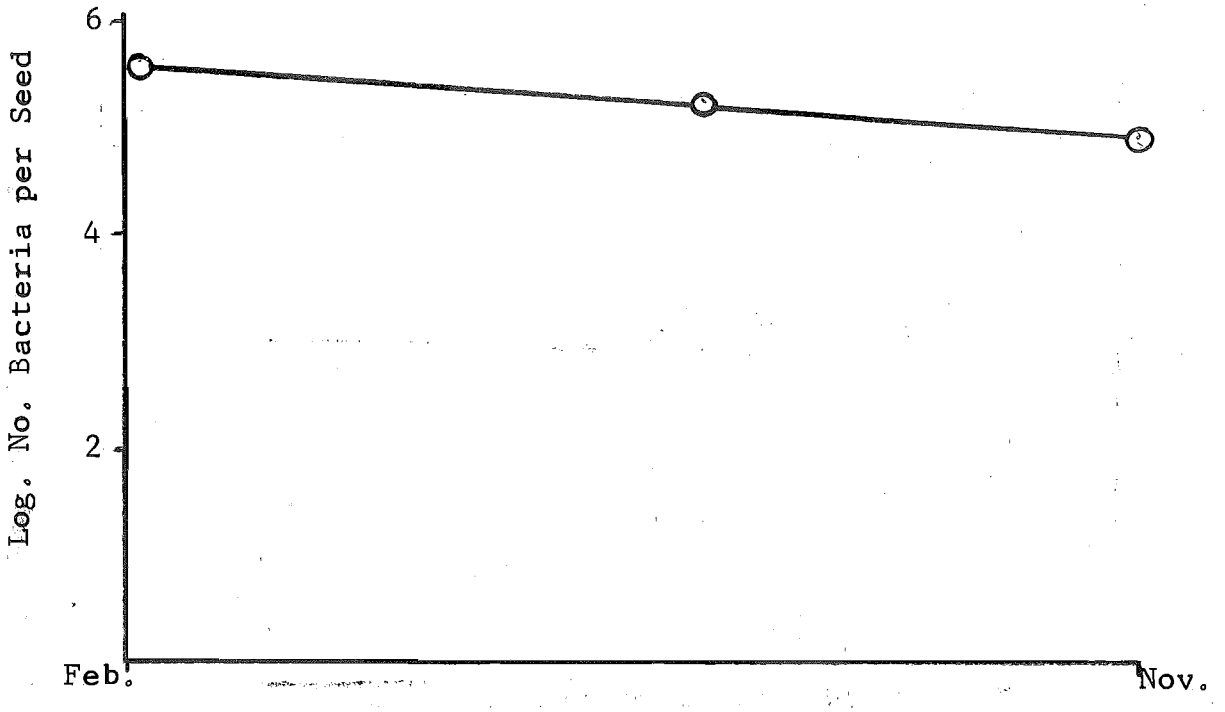


Figure 9. BACTERIAL NUMBERS ON BARLEY STORED FOR NINE MONTHS

4. DISCUSSION

The kinds of bacteria found on stored barley were in general like those found on ripe seed in the field. In the field the three main groups were I, II and IX. In store, these together with IV and XI, formed the dominant types although with the exception of Group II (Erwinia herbicola) they were not present in every sample.

Although the kinds of bacteria present were limited, their total and relative numbers varied a great deal from sack to sack, and with time. On sample N8 for instance, bacteria of Groups II and IX both numbered about 300,000 per seed, whereas on N9 they numbered 19,000 and over 500,000 respectively, and on N3 only 2,000 and 800 were found. The total count of bacteria varied from 600,000 to 13,000 per seed in July, and from 850,000 to 1,500 in November.

The persistence of large numbers of bacteria on grain stored for nine months indicates that these bacteria are adapted to survival for long periods under conditions inimicable to growth. It is of course impossible to deduce the extent to which numbers declined during storage except in the case of the sack stored under abnormal conditions in the laboratory. In this case, the higher mean temperatures and the relative lack of fluctuations in temperature and R.H. might be expected to lead to a more rapid fall in

numbers. It is noteworthy that even in this sack, after a nine months exponential decrease in numbers, each seed carried 97,000 bacteria.

Rhodotorula, which formed a conspicuous part of the seed microflora in the field, was sometimes present in large numbers in store. The fact that it was detected on plates of nutrient agar in which it had to compete with bacteria probably means that yeast numbers were in fact higher than recorded. For the same reason, the fact that Rhodotorula was not found at all in November does not necessarily mean that it was not present on the seed at that time.

Storage moulds, too, were numerous in some samples and absent from others. There did not appear to be any correlation between the numbers of moulds and bacteria, as might be expected if a general antagonistic relationship existed between them. Nevertheless, the existence of a specific and numerous bacterial flora pointed to the value of determining whether or not specific antagonistic relationships existed between the microorganisms of the seed coat. If multiplication of bacteria occurs, as indicated by Perera (1966), then it is unlikely that this has no effect on the growth of other bacteria and fungi on the seed. Biological control of seed decay might then be achieved by the bacteria 'scavenging' nutrients which

would otherwise stimulate growth of the pathogens: or they might actively antagonize these fungi. On the other hand, bacterial activity might stimulate the growth of the pathogens.

There are several reports in the literature of bacteria on seeds antagonizing fungal pathogens. One of the first was by Simmonds (1947) who found bacteria antagonistic to Helminthosporium sativum (Cochliobolus sativus) to be common on wheat seed. He did not identify these bacteria, but in 1966 Gayed showed that Bacillus pumilus sprayed on to barley seed before inoculation with H. sativum reduced infection by this pathogen. Inoculation of maize seed with B. subtilis was shown by Chang and Kommedahl (1968) to give good control of Fusarium roseum, and the same bacterium was thought to be antagonistic to unspecified pathogens on peanut kernels by Pettit, Taber and Foster (1968). Roth (1959), in Germany, concluded that healthy barley seed contained bacteria with an antibiotic action against most fungi found on the seed; and Chranowska (1964) stated that in the U.S.S.R. rye developed a bacterial microflora which was 'anti-fungal'. Finally, two recent reports indicate that Erwinia herbicola may be antagonistic to other microorganisms. Ross and Burchill (1968) described experiments in which a 'yellow peritrichous rod' (E. herbicola?) strongly inhibited the formation of

perithecia by Venturia inequalis, in contrast to 'pink and orange chromogens' (coryneforms?) which stimulated growth of the pathogen. Chatterjee, Gibbins and Carpenter (1969) are more specific and point out a possible mechanism by which E. herbicola antagonizes the fire-blight pathogen E. amylovora.

The above papers, however, with the exception of that by Chranowska, describe antagonism either in vitro or on germinating seeds. For one organism to antagonize another, both must grow, and it is widely believed (e.g. Plotho, 1940) that bacteria do not grow in the absence of free water, and that they therefore play no part in the deterioration of grain stored under normal conditions. The evidence for bacterial activity on stored seeds is therefore slight and further work on this problem justified. Apart from practical considerations of biological control of pathogens on the seed, such studies might enlarge our scanty knowledge of the water relations of bacteria. The possibility of biological control of storage moulds is considered in the next two chapters, Chapter VI being particularly concerned with the growth of bacteria at low moisture levels.

Figure 10 (facing). GRAIN WEEVILS (*Sitophilus granarius*) AND DAMAGED BARLEY FROM INFESTED SACK

CHAPTER V

INTERACTIONS BETWEEN BACTERIA, GRAIN WEEVILS AND STORAGE MOULDS

1. INTRODUCTION AND METHODS

The aim of this experiment was to indirectly assess the effect of the saprophytic microflora on infection by storage moulds by comparing the degree of infection in sterilized seeds during storage. In order to provide sufficient sources of inoculum for the sterile seed, it was to be mixed with the untreated seed in a ratio of not more than 1:15. This required a method of sterilizing and subsequently identifying seeds without affecting them in any other way.

After evolving a satisfactory sterilization technique, the microflora of treated and untreated seeds was determined immediately after harvest, and after five and nine months storage in a sack kept in the laboratory. The untreated seed was that already mentioned in Chapter IV (February sample).

After seven months storage, the sack became infested with grain weevils, Sitophilus granarius L., (see Fig. 10) and the opportunity was taken to examine the effects of insect attack on the saprophytic microflora and on infection by storage moulds. It was possible to compare damaged and undamaged, sterilized and untreated seeds, and in addition

a brief investigation was made of the microflora of the weevil gut and exoskeleton.

Sterilization of Seed

The pericarp of the barley caryopsis is commonly invaded by saprophytic microorganisms (Hyde and Galleymore, 1951) and it is this sub-surface microflora which renders surface sterilization techniques ineffective. To test the extent of such infection, 100 seeds were surface sterilized by briefly wetting in 1.0% detergent solution followed by ten minutes shaking in 10% of a commercial hypochlorite solution containing 3.5% available chlorine as sodium hypochlorite. They were then plated on malt extract agar, and incubated at 25°C for seven days. The number of seeds still infected was 88, the majority yielding colonies of a Fusarium species and/or bacteria.

The method of sterilization finally adopted was a modification of that described by Bose (1956). It entailed adding to seeds in test tubes a 0.005% aqueous solution of methylene blue previously heated to 53°C, and keeping the tubes in a water bath at this temperature for ten minutes. The seeds were then rapidly cooled by adding them to a jar of 0.1% mercuric chloride solution, which was mechanically shaken for five minutes. This was followed by rinsing in five successive changes of sterile water.

One hundred hot water-treated seeds showed no infection

when tested by plating on malt extract agar. Nor was there any evidence of microbial growth when one gram of seed was comminuted in 100 ml. of diluent and the resulting suspension streaked on ten replicate plates of malt-salt agar, malt extract agar, and nutrient agar.

