Seedling Blight of Barley caused by Fusarium spp. and Helminthosporium spp. and its control with special reference to the Action of Ethyl Mercury Phosphate (New Improved Ceresan.)

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A thesis presented to the University of Glasgow in fulfilment of the requirements for the degree of Doctor of Philosophy, with three additional papers.

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C/o n t e n t s

1. THESIS.

Seedling Blight of Barley caused by Fusarium sp. and Helminthosporium sp. and its control, with special reference to the action of ethyl mercury phosphate (New Improved Ceresan).

With Three Appendices.

2. ADDITIONAL PAPERS.

a. The Degeneration of Metropolitan Bent, Agrostis stolonifera.

b. The Interaction of two Fruit-Rotting Fungi, Oospora citri-aurantii and Penicillium digitatum.

c. A study of the transfer of fixed Nitrogen from the nodule to the plant in the garden pea, Pisum sativum L.

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Introduction.

Diseases of plants have been recognised for many centuries, references to them being found in classical writings such as the Bible, e.g. in Genesis 41, verse 23, and also in writers such as Aristotle and Theophrastus. In such books plant diseases are generally referred to as 'blastings', 'mildews', 'blights' etc., but from the description one can recognise rusts and smuts and mildews. Since primitive society was so completely dependent on agriculture and because of the nature of the loss caused by these diseases in extreme cases, it was therefore only natural for some method of control to be sought, and its conditions fulfilled if possible.

This usually took the form of various religious rites and ceremonies, such as planting 'in the dark of the moon' and the ancient Roman invocations and ceremonies to the Rubigalia or Rust Gods, but Pliny in his books 17 and 18 describes a series of more practical measures for the ensurance of the safety of the crop from rust, such as

the early sowing of the grain to allow it to ripen before the disease appears. As time went on however, the experience of centuries evolved a series of practical, if empirical, measures of disease control and it is obvious that the question of seed and its bearing on the resultant crop would be one of the first to be asked. This problem of seed disinfection is still very incompletely understood, for not only is one faced with the action of the disinfectant, but the response of the host plant, of the pathogen, and of the host-plant-pathogen complex all enter the problem to complicate it still more.

It is obvious that one cannot expect a simple solution of so complicated a problem, and there will require to be much research done on the nature of parasitism and disease before very many seemingly aberrant results from seed disinfection can be fully appreciated and finally harmonised with theory.

In the present study an attempt will be made to obtain some information which might help to elucidate the mode of action of a typical dust disinfectant, New Improved Ceresan in this case, on a typical seed-borne disease, namely seedling blight of barley caused by Fusarium spp. and Helminthosporium spp.. The question as to what property of the disinfectant is responsible for its toxic action will not be considered, but the method by which it

comes in contact with the fungus protoplasm, e.g. as a gas or as a liquid will be examined, together with the effect of various environmental factors on the effectiveness of the disinfectant when used against the disease in question. This question of the mode of contact is very important as until it is finally answered, the manufacture of new disinfectants will continue to be a matter of trial and error, both costly and requiring much time.

Incidental to this study of the action of the disinfectant, an ecological study of the seedling blight of barley will also be undertaken, with particular reference to the disease under various experimental environments, for as the disease varies it is likely that the effectiveness of the seed disinfectant will vary in proportion.

History of Seed Disinfection.

One of the earliest methods of seed disinfection was to steep the seed in some so-called therapeutic liquid such as wine or urine. This method was used as early as the middle of the seventeenth century in England, where seed was dipped in brine to prevent 'smuttiness'. Tull (26) recorded how in 1673 a ship laden with corn had been wrecked near Bristol, and when the grain was salvaged and sown, it was found to be very clean and disease-free. This experience started the practice of dipping grain in the

sea before sowing, although Tull was himself suspicious of the practice and wrote that the grain "might not have been smutty the next year though it had not been soaked in sea water."

An advance on this method was made by Schulthuss (24) in 1761 when he recommended the use of copper sulphate in place of salt, and that this recommendation was sound experimentally was shown by Prevost (22) in 1807 who observed the germination of smut spores in water and showed that this germination could be prevented by the presence of copper sulphate.

As copper was found to have an injurious effect on the grain, a search was then made for a more efficient substitute. For a time sulphur was used as the best seed treatment following the experiments of Kellerman and Swingle (14) in 1890. In the same year Bolley (1) had advocated the use of mercuric chloride as a dip for seed potatoes for the control of potato scab, but some results of Kellerman and Swingle (14) as to the efficiency of mercuric chloride as a seed disinfectant for cereal crops mitigated against it, and the introduction of formaldehyde caused it to disappear from popular use.