Tests showed that the methylene blue effectively identified the seed, enabling 100% recovery from mixtures of treated and untreated grain. It was also shown not to inhibit growth of the microflora. A comparison of 200 stained (but unsterilized) seeds with 200 unstained seeds plated on malt extract agar showed that all seeds were infected. The same organisms were present in each sample, viz. Alternaria, Fusarium, Rhizopus, Aureobasidium, and bacteria.

Hot water treatment is known to decrease the germinative power of cereal seed when used to kill internal smut mycelium (Dickson, 1962) but the less drastic treatment necessary to sterilize the pericarp was shown to have no such effect. Samples of 200 treated and untreated seeds were kept on moist blotters at room temperature for five days: 97.0% of the treated and 98.5% of the untreated seeds germinated in this time. The fact that the hot water treatment did not affect germination does not mean that it did not alter the seed in any other way. Nevertheless, it was reasonable to think that no gross physiological differences existed between treated and untreated seed.

The method described above was used to sterilize 100g of seed from a cloth bag containing 1.5Kg. The treated and untreated seeds were then thoroughly mixed, and the bag stored among the bulk of the grain near the centre of the sack.

The Seed Microflora

In February, mould count dilution series were set up as previously described, using treated and untreated seed from the 1.5Kg sample. Duplicate plates were poured using malt-salt agar and nutrient agar. From the latter, 70 bacteria were isolated and characterized by the tests described in Chapter IV for the February isolates.

Five months later, 5g stained and 5g unstained seeds were picked out and the same procedure carried out. This was repeated after a further four months storage, but as noted above, separate counts were made of:

i) weevil damaged, sterilized seeds
ii) weevil damaged, untreated seeds
iii) undamaged, sterilized seeds
iv) undamaged, untreated seeds

From each of these categories, 40 bacteria were isolated and characterized.

Microorganisms Associated with Weevils

The extent to which weevils might transport micro-organisms on the surface of their bodies was assessed by placing single insects on the surface of malt-salt agar or

nutrient agar in petri dishes. Ten plates of each medium were used, and in each case the weevil was first laid on its back in the centre of the dish, then righted and left to walk around. Incubation was for five days at 25°C.

Dissemination might also be achieved by passage through the gut. Julseth et al. (1969) record the experimental transmission of Salmonella in this way by the Hide Beetle, Dermestes. However, investigation of the gut microflora of Sitophilus was difficult. Because of its small size, the dissection method described by Macduff (1966) for the N.Z. black field cricket was impossible. Surface sterilization was therefore employed on the assumption that most of the viable internal microorganisms would be intestinal. This was true of the field crickets investigated by Macduff. Individual weevils were dipped in alcohol, flamed, and then crushed in a drop of nutrient broth on a sterile glass slide. This drop was used to streak plates of malt-salt agar or nutrient agar, seven plates of each medium being used.

2. INFECTION OF STERILIZED SEED

Numbers of Microorganisms

The number of microorganisms per seed as estimated by the mould count method is shown in Table II. No fungi were present on any of the plates. However, taking the dilution factor of x 100 into account, a 'nil' mould count is better

interpreted as meaning that less than 100 viable reproductive units were present per seed.

As previously noted, bacterial numbers on untreated seeds showed an exponential decrease during the nine months in store. In contrast, numbers on sterilized seeds increased from 0 to 10,000 per seed during this period.

TABLE II. Microbial Numbers on Sterilized and Untreated Seeds

Treatment	Months in Store	Bacteria	Fungi
1. nil	0	3,900,000*	0
2. sterilized		0	0
3. nil	5	170,000*	0
4. sterilized		1,400	0
5. nil	9	53,000*	0
6. sterilized		10,000	0

* see also Figure 9, page 56

Identification of Bacteria

The results of the tests carried out to characterize the unsterilized February isolates have already been considered in Chapter IV. The test results for the other

treatments are shown in Tables XII and XIII in the Appendix. These results were analysed by the computer system, and all isolates identified with previously described groups. A summary of the results, including those for the February isolates, is given in Table III. It can be seen that throughout the period of storage, the microflora of the untreated seed was dominated by bacteria of Groups I and II. The sterilized seed was at first re-colonized by a wider variety of bacteria, but by the end of the nine-month period Erwinia herbicola (Group II) made up 95% of the total flora.

TABLE III. Kinds of Bacteria Isolated from Sterilized And Untreated Seeds

Treatment	Months in Store	Percentage of Total						
		I	II	III	V	VII	X	IIj
1. nil	0	18.6	78.6	1.4	1.4	0	0	0
2. sterilized	0	0	0	0	0	0	0	0
3. nil	5	21.4	78.6	0	0	0	0	0
4. sterilized	5	5.0	12.5	30.0	27.5	5.0	17.5	2.5
5. nil	9	20.0	80.0	0	0	0	0	0
6. sterilized	9	0	95.0	2.5	2.5	0	0	0

3. THE EFFECT OF INSECT DAMAGE ON THE SEED MICROFLORA

Numbers of Microorganisms

The damaged seed which was used in this investigation consisted of pieces of debris of various size, which were recognizably stained with methylene blue, or not stained. Such pieces were not numerous, only 0.3g of treated and 0.5g of untreated seed being collected. As whole seeds were not used, the mould count results, which are shown in Table IV, have been expressed on a 'per gram' rather than a 'per seed' basis. The figures for the undamaged seed are based on the same plate counts as those given in Table III for treatments 5 and 6.

TABLE IV. Microbial Numbers on Damaged and Sterilized Seed.

(Number per gram)

Weevil Damage	Seed Sterilization	Bacteria	Storage Moulds
-	-	25,000,000	0
+	-	600,000	100
-	+	52,500	0
+	+	8,500	31,000

The untreated, undamaged seed can be regarded as the control in this trial. On this seed, bacterial numbers in all the groups identified fell throughout the period of storage. Infection by storage moulds did not occur, or was so slight as to escape detection by the dilution plate

technique.

When this seed was damaged by weevils, fewer bacteria and more fungi were found. This effect was much more marked in the case of previously sterilized seed, however, as is evident when the data in Table IV is laid out in the form of two-way tables.

Number of Fungi per Gram of Seed

	weevil damaged	sound	
sterilized	31,000	0	31,000
untreated	100	0	100
	31,100	0	

Number of Bacteria per Gram of Seed x 10^{-4}

	weevil damaged	sound	
sterilized	0.9	5.3	6.2
untreated	60.0	2500.0	2560.0
	60.9	2505.3	

Identification of Fungi

The single colony which developed on the malt-salt agar plates from untreated seed was of a Penicillium species. The 62 colonies of storage moulds derived from sterilized seed were identified as follows:

Penicillium sp.	5
Aspergillus glaucus	50
A. candidus	2
A. versicolor	5

Identification of Bacteria

The kinds of bacteria present on the undamaged seed have already been noted. Isolates from damaged seed were similarly characterized with the results shown in Table XIV (Appendix). All isolates were identified with previously described groups, and a summary of the results is given in Table V.

Erwinia herbicola (Group II) was dominant on the untreated, damaged seed: types V, VI, and VIII were also present. In the case of the sterilized seed, two other groups were represented, III and VII, and the latter was in fact the most numerous.

TABLE V. Kinds of Bacteria Isolated from Weevil-Damaged Seed

Seed Treatment	Percentage of Total					
	II	III	V	VI	VII	VIII
sterilized	10.0	10.0	17.5	15.0	37.5	10.0
untreated	87.5	-	5.0	2.5	-	5.0

4. MICROORGANISMS ASSOCIATED WITH WEEVILS

Exterior

No fungi of any kind were found. Of the ten nutrient agar plates on which weevils had walked, two remained

sterile, six contained 1 - 3 bacterial colonies, one contained seven colonies, and one showed trails of confluent bacterial growth. Twenty isolates were obtained from this last plate and partially characterized by a more limited series of tests than those previously used. The results are shown in Table VI.

Although the tests were limited in number, they were sufficient to show that the particular weevil investigated was carrying at least six different kinds of bacteria on the exterior of its body.

TABLE VI. Results of Tests Carried Out on Isolates from Weevil

	Tentative Group	Result of Test					
		I	II	III	V	VII	VIII
Character	No. Isolates	1	11	1	3	1	3
rods		+	+	+	+	+	+
motile		+	+	+	+	+	+
'snapping' cell division		-	-	-	+	-	-
gram stain		-	-	-	+	±	±
utilization of glucose	oxidative	+	-	-	-	-	+
	fermentative	-	+	-	-	+	-
acid from lactose		-	±	-	-	+	+
	salicin	-	+	+	-	-	+
pigmentation		-	Y	Y	R	R	R
mucoid growth		-	±	-	-	+	-

Y= yellow R= orange

Interior

The malt-salt agar plates remained sterile, but 14-32 white bacterial colonies developed on each of the nutrient agar plates. Twenty isolates were taken and characterized. All proved to be gram-positive spore-forming bacilli.

5. DISCUSSION

Insect damage to stored cereals is known to increase infection by storage moulds. In 1958, Agrawal, Hodson and Christensen showed that the activities of insects increased moisture in local areas and created ideal conditions for fungal development. Moreover, Caldwell (1961) concluded that mechanical damage to the selectively permeable membrane formed by the testa might remove a physical impediment to hyphal penetration and, if sufficient moisture were available, yield extra nutrients for fungal growth. Insects feeding on grain must damage the testa as well as raise the humidity of the inter-seed air. As Sikorowski (1965) has shown that several insect pests of grain feed on aspergilli, and are excellent vectors of these fungi, it is not surprising that infestation by insects often results in the formation of 'hot spots' in which the temperature, seed moisture content and microbial activity are all high.