Formaldehyde was first used as a seed disinfectant by Geuther (10) in 1895, and it gained much popularity in the United States through the work of Bolley (2) in 1897.

Its use was very widespread during the War years when there was a great scarcity of copper and the use of copper sulphate was discouraged by the governments. Formaldehyde however suffers from some disadvantages in that it is very unpleasant to use, and that seed unless sown immediately after treatment is liable to a severe form of injury which prevents germination. This has been attributed by McAlpine (18) to a hardening of the seed coat caused by the formaldehyde, but Hurd (12) on the other hand, believes that this injury is the result of the formation of a toxic polymer of formaldehyde, namely paraformaldehyde. Thus in both cases, the seed must be sown at once or dried in order to prevent this injury.

In the years 1915-1918 two very important advances were made by Darnell-Smith (6), namely the development of copper carbonate and the use of this chemical as a dust. The copper carbonate of commerce is a very inexact description of a mixture of salts formed by the reaction of copper sulphate and sodium carbonate - the main constituent of this mixture being a basic copper carbonate with the formula $CuCO_3.Cu(OH)_2$. There are other methods of preparation such as the use of calcium carbonate instead of the sodium salt, but this gives a less effective end-product. Previous to the work of Darnell-Smith the use of copper carbonate had been advocated by von Tubeuf in 1902, but its

great disinfecting power was only fully realised when used as a dust by Smith to control stinking smut of wheat - Tilletia tritici (Bjerk.) Wink.

The advantages of a dust over a liquid steep for seed treatment are well known, but are worth emphasising. In the first place the disadvantages inherent in the use of large quantities of water are avoided, as well as the disappearance of farther hazards such as the freezing of damp seed and the extreme difficulty of storing it and drying it before sowing. There is a great practical difficulty in sowing damp seeds as they tend to stick together and so will not run through the drill. There is a tendency for dusted seed also to stick in the drill but the addition of a little graphite to the seed overcomes that difficulty, and with the advent of the extremely finely divided dusts such as New Improved Ceresan and Agrosan G this difficulty does not even arise. As a farther advantage of dusting over steeping, there is much less work required by the former, and dusted seed can be stored for a reasonable time without much injury ensuing.

The modern organic mercury compounds date back to 1914 when Riehm (23) published results on the control of bunt by the use of 'chlorphenol mercury'. This compound was used as a steep and in 1915 was placed on the market by Fr. Bayer under the trade name of 'Uspulun'. This

compound, according to Martin (17), has probably the formula $Cl(OH)C_6H_3.HgOSO_3Na$, and contains approximately 18.8 per cent metallic mercury. The immediate success of this fungecide led to the production of similar mercury salts by the larger chemical industries of the world, namely the Imperial Chemical Industry of Britain and Du Pont de Nemours of the United States.

It was an obvious advance when in 1924 Bayer introduced an organic mercury disinfectant as a dust under the name of Tillantin R in Europe and Bayer Dust in America. This dust is described as an ortho-nitro-phenol mercury derivative, possibly an hydroxide and containing 3.4 per cent of metallic mercury. Such dusts usually consist of a filler or carrier which increases the bulk of the compound, and being in a very fine state of division allows the active agent which it carries, the maximum of surface area from which to act. As the majority of such dust compounds are poisonous or very unpleasant to work with, an oily substance is added which prevents the dust from flying in the air while not interfering with its action on the fungus. There is usually also a trace of some dye in the mixture, possibly to enable the farmer to distinguish between treated and untreated seed, and where large quantities of seed are treated as in the grain elevators of Western Canada and the United States, the

colour of dusted seed unables an expert to estimate when the correct proportion of seed and dust have been used.

The most recently issued and the best dusts at present are New Improved Ceresan containing 1.3 per cent mercury in the form of ethyl mercury phosphate and sold by Du Pont in the United States, Agrosan G containing 1.5 per cent mercury in the form of tolyl mercuric acetate and produced by Imperial Chemical Industries, and a European Ceresan produced by the I.G.Farbenheit A.G. whose active constituent is described as 'an ether of a mercurated alcohol' by Bonrath.

That such dusts are extremely effective is not to be denied, but the advance has been made by the chemists of the great industries directly concerned, and it is a pity that the enormous amount of data which has been accumulated by these concerns is not available for the use of scientists in universities and scientific institutions, but is kept in secret in the files of the industries directly involved where it in no way promotes the cause of science. Such secrecy as this tends to defeat rather than promote its own ends as it is only by the use of many brains on the data that its full significance and possible implications can be discerned.

Literature Review.*

The beneficial effects of seed treatment with the

* See also Appendix A.

organic mercurial dusts are very fully described in the literatures by such workers as Lambert, Rodenhiser, and Flor (16), and O'Brien and Dennis (20), and the faith placed in such methods is examplified by the fact that such a type of disinfection is recommended in Government publications in this country, on the Continent of Europe and in the United States. Farmers themselves are able to recognise the value of such treatment and many seed firms sell their seed wheat, oats etc. after it has been treated.

While the action of the dust in disinfecting the seed is widely recognised, there has been very little research done on the mechanism of such an action. The problem of the actual avenue of approach of the killing agent is extremely difficult on account of the large number of complicating factors in the host-parasite complex, while the question of the mechanism of toxicity, although it has received more attention, is as yet unanswered. Of late in their explanatory leaflets, industrial concerns have been stressing the point of volatility, and indicating that their research shows that the action of the disinfectant occurs in the gaseous state. Thus New Improved Ceresan or ethyl mercury phosphate is more volatile than the older Ceresan which was ethyl mercury chloride, and the increase in efficiency in the former is explained on the volatility basis.

The spores of the fungus are usually carried on the inside of the glumes, and the coleoptile of the plant in the cereals pushes between these as it emerges, and in so doing becomes infected and diseased.

As this space in the seed is very small, it is postulated that the only way the disinfectant can gain access to it, and so to the fungus spores is as a gas, hence much emphasis is placed by manufacturers on the point of volatility.

The alternative theory to this is that the disinfectant goes into solution and gaining entrance to the chamber by capillarity can spread over the surface of the spore and so kill it. Practically no experimentation has been made to dettle this point, but Dillon-Weston and Booer (7) after a series of experiments designed to seek a relationship between chemical structure in the organic mercurials and toxicity, state that there is a killing action due to the vapour but that it is by no means the main agent. Cunningham (5) states that 'no fungicidal action occurs until the seed is sown in moist soil, when the soil moisture and organic acids present therein, render soluble quantities of the therapeutant sufficient to inhibit spore germination, or protect emerging roots or plumules from mycelial invasion from contiguous soil fungi.'

The problem is therefore far from settled, with the industrial firms stressing volatility on the one side and such authorities as Dillon-Weston and Cunningham laying great emphasis on solubility and the liquid phase. It is natural therefore that attempts be made to settle this dispute by showing one side, or may be both sides in the right.

Material and General Methods.*

The Barley seed used in this series of experiments was obtained from Professor J.J. Christensen of the Department of Plant Pathology at the University of Minnesota, and had been grown on a farm near Morris, Minnesota. It was of the variety 'Odessa' and was known by previous examination to be very highly infected with the organisms causing seedling blight. The New Improved Ceresan was purchased in the ordinary way from an agricultural dealer, and was applied to the seed at the recommended rate of one half ounce per bushel. The disinfection was done in lots of one pound weight, a proportionate amount of disinfectant being added, then the seed and disinfectant were shaken in a glass bottle for about 2 minutes before being used. As far as possible, and unless the experiment demanded otherwise, the seed was disinfected and sown on the same day.

* See also Appendix B.

The details as to methods used in the various experiments with such factors as temperature and moisture will be found in the sections of this paper which actually deal with those factors. The number of seed sown for each environmental state was at least two hundred and the experiments were each repeated twice and in some cases three times.

It must be noted that figures given for percentages of disease can only be compared within the experiment from which they were obtained as environmental conditions not only cause variation in the degree of control by the disinfectant but also cause marked variations in the amount of disease obtained in untreated controls. It follows therefore that the disease which does appear in disinfected seed is the result of two factors, viz. the effect of the environment on the efficacy of the fungicide and also the effect of the environment on the seedling blight. The ecology and epidemiology of the disease are therefore of major importance, and a considerable amount of time must be spent in each section describing the effects of the particular environment on the incidence of the disease, before essaying to draw any conclusions as to the action of the fungicide.

Seedling Blight of Barley.

(a) History and Causal Organisms.