In the present instance, a large increase in storage mould numbers was found only in insect-damaged seed which

had been previously sterilized. As the previous work reviewed above would lead one to expect increased infection in all damaged seed, it is likely that the large difference between sterilized and unsterilized seed reflected a difference in the rate of colonization by moulds rather than unsuitability of the latter as a substrate for their growth. The reason for this difference is not clear. Although it is possible that the presence of a saprophytic microflora rendered the untreated seed more resistant to attack because of antagonism or depletion of nutrients, the evidence for this is inconclusive. It is difficult to reconcile the notion of an antagonistic and therefore actively growing saprophytic microflora with the decline in bacterial numbers which occurred.

Bacteria have not previously been considered in relation to insect damage, but in the case of mechanically damaged wheat Spicher (1956) noted an increased ratio of bacteria to fungi compared with undamaged grain. This is the opposite of the present results with insect damaged barley. With increasing moisture content of wheat and rye, however, (which is one effect of insect damage) Spicher (ibid) found an increase in total microbial numbers with a decreasing proportion of bacteria to fungi. Neither the results of Spicher nor those presented here indicate

why this inverse relationship occurs, but the fact that it does occur suggests that the storage moulds antagonize the epiphytic bacteria rather than the converse.

Other explanations for the more rapid mould growth on sterilized seed are equally unsatisfactory. If, for instance, hot water treatment damaged the semi-permeable membrane of the seed, enabling A. glaucus and other moulds to invade sterilized seed when the atmospheric humidity was raised by weevil activity, then one would not expect these fungi to be present only in seed actually attacked by weevils. This experiment therefore failed in its primary object of assessing the effect of the saprophytic microflora on infection by storage moulds. The possibility of interaction is not ruled out by the results; but the fact that only insect damaged seeds became infected obscures their interpretation.

This work did confirm the conclusions of Agrawal, Hodson and Christensen (1958) in that weevil activity started under environmental conditions unsuitable for fungal growth, and this activity resulted in the infection of damaged seed by Aspergillus species, particularly A. glaucus. The insects did not appear to be carrying fungus spores on their bodies. Sporing fungi were probably present only on the damaged seed pieces, however, and the number of spores present in the grain as a whole therefore

not great. Had the sampling been done later, when a higher proportion of the grain had become infected, it is possible that external contamination of the weevils would have been more readily found.

As was to be expected, the sterilized seed did not remain sterile. The extent to which it became contaminated was interesting however. Even before the weevil infestation, 1,400 bacteria were present per seed, and this number increased to 10,000 during the final four months. During this time, numbers of bacteria on unsterilized seed steadily dropped, so it is unlikely that conditions were suitable for multiplication. Contamination of the seed from the dusty environment found in a sack of dry grain would certainly account for some of the bacterial population, and it is noteworthy that after the first five months, the bacteria found were more varied in kind than those present on the untreated control. The large increase noted latterly, however, resulted in the dominance of the Erwinia herbicola group, the commonest type present on the control seed. It is likely that this was the result of dissemination by the weevils present during this period. Although most of the insects were surprisingly free from microbial contamination, they were shown to be capable of carrying externally on their bodies viable bacteria of the kind commonly found on

seed. There was no evidence that these bacteria might also be disseminated in egested wastes after passage through the insect gut.

CHAPTER VI

THE EFFECT OF HUMIDITY ON BACTERIA AND STORAGE MOULDS

1. INTRODUCTION

Living cells contain 75 to 85 per cent water, and the metabolism of all living things is dependent on its presence. Yet as recently as 1965, J.D. Bernal emphasized that the way in which water functions in biological systems is largely unknown. He showed that it does not serve merely as a solvent, and quoted work which proved that proteins are sheathed in a layer of water molecules closely bonded in something like an ice structure. Bernal was of the opinion that beyond this ice layer, the cell water was not so tightly bound, but was nevertheless present in polarized multilayers oriented on the protein surfaces. Such water has properties very different from ordinary water. For example it may accept a much lower concentration of solutes than the extracellular water with which it is in contact (Ling, 1965) and it may still be liquid at -57°C (Toledo, Steinberg and Nelson, 1968).

Derjaguin (1965) has reviewed the evidence showing that water adsorbed to non-living surfaces also exhibits properties different from normal liquid water. He concluded that it too is strongly bound by hydrogen bond formation and by dipole-dipole interactions, and that these forces orient more than one layer of water molecules. The strength

of binding lessens with increasing distance from the surface, however, and it is impossible to draw a hard and fast line between 'bound' and 'free' water.

While not disputing the properties exhibited by adsorbed water, Sharma, Uehara and Mann (1969) produced evidence that the structure given to liquid water by hydrogen bonding is broken on adsorption and that it is therefore more disordered, rather than less so, at the molecular level. Whatever the physical basis may be, however, the thermodynamic properties of adsorbed water make it clear that the water relations of a bacterial cell adsorbed on a nutrient substrate are likely to be complex, particularly as the substrate and the bacterial cell both contribute to the solute concentration of the water film.

The primary effect of a lack of available water in the environment of a cell is cessation of metabolism, presumably because there can be no transport of materials across the membrane. Loss of water to the environment may then result in death of the cell. Webb (1964) has shown that death is caused by the removal of water bound to cell macromolecules, resulting in a change in their structure. This does not necessarily happen, however, and numerous studies have been made of the ability of bacteria and other organisms to survive under conditions

of dehydration. Brock (1966) notes that "in bacteria, the slime layer is thought to promote resistance to drying by enabling the organism to retain moisture." This resistance would be short-lived, however, and is certainly quite difference from that of organisms in a state of cryptobiosis.

> "In cryptobiosis all metabolic processes have ceased, and such chemical reactions as occur are entirely adventitious: the organism is reduced to a purely morphological state, the successful maintenance of which does not require interactions between cells or between constituents of cells but only requires that certain spatial reactions be preserved."
>
> (Hinton, 1968)

In such a state, an organism would theoretically survive indefinitely in the absence of adventitious damage, and Hinton quotes authenticated records of plants, animals, and microbial cells surviving for periods of up to forty years. The fact that numerous bacteria, yeasts, and moulds remain viable for several months on the surface of dry grain is therefore not surprising.

The growth of microorganisms under conditions of restricted water availability has been studied less than their ability to survive desiccation. Moreover, much of the work which has been carried out concerns fungi rather than bacteria, probably because the ability of some fungi

to grow in the absence of liquid water (first demonstrated by Tomkins in 1929) is an important factor in the deterioration of stored products.

Pelhate (1968) considered that the xerophytic nature of moulds on grain is best shown by the fact that the optimum R.H. for their growth is less than 95%. The more generally accepted view, however, is that a xerophytic microorganism is one with a minimum requirement for growth of less than 80% relative water-vapour pressure ($=0.80a_w$). By this standard, aspergilli and penicillia are the commonest xerophytic moulds, Aspergillus glaucus in particular being well known for its ability to grow on a variety of substrates where the activity of water (a_w) is as low as 0.65 to 0.70 (Vallentyne, 1963). The lowest activity at which fungal growth has been reported is $0.605a_w$, recorded by Pitt and Christian (1968) for Xeromyces bisporus.

The paucity of comparable work on bacteria is possibly due in part to the fact that such experimental evidence as is available supports Semeniuk's (1954) conclusion that bacteria are hydrophytes i.e. their minimum requirement for growth is $0.90a_w$ or greater. In 1935, Shaw showed that in the intercellular spaces of apple leaves, growth of Erwinia amylovora was suppressed at 97% R.H. and was slight even at 98% R.H. Stotzky (1968), reviewing studies

in soil microbiology, stated:

> "Microbes may persist for long periods of time in the absence of water ..... but free water is necessary for reproduction and metabolism."

It is true that both American and Russian work undertaken in connection with their respective space exploration programmes has indicated the existence of a xerophytic bacterial flora in desert soils (Cameron and Blank, 1965; Imshenetsky et al., 1964) but no critical work on the water relations of bacteria appears to have been done since that of Scott (1953) and Christian and Scott (1953). These authors showed that the lowest a_w at which salmonellae would grow was 0.96, the corresponding figure for Staphylococcus aureus being 0.92.

Scott (ibid) also pointed out the relationship between solute concentration and water activity. The a_w of, for example, nutrient broth containing 12% NaCl, is 0.92; and the water relations of a cell growing in this medium are the same as one growing on a nutrient substrate in equilibrium with an atmospheric relative humidity of 92%. This concept cannot be reconciled with the view that all bacteria are hydrophytes, for bacteria are found which can grow in activities of water ranging from the very low levels described for extreme halophiles by Kushner (1968) to the very high levels found in the waters

of oligotrophic lakes.

As mentioned in the Introduction, Perera (1966) presented evidence that under certain undetermined conditions, bacteria multiply on barley in store. His data have been used to compile Figure 11, which shows that the population growth curve for bacteria during nine months of storage was similar to that for storage moulds, and unlike that for Alternaria tenuis, the predominant field fungus.

To confirm this result, and to obtain more precise data on the use of water vapour by bacteria, an experiment was designed in which seeds were stored for eight weeks at known atmospheric relative humidities: periodically, the numbers of associated bacteria and fungi were determined. The relative humidities used were 100%, 95% and 75%; the higher levels because it was thought that if bacteria do make use of water vapour for growth it would be most apparent in these treatments. The lower level of 75% R.H. was included because if bacteria are implicated in seed deterioration, they must be capable of growth at activities of water similar to those at which the xerophytic moulds grow.

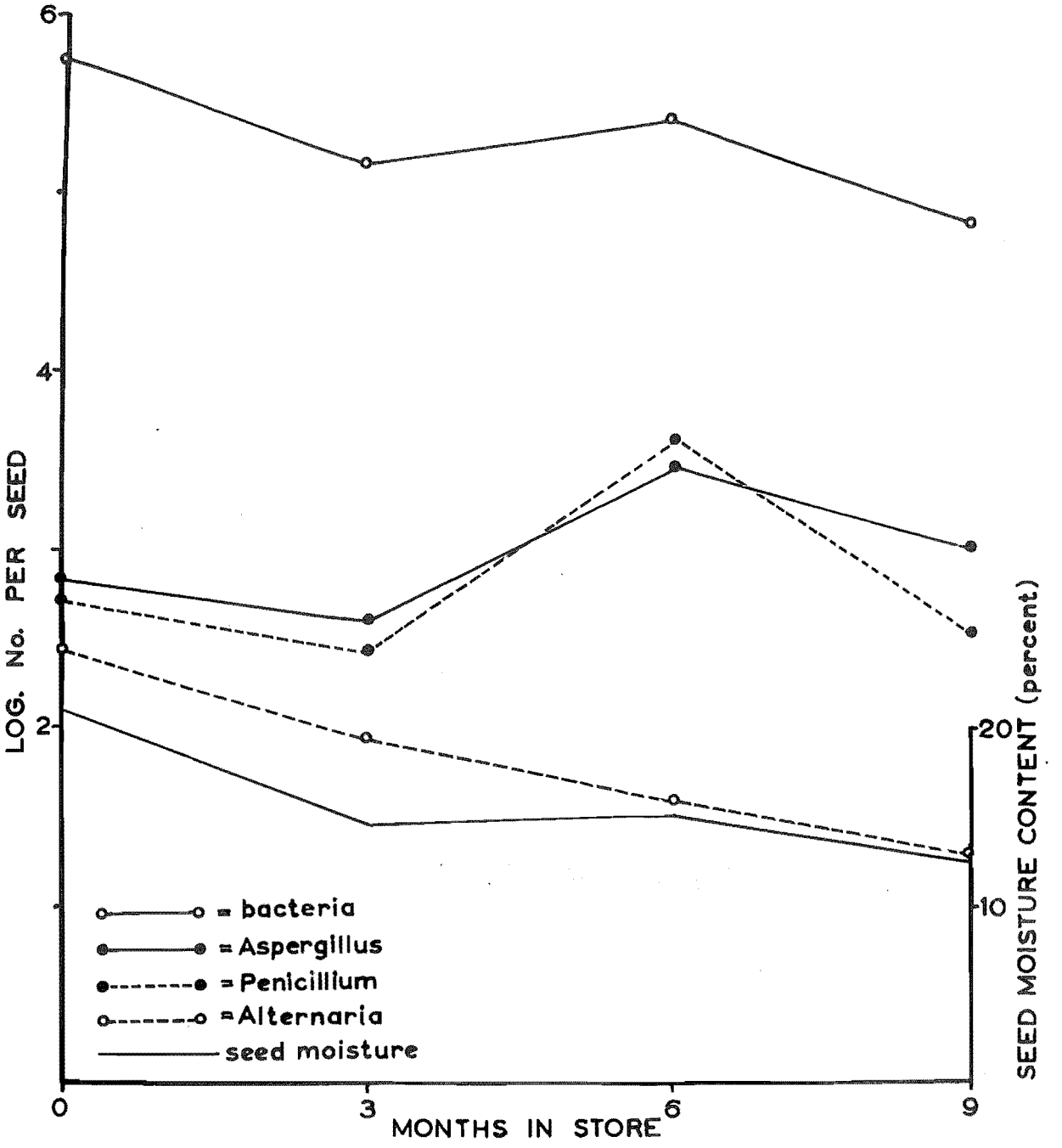


Figure 11. MICROBIAL POPULATIONS ON STORED BARLEY
(Data from Perera, 1966)

2. APPARATUS AND METHODS OF STUDY

Hugh and Oxley (1960), studying the hermetic storage of grain, concluded that at high levels of humidity in a closed system, CO_2 from the respiration of seeds and their associated microorganisms builds up to levels toxic to some portions of the microflora, while the corresponding depletion of oxygen eventually leads to anaerobic conditions. In order to avoid such an effect, the seed in this experiment was kept in a constant flow of pre-humidified air.

Several methods are available for obtaining air of a known relative humidity. The most widely used employ:

(i) solutions of glycerol of specified concentration

(ii) solutions of sulphuric acid of specified concentration

(iii) saturated solutions of different salts which are selected for the humidities required.

The latter method was chosen, primarily because progressive dilution of the humidifying solution occurs as air is passed through it, and it is easier to keep a solution saturated than to maintain it at any other concentration.

Humidity Chambers. A series of units was constructed in each of which air from a compressed air line was humidified by slowly passing it through a sintered glass gas distribution bottle containing the desired salt

solution. This air was then led into a container of the same solution over which the seed was stored under a bell jar. A small outlet on top of the bell jar allowed the air to escape. This apparatus, which is illustrated in Figure 12, has worked satisfactorily for periods of up to three months, the only maintenance required being the replenishment of salts as they were taken into solution (i.e. ensuring that undissolved crystals were always present) and occasional adjustment of the rate of air flow. It proved impossible to keep this flow at a constant rate because the gas distribution tubes became progressively blocked by salt crystals and required to be washed about once every two weeks.

The salts used in this experiment, and the resulting relative humidities, were:

i) NaCl. . . . . 75.5% - 76.0% R.H.

ii) KNO_3. . . . . 92.5% - 95.5% R.H.

iii) nil (distilled water) . . . . 100% R.H.

The relative humidity obtained is of course influenced by temperature, and NaCl and KNO_3 were chosen from several possible salts because they equilibrate at the above relative humidities over a relatively wide range of temperatures. Winston and Bates (1960) give the relative humidity value of saturated NaCl at 15°C as 76.0% and at 25°C as 75.5%; the corresponding values for KNO_3 are

95.5% and 92.5%. Temperatures in the laboratory during the period of this experiment did not exceed these limits.

Seed. The seed used was of the variety Kenia, and had been sack-stored for five months at the Heathcote plant of the Canterbury Malting Company. The 3 Kg sample taken was sub-divided into portions for immediate examination and for storage under the conditions of the experiment.

Treatments. The storage conditions consisted of humidity chambers maintained at relative humidities of

1. 100%
2. 95% (nominal)
3. 75% (nominal)
4. A control sample was kept in a cloth bag on the laboratory bench beside the humidity chambers. The relative humidity of the air to which this seed was exposed was not accurately determined but periodic readings with a hair hygrometer gave readings varying from 61% to 89%.

Microflora. The mould count method was used as before to obtain estimates of numbers of bacteria and storage moulds using nutrient agar and malt-salt agar respectively. Samples of seed from each treatment were taken for examination after 2, 5 and 9 days, and 2, 3, 4, 5, 6 and 8 weeks. All plate counts were made after 10 days incubation at 25°C.

Figure 12 (facing). APPARATUS USED TO STORE SEED IN AIR OF CONSTANT RELATIVE HUMIDITY

A = air outlet

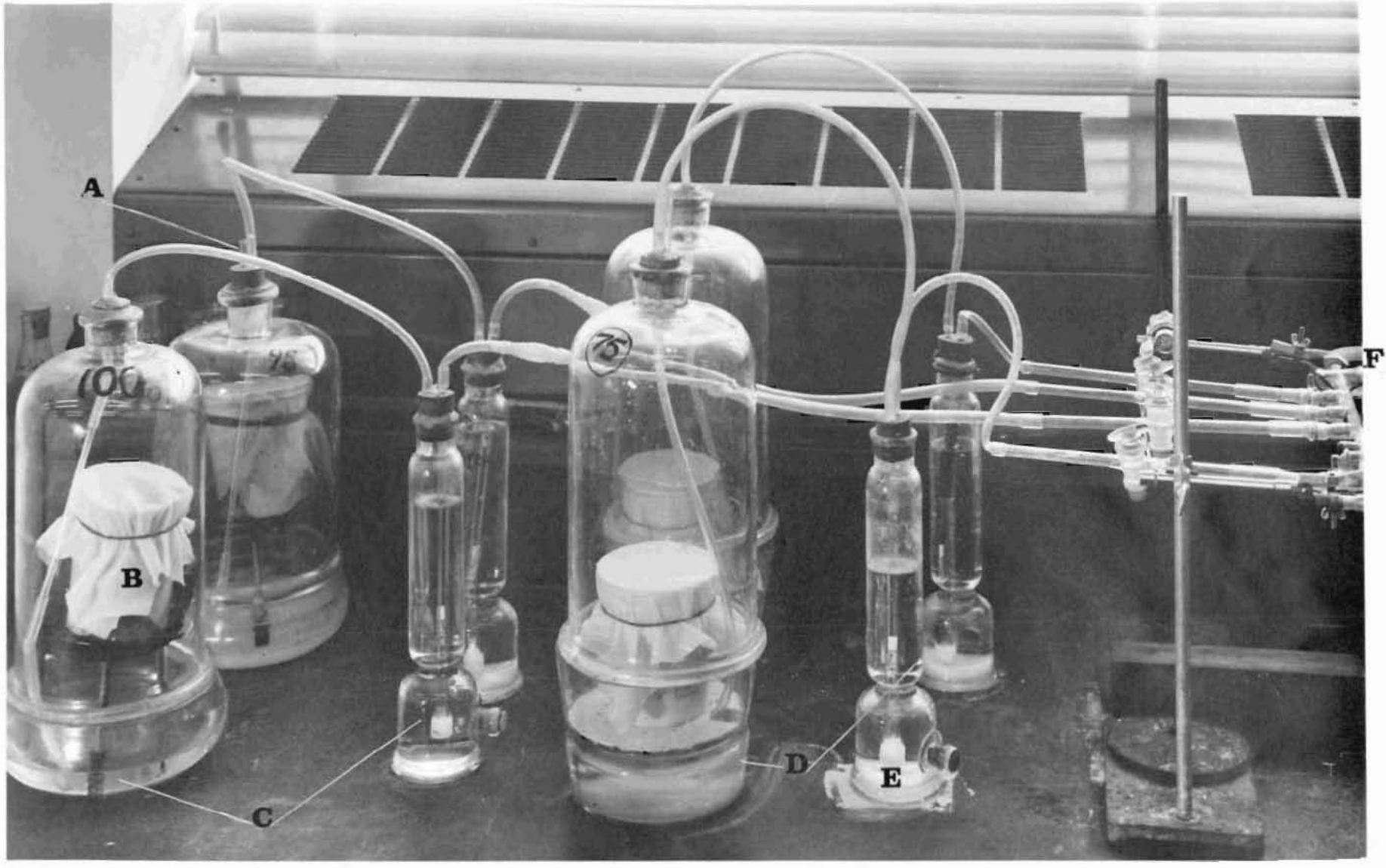
B = waxed container with seed

C = distilled water

D = saturated solution of NaCl

E = aerator

F = compressed air line


A
B
C
D
E
F

Bacteria were not identified, but separate counts were made of yellow and non-yellow colonies. The only fungi counted were species of Aspergillus and Penicillium. Separate counts were made of the different aspergilli found, and representative isolates were grown on Czapek-Dox medium and identified to species group level by the methods of Raper and Fennel (1965).