That some barley seed when sown would only germinate very poorly and that most of the emerging plants would be weakly, has been known for a long time. When attention of scientists was drawn to this fact, not only true for barley but also for other cereals, it was soon shown that the lack of germination and the weak plants were the direct result of the attack of various fungi. Pammel, King, and Bakke (21) in 1910 isolated some species of Helminthosporium from blighted barley and showed that a large percentage of the blight or foot rot was due to one of these which they called Helminthosporium sativum, P.K.B.. Since 1910, many graminicolous species of Helminthosporium have been described, especially by Nisikado (19) and Drechsler (9), and the perfect stage of H.sativum has been described by Kuribayashi in 1929 as Ophiobolus sativus (15).

Other fungi have been isolated from diseased plants, for in many parts of the world species of Fusarium are very common parasites of barley, and in Russia the presence of these fungi on the grain causes the appearance of symptoms of intoxication and poisoning in those who chance to eat bread made from the flour. The most common organism causing seedling blight in Russia is Fusarium avenaceum

(Fr.) Sacc., while in Finland and South Russia it is F. graminearum Schwabe (8).

A critical examination of diseased seed and plants by Christensen and Stakman (4) in 1935 revealed the presence of many other fungi and of other species of Fusarium and Helminthosporium. Some of these other fungi, such as Rhizoctonia, Sclerotium, Gliocladium, could actively parasitise the plants under suitable conditions, while many species of Alternaria and Penicillium etc. were seemingly an ever-present fraction of the microflora of the seed but an essentially saprophytic one and here almost non-injurious. As well as the fungi named above, these workers isolated many species of Trichoderma, Mucor, Rhizopus, Pythium, Aspergillus, Cepholothecium, and Verticillium, many different bacteria, Actinomycetes, and yeasts.

(b) Symptoms and Etiology.

As barley seedling blight is often referred to as 'root rot' or 'foot rot', it is only to be expected that one of the most typical symptoms would be a rotting of the roots and the base of the stem. The young seedling as it emerges from the seed is attacked by the fungus or fungi which results in a brown discoloration of the stem base. This may be very severe, in which case the stem rots through and the young plant dies, or in exceptionally bad attacks

the seedling may never get above ground if the actual growing point is parasitised and killed. Usually the attack is not so deadly, but in all cases the base of the plant is weakened, roots are also attacked and their function impeded if not destroyed, and the resultant plant is very unthrifty.

It is difficult to distinguish between the symptoms of Fusarium attack and those of Helminthosporium attack, however if the disease is not too marked and all the tissues are not browned, then it is possible to effect a rough separation on the basis of colour and appearance of the lesions. The Fusarium rot is usually of a lighter brown colour, a little softer in outline and gradually fading into the normal white colour of the stem, while the Helminthosporium causes a more chocolate brown discolouration, usually sharply delimited and not so generally distributed as that of the Fusarium. There is also a tendency for the lesions caused by Helminthosporium to occur in streaks or lines running parallel to the stem axis, as opposed to those of the Fusarium spp. which are more general in their nature and usually encircle the whole stem or a large part of it. See Fig.1.

Very often, especially if growth is slow, leaf lesions may be developed. These are usually on the first or

second leaf and are purplish-brown in colour, with a reddish or light brown margin. Such lesions are elongated in the direction of the veins of the leaf and are usually caused by the Helminthosporium spp. in the complex, especially H.sativum.

The major source of infection in all cases is the seed, which is itself infected by wind blown conidia from secondary leaf lesions at, or just prior to, the time of harvesting. There can be infection from the soil but the likelihood of this is not very great on account of the antibiotic effect of the natural microflora and microfauna of the soil limiting the growth of these foot rotting fungi to a very marked degree. This was demonstrated by Henry (11) who observed that seedling injury caused by Fusarium and Helminthosporium spp. was much reduced by reason of the antibiotic effect of the soil organisms, while O'Brien and Dennis (20) showed that Helminthosporium avenae Eidam could only grow on sterilised soil but not on naturally occurring field soil, and gave the same reason.

The grain produced on such plants as have survived an attack of the disease is very light in weight, and yields are reduced to a minimum. Although the disease is not systemic and has therefore no way of getting from the seed in the ground to the ear, still the presence of so

many centres of possible infection around the developing grain will probably result in a more highly infected yield of seed than that grown in a field of clean barley.

When a disease such as seedling blight is caused by a complex of fungi, it is more likely that it should be able to develop under a wider range of environmental conditions than would be the case were it the result of the attack of a single pathogen. This is true where seedling blight of barley is concerned for it could develop its symptoms under all the environmental conditions produced, although these symptoms were not always of the same kind. In plants grown at lower temperatures there is a predominance of the symptoms of the Helminthosporium with a noticeable increase in the amount of leaf lesion, while at high temperatures, leaf lesions are practically absent and there is the severe general rotting of the stem base caused by the species of Fusarium.