Seed Moisture. Each determination of seed moisture content was based on three replicate samples weighing 25 g to the nearest seed. These were dried to constant weight in a drying oven at 105°C, then cooled in a desiccator over calcium chloride and re-weighed. The moisture content was calculated on a wet weight basis.

3. THE SEED MICROFLORA AT DIFFERENT ATMOSPHERIC RELATIVE HUMIDITIES

Bacteria. The ratio of non-yellow to yellow-pigmented colonies varied from 0.80 to 1.87, the greatest variation being found in the control series of plate counts. Any change in the total number of bacteria was therefore the result of an increase or decrease in both types, and the counts have not been considered separately.

Population growth curves based on the plate counts made during the eight-week period of the experiment showed marked differences between treatments. This is illustrated in Figures 13 to 16. At the three relative humidities

tested (but not in the control series) bacterial numbers increased by a factor of five. This occurred within two days at 100% R.H. but not until four weeks at 95% R.H. and five weeks at 75% R.H. In each case, this peak was followed by a drop in numbers during the ensuing three to seven days to a figure approaching that of the control. At 100% R.H. they fluctuated about this level for five weeks, and then declined to a very low level (0.1×10^5 per seed as compared with 1.1×10^5 for the control). At 95% R.H. bacterial numbers dropped to the same low level, but at 75% R.H. they fell only to the level of the control.

Fungi. The mould count made at the start of the experiment showed that a species of Penicillium was present on the seeds, and for five weeks a low incidence of penicillia and aspergilli was recorded on all samples. Thereafter, very high counts of both Penicillium and Aspergillus glaucus were recorded at 95% and 100% R.H. The results of the mould counts are shown in Table VII.

Seed Moisture Content. The results of the seed moisture determinations are included in Figures 13 to 16. They show that even after eight weeks, the seed had not equilibrated with the atmosphere. In each case, however, the moisture content was near that given as the equilibrium content of wheat by Best and Hullet (1968), and according to Hlynka

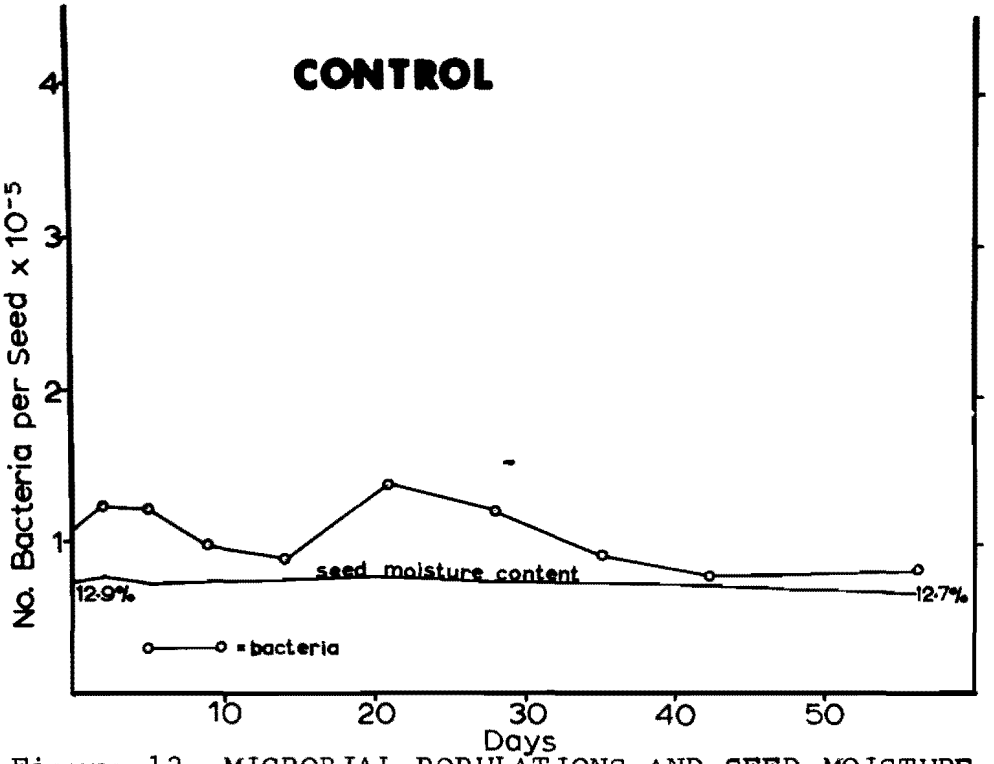


Figure 13. MICROBIAL POPULATIONS AND SEED MOISTURE CONTENT OF BARLEY STORED ON LABORATORY BENCH

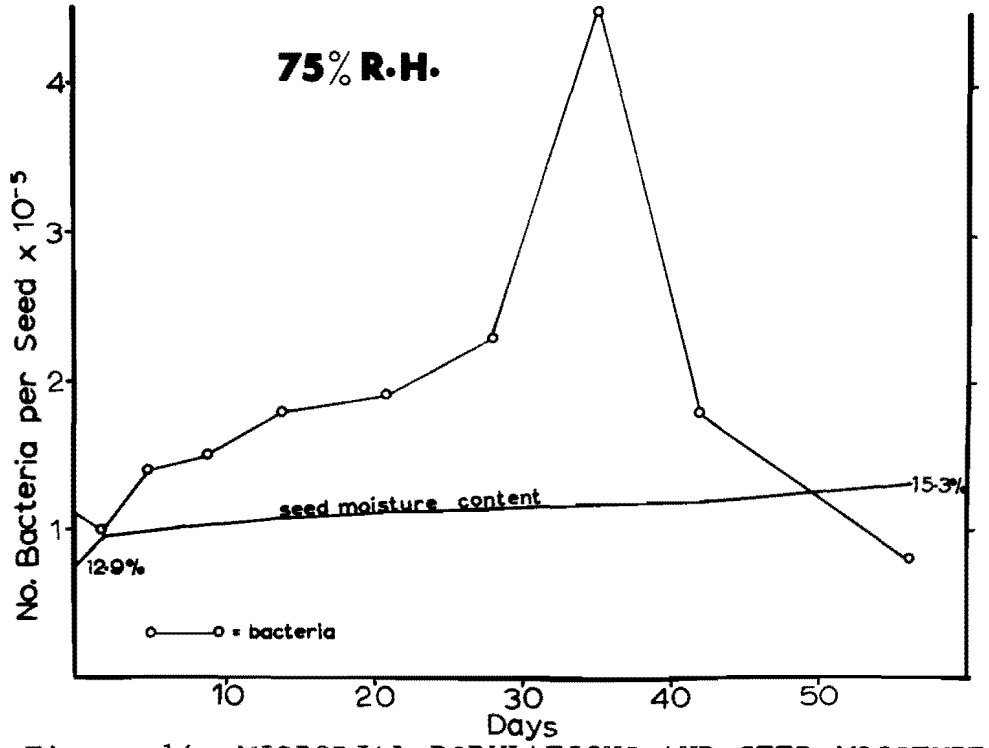


Figure 14. MICROBIAL POPULATIONS AND SEED MOISTURE CONTENT OF BARLEY STORED AT 75% R.H.

Figures 13 and 14: see over

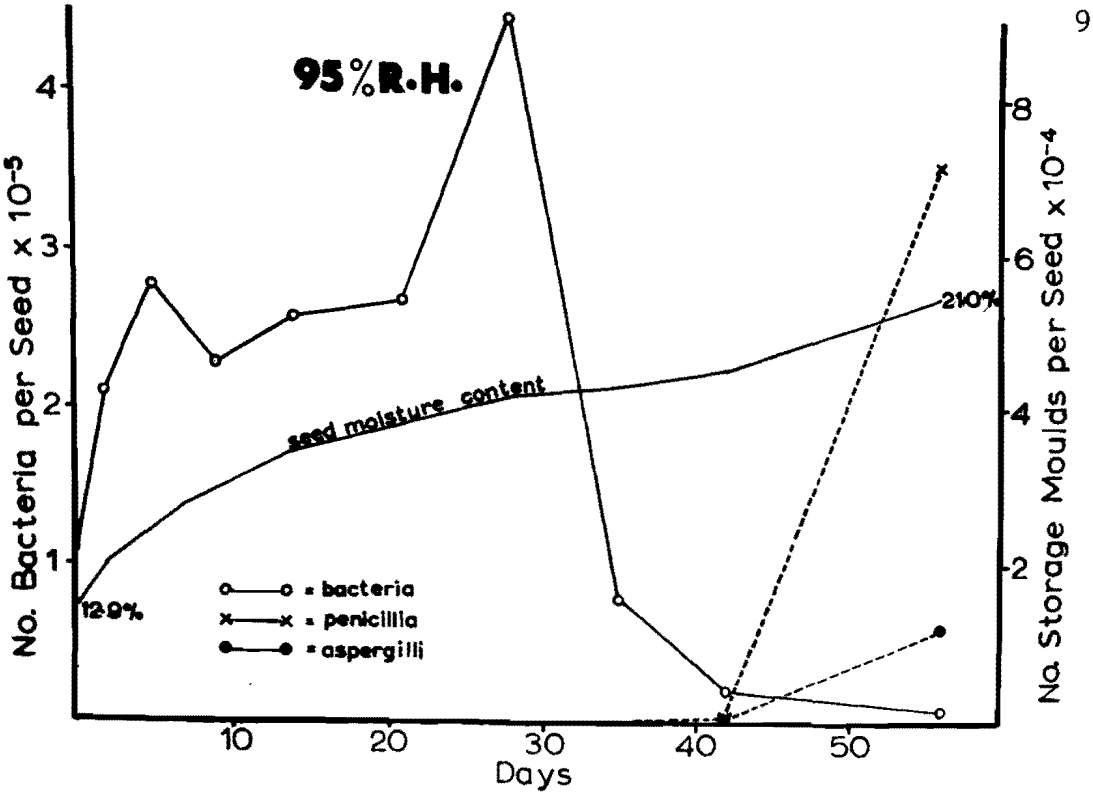


Figure 15. MICROBIAL POPULATIONS AND SEED MOISTURE CONTENT OF BARLEY STORED AT 95% R.H.

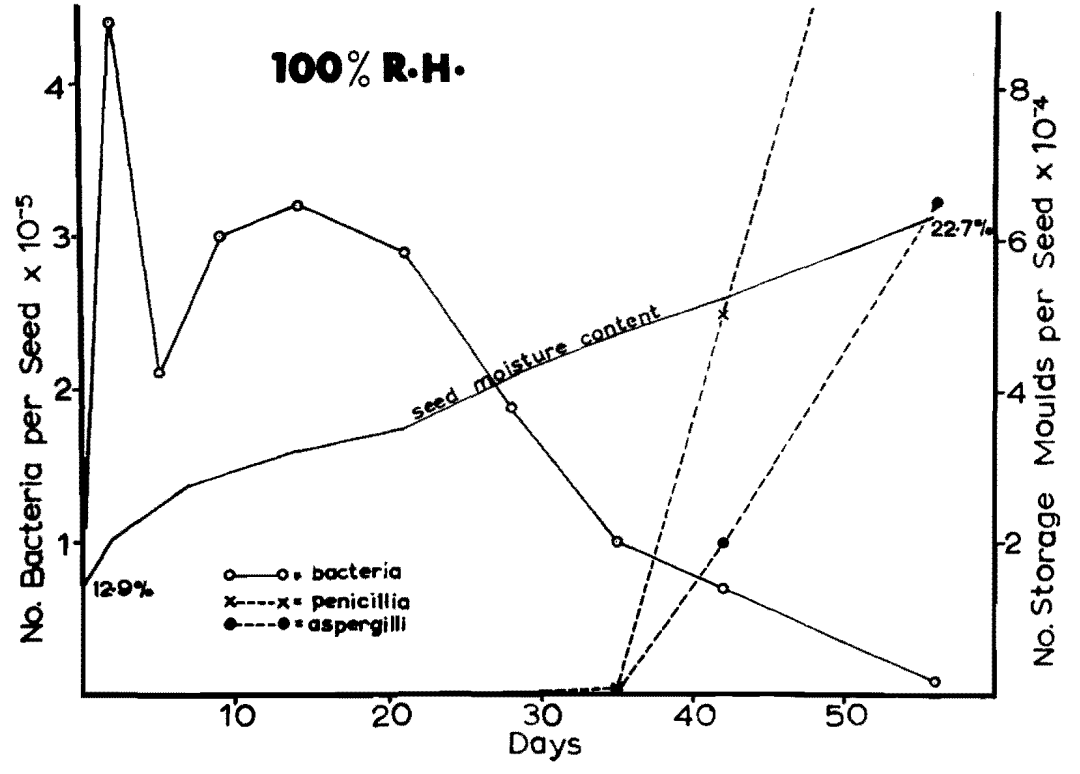


Figure 16. MICROBIAL POPULATIONS AND SEED MOISTURE CONTENT OF BARLEY STORED AT 100% R.H.

TABLE VII. Number of Storage Moulds on Barley Kept At Different Atmospheric Humidities

Days	Relative Humidity	Mould Count (No. per seed)	Fungi
0	Control	48	*Penicillium* sp.
	Control	24	*Penicillium* sp.
	75	57	*Penicillium* sp.
2	95	8	*Penicillium* sp.
	100	0	
	Control	9	*Aspergillus glaucus*
	75	0	
5	95	0	
	100	0	
	Control	0	
	75	0	
9	95	0	
	100	0	
	Control	18	*Penicillium* sp.
	75	24	*A. flavus* (13), *Penicillium* sp.
.14	95	0	
	100	0	
	Control	0	
	75	148	*Penicillium* sp.
21	95	11	*Penicillium* sp.
	100	0	
	Control	40	*Penicillium* sp.
	75	26	*Penicillium* sp.
28	95	0	
	100	0	
	Control	0	
	75	18	*A. glaucus*
35	95	0	
	100	308	*A. glaucus* (116), *Penicillium* sp.
	Control	0	
	75	0	
42	95	406	*A. flavus*
	100	70,000	*A. glaucus*(20,000), *Penicillium* sp.
	Control	24	*Penicillium* sp.
	75	65	*A. glaucus*(37), *Penicillium* sp.
56	95	84,000	*A. glaucus*(12,000), *Penicillium* sp.
	100	190,000*	*A. glaucus*(70,000), *Penicillium* sp.

* Estimated numbers only:- plate count too high.

and Robinson (1954) the equilibrium moisture contents of all cereals are similar.

4. DISCUSSION

The results clearly show an increase in bacterial numbers at all three relative humidities tested, and it therefore appears that bacteria can metabolize and grow in the absence of liquid water. Although the bacteria which multiplied were not identified, more than one kind did so, as indicated by the fact that the ratio of yellow to non-yellow colonies remained constant. The growth response was slower at 95% R.H. than at 100% R.H., and slower still at 75% R.H. No comparable work on bacteria has been previously reported, but these results are in accord with those given by Snow (1949) for various fungi, including Aspergillus glaucus. He showed that the rate at which spores of these fungi germinate is hyperbolically slower as the relative water-vapour pressure is further from the optimum.

The results of this experiment present some features which are not readily explained. Briefly, these are:

i) if moisture was no longer a limiting factor, why did the bacteria stop growing in each case when the population reached a level of 5×10^5 cells per seed?

ii) why, having increased to this level, did numbers

immediately fall again? The bacteria were derived from cells well adapted to survival on the seed coat.

iii) at 100% R.H., the bacteria multiplied within two days. Why was the growth of storage moulds not apparent until five to six weeks later, and why did they not increase at all at 75% R.H.?

Obviously, further work is required to answer these questions satisfactorily. This might involve the use of simplified models of the ecosystem using cellulose pads and pure cultures of bacteria: the S-shaped adsorption isotherm is common to cellulose and cereal grains (Hlynka and Robinson, 1954). Direct microscopic examination of the seed coat with a high power incident light system might serve as a useful check on plate counts besides giving information on micro-colony formation. Identification of the bacteria is required, and Scott's (1953) method might be useful in determining the limits of a_w at which growth of these bacteria occurs. Preliminary tests have shown that Erwinia herbicola, Pseudomonas sp., Rhodotorula sp., and three different coryneform bacteria (all from stored barley) are more tolerant of high salt concentrations in the growth medium than are a variety of bacteria from other environments.

Such experiments are best designed to test a hypothesis

which fits the facts already established, and one which answers the queries posed above might be stated thus:

i) The early stages of imbibition of water by cereal seeds (corresponding to the first curve in the S-shaped adsorption isotherm) represent chemisorption by proteins and other macromolecules, and according to Hlynka and Robinson (1954) this water is not available for the metabolism of the seed nor of its microflora. If this is so, then the very early growth of bacteria at 100% R.H. was not stimulated by nutrients from the seed coat, as these would still be only partially hydrated and tightly bound. The nutrient source may have been intracellular food reserves, or cryptic growth, as described by Postgate (1967), may have occurred. As the microbial population of the seed coat had declined steadily during storage, and as lack of moisture would prevent microbial degradation of the dead cells, the survivors may well have used leakage and lysis products from these cells as nutrients for growth. Only limited growth would be likely, however, as Postgate (ibid) noted that the death of fifty Aerobacter aerogenes cells allowed the doubling of only one survivor.

ii) Saidel (1968), describing a mathematical model relating the development of bacterial cell populations to their environment, states:

> "From this model we find that if cells die of the toxic product, the stationary phase no longer appears; however if the cells die of a lack of nutrient, the stationary phase is present."

and "the rate of cell death is a function of ..... toxic product concentration."