Effects of New Improved Ceresan* on the Incidence of the Disease.

(a) Plating experiments.

Generally speaking the percentage of disease can be very materially decreased by the treatment of infected seed with New Improved Ceresan. Over a large number of experiments involving some hundreds of thousands of plants,

* See also Appendix C.

the average percentage disease in untreated seedlings was 47 per cent while in the treated seed the disease was 1.8 per cent on the average.

That the disinfectant lowers the actual amount of fungal growth either in or on the seed has been demonstrated from a series of plating experiments. Christensen and Stakman (4) as has been described above, plated a large number of barley seeds and diseased stems, roots, etc., and demonstrated that while species of Alternaria were the most common constituent of the microflora of the disease-carrying seed, imparting a blackish green colour to the tips of the husk, still the percentage of disease in the crop grown from such seed was directly proportional to the percentages of Fusarium and Helminthosporium on the seed. By growing crops of plants from diseased seed to maturity, and by gathering and weighing the crop, they also showed that not only was the amount of disease proportional to the percentages of Fusarium and Helminthosporium on the seed, but the yield of grain and of straw bore a similar relationship.

Platings of the seed used by the writer in his experiments were accordingly attempted, and not only was the variety 'Odessa' used in the experiments, but other two varieties also, namely 'Swansota' and 'Minsturdi'. Neither

of the latter two samples was as heavily infected as the original Odessa, but it was thought advisable to use more than one variety to test the universality of application of the results.

In all cases platings were made on acidified potato-dextrose agar, as this medium was found to be the most suitable for abundant growth of most fungi, and the addition of a little lactic acid served to keep the numbers of bacterial colonies within reasonable limits. A farther advantage of this medium was that most fungi would sporulate very readily on it, and could thus be recognised in situ obviating the necessity for transfer of the colony to some other agar where sporulation could be more easily induced.

The seeds were selected at random from the sample of barley used throughout the entire experiment, and were first of all dipped in absolute alcohol to clean them and to aid penetration of rough places on the surface by the next wash. From the alcohol they were then soaked in corrosive sublimate-(1-1000 mercuric chloride) - for two or three minutes, followed up by a wash in a solution of sodium hypochlorite which had about 5 per cent of available chlorine. The purpose of this last wash was to remove the mercuric chloride and so allow the fungi underneath the

seed coat and between the glumes to grow out. Originally, sterile distilled water was used for this last wash, but it was found that many colonies of bacteria grew from the seeds, and it was very difficult to keep the water sterile for the length of time required to plate some hundreds of seeds. The solution of hypochlorite acts as a wash for removing the corrosive sublimate and being a mild disinfectant in itself keeps the seeds quite surface sterile until they are placed on the agar. The plating was done in ordinary eight centimetre petri plates, ten seeds being placed on each plate with sufficient space between them to allow the development of the fungal colonies, and twenty plates, i.e. two hundred seeds in all constituting a sample.

The fungi were identified when the colonies were about four centimetres in diameter, that is after about five days of incubation at 70°F.. For identification the lid of the petri-plate was removed and the colonies examined under the low power of the microscope. Where the fungus could not be named on first sight, subcultures were taken from the original colony and grown in tubes on a variety of media until sporulation had been induced on one or more of them. In this way many fungi were identified and it was found that very often there was more than one fungus per seed so that figures for percentage infection total to more than one hundred per cent.

At first an effort was made to identify all the fungi thus isolated, but latterly only the numbers of colonies of Fusarium and Helminthosporium were noted specifically, the remaining colonies simply being counted and classed as 'other fungi' and 'bacteria'. This was done, as Christensen and Stakman (4) had shown that for practical purposes and under field conditions, the other fungi could be regarded as non-parasitic.

The plating technique was used for both disinfected and natural seed, and in this experiment a quantity of Swansota seed was treated with the equivalent amount of old Ceresan or ethyl mercury chloride, for the purpose of comparison. The results are tabulated in Table 1.

Table 1.

Colony counts on treated and untreated barley seed.