The growth curves obtained in this experiment are therefore typical of populations in which there is a rapid accumulation of toxic waste products in the environment. In the absence of liquid water, this is just what one would expect. Griffin and Quail (1968) could detect no movement of Pseudomonas aeruginosa in soil or aluminium oxide at pF3.0, and concluded that bacteria need continuous water pathways for movement. Bacteria growing on a dry surface would therefore be unable to move away from their metabolic wastes, nor would these wastes be carried away from the immediate vicinity of the metabolizing cells. Staling is therefore the likeliest explanation of the rapid decline in numbers following growth, and may also be postulated as the factor limiting growth.

iii) At 95% and 100% R.H., bacterial numbers fell to very low levels at the time that Aspergillus and Penicillium populations were increasing. It is possible that an antagonistic relationship existed. Whether or not this was so, the fact that conditions of high humidity depressed bacterial numbers is unexpected in view of the following

statement by Semeniuk (1954):

> "Molds are the important agents of deterioration in grain .... at below 90% relative humidity ..... When the relative humidity is higher, bacteria become as important as molds. Bacteria, however, may also be important at seemingly lower moisture when the metabolic water from molds creates pockets of higher moisture."

iv) The mould count method did not show any significant increase in storage mould numbers for five to six weeks, but as the counts are very much affected by spore numbers, it is possible that spore germination and mycelial growth occurred much earlier. Moreover, even if the humidity enabled growth to take place, lack of nutrients might prevent extensive growth and sporulation until the seed moisture content reached a critical level. The relationship between relative humidity and seed moisture has led some authors to equate the two when discussing the deterioration of seed (Christiensen and Kauffman, 1965). However, the time lag between them might well result in the stimulation of microbial growth before the seed were sufficiently hydrated to act as a nutrient source. The 'critical level' of seed moisture for safe short term storage of cereals is said to be 15% (Milner and Geddes, 1954) and this level was not reached in the present instance until seven days had elapsed even at 100% R.H.

To summarize, the hypothesis is that as far as metabolism and cell growth are concerned, bacteria occur which are as xerophytic as the aspergilli commonly found on stored grain. In the absence of liquid water, however, these bacteria cannot be dispersed on the seed surface. Moreover, toxic wastes soon accumulate and the colony stales.

If this can be verified, it means that in the absence of liquid water bacteria are at a competitive disadvantage with fungi, regardless of their physiological capabilities. Ecological dominance requires direct or indirect occupation of space, and bacteria are inherently unable to colonize new substrates - to move away from their own wastes - other than in a film of water. This is not true of fungi. As J.S. Waid notes in a recent review (1968):

> "The uptake and accumulation of materials from the environment is achieved by the growth of hyphae from regions where nutrients are being depleted, or where staling substances are accumulating, to other regions."

It is possible, then, that when xerophytic storage moulds dominate the ecosystem of the cereal seed coat, they do so not only because they are xerophytic but because they are mycelial.

CHAPTER VII

SUMMARY AND CONCLUSIONS

This work was initiated by the finding of Perera (1966) that bacteria on stored barley were capable of multiplying under conditions which also favoured the growth of storage moulds. The possible existence of xerophytic bacteria had obvious practical implications, as well as being intrinsically interesting, and the primary object was to confirm Perera's result and establish the interrelations of the two groups on the seed surface. An ecological study should, however, deal with defined taxonomic units, and the nature of the bacterial flora was largely conjectural. Much of the work therefore concerned the identity of the bacteria found on barley seed.

The bacteria were characterized by a selection of morphological, cultural and biochemical tests, and the resulting data subjected to analysis by a computer system to derive the overall similarities of each isolate. The resulting groups were not all taxonomically valid, but they formed clusters of strains which were recognizable in different populations. As conventional classification would have resulted in fewer groups, including a collection of 'unknown' isolates, the use of the computer in this way was thought to be justified.

A survey of the kinds of bacteria present on sack-stored barley showed that the seed carried a limited and characteristic microflora. Erwinia herbicola was present in every sample, and two groups of coryneform bacteria (IV and IX) were also common. The only other bacteria of any significance were a third group of coryneforms (XI) and a loosely defined 'pseudomonad' group. Of the 1,106 bacteria isolated from stored seed, 44.6% were classed as E. herbicola, 37.7% as coryneforms, and 5.8% as 'pseudomonads'.

An ecological study of the seed microflora should take into account the establishment of this microflora - its development on the substrate from the time this first becomes available for colonization. This was studied in a plot of barley from the time of ear emergence until harvest. Yeasts tentatively identified as Rhodotorula were the first microorganisms to colonize the seed in large numbers, and they persisted on the seed coat until harvest. They were also numerous in some sacks of barley after five months in store.

As the yeast growth stopped, there was a second wave of colonization resulting from the rapid growth of bacteria. This reached its peak one week before the seed was judged to be field ripe, and numbers had fallen somewhat by harvest. The kinds of bacteria found were in general the same as

those found in stored seed although a group of gram-negative chromogens ('flavobacteria') and micrococci were much more numerous on the ripening seed. The 'pseudomonads' were comparatively late in developing on the seed, but were the most xerophytic in that they, and a small group of coryneforms, were the only bacteria to multiply in the final week of ripening.

Characterization of bacteria from ripening seed and from stored seed therefore showed the presence of numerous bacteria adapted to growth on this substrate and capable of surviving there. These bacteria were of two main kinds: a homogenous group of yellow, fermentative, gram-negative rods (E. herbicola), and an extremely variable coryneform group.

An attempt was made to study the interrelations of these bacteria and storage moulds by comparing the degree of mould infection in sterilized and unsterilized seeds during storage. An infestation of granary weevils made interpretation of the results equivocable, however. On previously sterilized seed attacked by the insects there was a decrease in bacterial numbers and a corresponding increase in Aspergillus glaucus. A. glaucus may have antagonized the bacteria on this seed but because of the apparent inactivity of the bacteria, the converse was thought to be unlikely on the unsterilized or the undamaged

seed, which was not infected by aspergilli.

This experiment confirmed that Sitophilus granarius can incite deterioration of stored grain by A. glaucus. Dissemination of the fungus by weevils was not found, but numerous bacteria of the kinds common on the seed coat were found on a minority of the insects.

Finally, the interaction of the bacterial and fungal populations was studied on barley kept at different atmospheric humidities. The results showed an increase in the numbers of bacteria at 75%, 95% and 100% R.H. Although the rate of increase was inversely proportional to the humidity, the maximum population attained was the same in each case, and at each humidity level it was immediately followed by a drop in numbers to levels approaching that of the control. After five to six weeks, at 95% and 100% R.H., the numbers of bacteria declined still further, and there was a corresponding increase in the numbers of Penicillium sp. and A. glaucus. These results were interpreted as showing that xerophytic bacteria do occur on stored barley, and that these can grow at activities of water as low as those required for growth of aspergilli. This ability does not enable them to compete with the moulds, however, as in the absence of liquid water toxic waste products probably accumulate and bring about the rapid decline in population noted in the

experiment.

In a broad sense, the projects described in this thesis achieved their aims insofar as the kinds of bacteria found on the barley caryopsis were identified, and the bacterial flora shown to be capable of growth in the absence of liquid water. As noted above, this ability appears to be of no advantage to the bacteria under the conditions of storage used, and their short-lived activity appeared to have no effect on the development of the fungi. However, some questions remain unanswered and new questions are posed by the results of this work. Perhaps the most obvious is the fact that the bacteria were only partially identified. Much work could still be done to further characterize those already isolated, and it is not known to what extent other methods of isolation would reveal completely different kinds of bacteria. Moreover, the bacteria enumerated in the controlled humidity experiment were not identified at all, and although it was clear that more than one kind was xerophytic, the lower limit of humidity at which they will grow is not known.

Other lines of work suggested by these results include a comparison of the saprophytic bacterial flora of seeds with those of other environments. Bacteria of the E. herbicola group are believed to be widespread in nature,

occurring on or in plant surfaces, decaying plant material, blood, urine, water, grass, air, human throats and the internal organs of deer (Graham and Hodgkiss, 1967). The fate of bacteria which persist on seeds is therefore of interest. Is this ecological niche normally a blind alley? Or are some organisms capable of growing with the germinating seed and colonizing the phyllosphere of the young plant? The work of Leben and Daft (1966) suggests that this might be the case.

In the particular case of malting barley, we do not know the significance of the bacterial flora in the malting process. Anderson, Gjertsen and Trolle (1967) point out that microbial growth on barley in the field, during storage, and in the malt house, is known to affect the quality of wort and beer. Fusaria in particular have been implicated in the production of 'off-flavour' and 'gushing' in beer but bacteria are known to increase considerably in number during steeping and subsequent germination of the grain, and the effect of this on the quality of the malt is not known.

The possibilities for further work of an ecological or physiological nature are indeed numerous, but this is not so in the field of plant pathology. The possibility of the saprophytic microflora of cereal grains interfering with the process of infection by pathogenic fungi is not ruled out by the results of the work described in this thesis.

Antibiosis might occur under certain conditions. The likelihood of its being a common occurrence is not great, however, and it appears that post-war North American work on the microbiology of grain storage has been correct in stressing the importance of seed/storage mould/environment interactions, and in ignoring the epiphytic bacterial flora as a factor in that environment.