Variety	Treatment.	Percentage number of colonies of -			
		Helmintho-sporium.	Fusarium	Other fungi	Bacteria.
Odessa	None	60	5	88	22
"	New Imp. Ceres.	23	4	29	31
Minsturdi	None	6	7	95	8
"	New Imp. Ceres.	0	0	9	14
Swansota	None	6	6	68	19
"	New Imp. Ceres.	0	1	9	17
"	Old Ceres.	4	2	38	29

A number of conclusions may be drawn from the above figures which are of some interest. It is only to be expected that the amount of infection would vary from sample to sample, and of the varieties of barley used, Odessa was by far the most heavily infected with Fusarium and Heminthosporium, followed by Minsturdi which however was not much different from Swansota. In all these samples, treatment with New Improved Ceresan materially reduced the number of resultant pathogenic colonies, notwithstanding the facts that the seed had been treated only two days before plating in the case of Odessa and one day in the case of the other two, and furthermore that the surface disinfection with alcohol, corrosive sublimate, etc. had removed all of the disinfectant and only the little that was carried in as solution in the washing or that obtained entry as particles of gas would be able to carry out the disinfective action.

In the case of Odessa where the amount of Helminthosporium and Fusarium was reduced from 65% to 27%, the seeming lack of complete control was not shown in seed sown in the greenhouse, in which the amount of disease was reduced from 62% to .5%. The difference is probably due to the longer period of action of the fungicide when the seed is sown in damp earth and the higher concentration of

the solution of New Improved Ceresan in the soil coupled with its presence as a disinfecting agent for the growing point of the stem as it emerged from the seed carrying the young mycelium of the fungus.

It must also be noticed that the number of saprophytic fungal colonies is also materially reduced and the importance of this fact will be discussed later in the paper. One rather strange fact was that the number of bacterial colonies from treated seed showed a marked increase over those from untreated seed. Very careful examination, however, showed that where fungus colonies were growing strongly the bacterial colonies were practically suppressed and could only be seen as a faint clouding of the agar while the fungus was very young. Thus it was possible to miss the presence of bacteria in plates where the fungi were growing rapidly and well, and it is certain that could all the bacteria have been seen equally well in all the plates, this apparently anomalous increase would not have occurred. This masking effect could have occurred with the fungi also, but Helminthosporium and Fusarium both form such distinctive colonies and spores, that although a small colony of one of the non-sporulating fungi might have been missed, the examination of the whole colony from each seed was sufficiently thorough for absol-

ute reliance to be placed on the figures given for the pathogenic fungi.

The difference in the efficiency of New Improved Ceresan and old Ceresan is quite marked especially in the column headed 'other fungi'. That the old Ceresan has a disinfective action is quite clear, but it is also clear that it is by no means as effective as the more recently issued fungicide, New Improved Ceresan, and does not afford such complete protection to the seed. This may be due to greater volatility of the latter product as the makers claim, or it may be due to a greater solubility, or, most obvious of all, the New Ceresan may be actually more toxic than the older product. The last supposition is contrary to the findings of Dillon-Weston and Booer (7), who using a large number of organic mercury compounds came to the conclusion that the toxicity was independent of the acid radicle. They represented all organic mercury salts with the general formula R-Hg-X, where 'R' was the organic radicle and 'X' the acid radicle, and stated that toxicity increased with decrease in the molecular weight of 'R' but was independent of 'X'. New Improved Ceresan is ethyl mercury phosphate, and Ceresan is ethyl mercury chloride, therefore if Dillon-Weston and Booer are correct they should be equally toxic, which obviates the third argument above.

This work on toxicity of organic mercury salts has not been repeated so far, and so is not quite accepted yet, but it does seem likely that other properties such as solubility and volatility would enter into the question of the relative toxicity of these compounds to fungi, for if a substance is to be toxic it must come into intimate contact with the protoplasm and this can be accomplished only as a solution or as the particles of a gas.

(b) Temperature experiments.

When it was decided to conduct research on the action of New Improved Ceresan under various environmental conditions, temperature was naturally one of the first factors to be given consideration. L.R.Jones, Johnson, and Dickson (13), working with extremely accurately controlled soil temperatures in the Wisconsin temperature tanks have shown the great importance of soil temperature when dealing with practically all plant diseases. Not only is the soil temperature important in the initiation of infection, but the development of that infection is often governed by the condition and growth of the plant which in turn can be very much modified by the soil temperature.

In the case of the problem of seed disinfection, a farther factor complicates the question, namely the temper-

ature at which the treated grain is stored.—Usually a farmer buying barley for seed does not wish to have the trouble of treating it in the spring which is his busiest time of the year, with the result that it is very often disinfected in the autumn or early winter and stored until the time for sowing. This storage is often carried out at very low temperatures especially in the North Western United States where the thermometer may fall as low as forty degrees below zero. It was therefore decided not only to carry out soil temperature tests but also to carry out storage temperature tests.

For the storage experiments a series of temperatures were used ranging from $-5^{\circ}C.$ to $+30^{\circ}C.$ These temperatures were electrically controlled in incubators and the seeds were stored for 25 days, taken out and sown at once. The seed, Odessa, was disinfected with New Improved Ceresan immediately before it was put into the storage chambers, about one thousand seeds of both treated and untreated samples being stored in each chamber. In the chamber the seed was spread out in a thin layer on the bottom of a large petri-plate to overcome any tendency for the seed to vary in temperature as it might have done, had it been stored in a heap.

On removal from the storage chambers the seed was

sown in sterilised soil in sterilised pots 10 cms. in diameter, at the rate of 20 seeds per pot, 20 pots per sample, i.e. 400 plants were grown from each set of seed, treated and untreated from each temperature chamber. The plants were grown in a greenhouse at 75°F. and were watered every day. The pots were randomised in their distribution in the greenhouse so that any irregularity in the watering would be obviated.

The plants were uprooted 20 days after sowing when they were about six inches high and when symptoms of seedling blight were apparent on the controls but before there was a chance of secondary infection of healthy plants spreading from diseased ones and so spoiling the counts. The plants when uprooted were washed individually, the coleoptile, stem base and roots were examined for root rot discolouration and results tabulated, see Table II.

Table II.

Seedling Blight on disinfected and undisinfected Odessa barley seedlings after storage at different Temperatures.

Temp.of Storage. °C.	UNTREATED % Emergence	% Disease*	TREATED % Emergence	% Disease*
-5	61	59.7	74	1
+2	62	64.9	74	0
10	63	64.4	74	0
20	63	60.9	69	.3
30	54	67	71	1.

* This is calculated on basis of emergence figures, i.e.

$$\% \text{ Disease} = \frac{\text{Number Diseased}}{\text{Number Emerged}} \times \frac{100}{1}$$

From this table there are some obvious conclusions to be drawn, with respect to both the effect of storage temperature on the incidence of the disease and on the efficiency of the fungicide. Of these the first is that the temperature of storage in no way affects the efficiency of New Improved Ceresan. This is evidenced by the very little disease in the treated seed, and by the increased emergence of it also, the variation in which falls well within the range of experimental error.

In the untreated seed however there may be a slight effect of temperature on emergence at the 30° point, although there is no appreciable difference at lower temperatures. It will be noted that the emergence of seeds stored at 30°C. is the lowest by a considerable percentage while the disease on seed stored at this temperature is at the maximum of 67%. This may be a coincidence but it seems also feasible that the higher temperature benefited the fungus on the seed coat with the result that fewer seeds were able to emerge above the ground and of these more had symptoms of the seedling blight than had those seeds stored at lower temperatures.

It is to be concluded therefore that the temperature of storage has little or no effect on the emergence or percentage disease of plants sown from either treated or

untreated seed, until such temperatures are reached as 30°C. For such a temperature to be reached in storage either by farmers or by larger wholesale dealers using elevators is extremely unlikely especially if well dried seed is being stored and there is little likelihood of heating in the piles. It was however important to test this point, as in experimental work, seed is often stored at temperatures higher than that used by farmers and the results might have been affected thereby and not have been applicable under field conditions.

The other temperature factor mentioned above was that of the soil temperature at which the plant and pathogen were growing. Such a factor as this is extremely difficult to control while keeping the other factors constant, for there is much more evaporation from the hot soil surface than from a cold one and so the plants grown at high temperatures tend to be exposed to more xerophytic conditions than the others. In the experiments to be described below an effort was made to overcome this difficulty but that it was only partially successful could be seen by comparing the dryness of the soil in the tanks in the morning after the process of evaporation had been going on all night.

The method of obtaining various soil temperatures was to use large water baths in area about 12 sq. feet with

the temperature of the water electrically controlled and adjusted. This temperature was very well maintained and in no case was the variation more than ± 1°C.. In these baths zinc cans containing the soil were suspended and these cans and the soil in them soon reached the temperature of the surrounding water and stayed there with a few very minor fluctuations. There were eight such cans in each bath and in four of these was sown treated Odessa seed while untreated seed was sown in the other four. Fifty seeds were used per can so that two hundred seeds formed a unit in the experiment. There were five temperature tanks available, with the minimum temperature being that of running water which was remarkably constant in winter at 13°C..

The cans were watered to constant weight every night and morning, water from the tank in which the can was placed being used for this purpose in order to avoid variations in temperature in the soil due to the addition of water at a higher or lower temperature. The plants of course grew at different rates so that it was not quite feasible to uproot them all for examination on the same date. They were therefore uprooted when they were all at the same stage in development namely the appearance of the third leaf. This was fourteen days after planting for all but those grown at 13°C. when it was eighteen days.