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APPENDIX

Results of Tests Used to Characterize Bacteria

TABLE VIII. Results of Tests Carried Out on Isolates from Ripening Barley

	Percent. Isolates Positive for Character								
Group	I	II	III	IV	V	VI	VII	VIII	IX
CHARACTER / No. Isolates	42	112	56	27	9	21	1	9	68
rods	100	100	100	100	100	0	100	100	99
pleomorphic	0	0	0	0	0	0	0	0	34
'snapping division'	0	0	7	0	22	0	0	0	21
motile	100	100	100	100	100	0	0	0	18
gram stain	0	0	0	85±,15+	100	100	100±	100	50±,50+
Hugh & Leifson oxidative	100	0	0	4	0	0	0	100	0
Hugh & Leifson fermentative	0	100	0	0	100	62	100	0	0
catalase	100	100	100	100	100	38	0	100	100
acid from lactose	7	18	71	100	0	0	100	0	0
sucrose	11	89	75	100	0	0	100	100	100
salicin	10	80	25	100	0	0	0	0	0
inositol	10	79	56	85	0	0	0	0	100
rhamnose	10	80	67	100	0	0	0	0	72
effect on milk	81K	93A	44A,52K	85A	44K	38K	100A	56K	85K
gelatin liquefaction	95	89	86	85	44	0	0	0	72
reduction of nitrate	0	100	56	33	0	0	100	0	16
pigmentation	7Y,7P	100Y	84Y,7P	96Y	22Y,33R	57R	100P	100R	19Y,31R,6P
mucoid growth	0	29	0	0	0	0	100	78	13

K= peptonization A= acid production

Y= yellow R= orange P= pink

TABLE IX. Results of Tests Carried Out on Isolates from Store: February

CHARACTER	Percent Positive / Result of Test			
Group	I	II	III	V
No. Isolates	13	55	1	1
rods	100	100	+	+
motile	100*	100**	+	+
gram stain	0	0	-	+
oxidative utilization of glucose	100	0	-	-
fermentative	0	100	-	+
catalase	100	100	+	+
oxidase	0	0	-	-
acids from lactose	0	0	-	-
sucrose	0	100	-	-
salicin	0	100	-	-
inositol	0	63	-	-
rhamnose	0	100	-	-
effect on milk	100K	87A	-	-
gelatin liquefaction	100	100	-	+
reduction of nitrate	0	100	-	-
H_2S production	0	0	-	-
starch hydrolysis	0	0	-	-
growth in NaCl: 6%	100+	100	+	+
10%	0	100	-	-
growth in Ayre's medium	100	100	+	+
pigmentation	0	100Y	Y	-
mucoid growth	0	27	-	-
water soluble pigment	100	0	-	-

* 3 isolates shown to have single polar flagella

** 5 isolates examined and shown to be peritrichously flagellated

K= peptonization
A= acid production
Y= yellow

TABLE X. Results of Tests Carried Out on Isolates from Store: July.

CHARACTER		Percent. Isolates Positive for Character						Result of Test		
	Group	I	II	III	IV	V/VI	IX	IIj	X	IIIj
	No. Isolates	33	134	14	106	43	7	1	2	3
rods		100	100	100	100	91	100	+	+	+
pleomorphic		0	0	0	0	0	100	-	-	+
'snapping division'		0	0	0	4*	9	14	-	+	-
motile		97	100	100	77	40	57	+	±	-
gram stain		0	0	29±	53±,38+	40±,60+	100+	-	+	-
Hugh & Leifson	oxidative	94	0	0	0	25	43	-	+	-
	fermentative	6	100	36	0	7	0	+	-	-
catalase		100	100	100	100	95	100	+	+	+
oxidase		9	0	0	48	2	29	-	-	+
acid from lactose	P.R.	24	41	100	81	86	86	+	+	-
	B.B.	3	3	•	•	•	•	•	•	•
sucrose	P.R.	33	100	100	97	95	100	+	+	-
	B.B.	9	92	•	•	•	•	•	•	•
salicin	P.R.	0	100	100	73	78	86	+	+	-
	B.B.	0	80	•	•	•	•	•	•	•
mannitol	P.R.	39	100	100	76	86	100	+	+	-
	B.B.	9	100	•	•	•	•	•	•	•
effect on milk		100K	92A	29K,57A	25A	35K,30A	100K	A	A	K
gelatin liquefaction		88	82	100	57	42	100	-	+	+
reduction of nitrate		15	100	0	27	12	0	+	+	-
pigmentation		21Y	100Y	57Y,43P	49Y,39R,11P	28Y,58R,12P	29Y,71R	-	P	-
mucoid growth		6	15	100	57	42	71	-	+	-

K= peptonization A= acid production Y= yellow R= orange P= pink

PR= PhenolRed indicator BB= Bromthymol Blue indicator •= not tested

* 42% showed palisade formation in gram-stained smear but not in wet mount.

TABLE XI. Results of Tests Carried Out on Isolates from Store: November.

CHARACTER	Percent. Isolates Positive for Character			
Group	I	II	XI	IX
No. Isolates	15	140	13	127
rods	100	100	100	100
pleomorphic	0	0	0	1
'snapping division'	0	0	100	14
motile	93	96	100	40
gram stain	0	0	46+,54±	78
utilisation of glucose oxidative	93	0	38	5
utilisation of glucose fermentative	0	100	23	0
catalase	100	100	100	100
oxidase	33	0	23	0
acid from lactose	0	27	8	0
acid from sucrose	7	100	8	5
acid from salicin	7	99	0	0
effect on milk	93K,7A	90A	92K	80K
gelatin liquefaction	100	92	85	77
reduction of nitrate	0	99	23	15
pigmentation	13Y,7R	89Y	46Y,15R	45Y,30R,16P
mucoid growth	20	6	0	46

K= peptonisation A= acid production
Y= yellow R= orange P= pink

TABLE XII. Results of Tests Carried Out on Isolates from Untreated Seed

CHARACTER	% Positive July		% Positive November	
Group	I	II	I	II
No. Isolates	15	55	8	32
rods	100	100	100	100
motile	97	100	100	100
gram stain	0	0	0	0
utilization of glucose oxidative	100	0	100	0
utilization of glucose fermentative	0	100	0	100
catalase	100	100	100	100
oxidase	53	0	0	0
acid from lactose	7	9	0	6
acid from sucrose	7	98	0	100
acid from salicin	0	100	0	100
acid from inositol	0	91	0	94
acid from rhamnose	7	100	13	94
effect on milk	87K	91A	100K	100A
gelatin liquefaction	100	95	100	94
reduction of nitrate	13	100	13	100
starch hydrolysis	20	0	0	0
growth in NaCl 6%	100	100	100	100
growth in NaCl 10%	20	89	25	97
growth in Ayre's medium	67	100	50	97
pigmentation	0	100Y	0	100Y
mucoid growth	0	15	0	25
water sol. pigment	34	0	50	0

K= peptonization A= acid production

Y= yellow

TABLE XIII. Results of Tests Carried Out on Isolates from Sterilized Seed

CHARACTER	Percentage Positive Results: July							Percent Positive: November		
Group	I	II	III	V	VII	X	IIj	II	III	V
No. Isolates	2	2	12	11	2	7	7	38	1	1
rods	100	100	100	100	100	100	100	100	100	100
motile	100	100	58	0	0	71	100	100	100	100
'snapping cell division'	0	0	0	100	0	100	0	0	0	100
gram stain	0	0	0	100	100±	100	0	0	0	100
utilization of glucose: oxidative	100	0	0	0	0	43	0	0	0	0
utilization of glucose: fermentative	0	100	0	0	100	0	100	100	0	0
catalase	100	100	100	100	50	100	100	100	100	100
oxidase	100	0	17	0	0	14	0	0	0	0
acid from lactose	0	20	50	0	50	71	100	58	100	0
acid from sucrose	50	100	100	0	100	86	100	100	100	0
acid from salicin	0	100	8	0	0	57	100	100	0	0
acid from inositol	0	100	50	0	0	86	100	95	0	0
acid from rhamnose	0	100	50	0	50	86	100	92	0	0
effect on milk	K	A	66A	K	0	86K	A	A	0	0
gelatin liquefaction	100	100	58	100	0	43	100	95	0	100
reduction of nitrate	0	100	0	0	0	100	100	97	0	0
starch hydrolysis	0	0	0	0	0	14	0	0	0	0
growth in NaCl: 6%	100	100	83	100	100	100	100	100	100	100
growth in NaCl: 10%	0	100	0	100	0	71	0	76	0	0
growth in Ayre's medium	50	100	0	0	0	86	100	97	0	0
pigmentation	0	Y	75Y	R	P	57R,43P	0	Y	Y	R
mucoid growth	0	0	8	100	0	57	0	5	0	100
water-soluble pigment	0	0	0	0	0	0	0	0	0	0

K= peptonization A= acid production Y= yellow R= orange P= pink

TABLE XIV. Results of Tests Carried Out on Isolates from Weevil-Damaged Seed

CHARACTER	Percentage Positive Results									
	Untreated Seed				Sterilised Seed					
Group	II	IV	VI	VIII	II	III	V	VI	VII	VIII
No. Isolates	35	2	1	2	4	4	7	6	15	4
rods	100	100	0	100	100	100	100	0	100	100
motile	100	100	0	0	100	100	71	0	0	50
'snapping cell division'	0	100	0	0	0	0	71	0	0	0
gram stain	0	100	100	100	0	0	100	100	40+,60±	100
utilization of glucose oxidative	0	0	0	100	0	0	0	0	0	100
utilization of glucose fermentative	100	0	0	0	100	0	14	0	100	0
catalase	100	100	100	100	100	100	100	100	93	100
oxidase	0	0	0	0	0	25	14	30	20	0
acid from lactose	14	0	0	0	0	100	0	0	100	50
acid from sucrose	97	50	0	100	100	100	29	0	100	75
acid from salicin	97	0	0	50	100	100	0	0	66	0
acid from inositol	91	0	0	50	100	100	0	0	66	25
acid from rhamnose	91	0	0	50	100	100	14	0	80	75
effect on milk	86A	50K	0	50A	A	A	K	0	53A,40K	50A
gelatin liquefaction	86	0	0	0	100	100	29	0	13	25
reduction of nitrate	100	0	0	0	100	100	14	0	27	0
pigmentation	Y	R	R	R	Y	Y	29Y,71R	57R	93P,7R	50R
mucoid growth	20	0	0	50	0	0	0	0	53	0

K= peptonization A= acid production Y= yellow R= orange P= pink