The amount of disease was estimated in the usual way and the results expressed as percentages are in Table III.

Table III.

Seedling Blight on disinfected and undisinfected Odessa Barley Seedlings grown at different soil Temperatures.

Temp. of soil °C.	UNTREATED		TREATED	
	% Emergence	% Disease*	% Emergence	% Disease*
13	85	43.5	92	1.1
18	85	35.3	88	2.4
23	82	58.5	88	0.
30	74	61.4	88	1.7
35	71	56.7	77	6.1

* Calculated as in Table II.

In the above Table there are a number of interesting features bearing both on the disease and on the action of the disinfectant, but these features are complicated by secondary factors such as soil moisture entering the problem. The first general trend can be seen in the percentage germination columns both of the treated and untreated plants, and that is a tendency for a reduction in germination of the seed with increase in soil temperature. It is interesting to notice that the treated seed always germinated better than the seed which was not disinfected, which is doubtless due to the benefits of the New Improved Ceresan itself. Notwithstanding this, there is a general

trend towards a decrease in germination as the temperature rises which as it occurs in both treated and untreated seed must be due to some factor other than disease and it is only reasonable to assume that this factor is temperature itself. It is common knowledge that at higher temperatures seeds of normally low-temperature plants such as barley, do not produce such strong seedlings as at the more intermediate temperatures, and therefore what at a lower temperature might germinate and emerge as a weak plant, at a higher temperature will not be able to penetrate the soil.

The amount of disease in the untreated seed is a matter of great interest, and although the figures do not form a perfect series, still it is thought they show the general trend in the development of the disease. The work of Jones, Johnson and Dickson (13) on Helminthosporium sativum P.K.B. on wheat and barley seedlings indicate a gradual increase in the amount of seedling blight until an optimum temperature of 28°C. is reached, after which point the amount of disease begins to decrease. These workers worked with pure cultures of the fungus and artificially inoculated seed, but in the work being described here there was also the Fusarium part of the complex present on the naturally infected seed. There is however a similar curve shown by the disease figures in the case

on hand for it is believed that the aberrant figure of 35.3% disease at 18°C. does not really indicate a fall in the amount of disease but is an experimental error due to extraneous circumstances. Whether or not this is so, the percentages show a maximum amount of disease at, or about, 30°C with the consequent falling off as is described by Jones, Johnson and Dickson.

The final point to be discussed with regard to the effect of soil temperature on the disease and its control is the actual effect of temperature on the action of the New Improved Ceresan. The amount of disease occurring in the treated seedlings is very low and over the major part of the experiment, 13°C - 30°C is very constant at about 1%. At a temperature of 35°C. however there is a sharp rise in the percentage disease to 6.1%. This is not a great deal of disease but it is almost five times as much as in found in the other treated plants, and it is occurring at a temperature at which the percentage disease is falling in the untreated controls from 61.4 to 56.7%, and so is possibly of some significance.

There are some factors which might explain this fall in efficiency, such as a sudden increase in the amount of disease but this is not the case as is indicated above. It might be that the disinfectant was not so toxic to protoplasm at higher temperatures but this is extremely unlikely

and since both the Helminthosporium spp. and the Fusarium spp. have optimum growing powers at 28-30°C. at this temperature of 35°C. they are almost at their maximum and so should be much more easily killed by a toxic substance such as ethyl mercury phosphate.

The other explanation would be that the toxic substance could not obtain a perfect contact with, or access to, the protoplasm of the fungus at the high temperature and so was not such an effective killing agent. If the means of obtaining access were as a gas and the disinfection were largely complete before the seed were sown in the soil then it should be imagined there would be no such drop in efficiency. If also the disinfection by a gaseous agent were to take place in the soil as has been postulated, then the increase in temperature should have favoured it for the volatility and vapour pressure of a solid increase with a rise in temperature. If however the disinfection of the seedling took place as a solution, then this result could be explained, for, as has been stated above, the soil in the high temperature tanks was drier than in the lower temperature ones and consequently there might be a lack of water to make the disinfection absolutely perfect.

It is thought that this last explanation is the most likely as it seems to answer all possible objections.

(c) Soil moisture experiments.

In the above-mentioned experiments regarding the effects of soil temperature on the action of New Improved Ceresan and on the incidence of seedling blight, one of the results was explained on the basis of a variation in the soil moisture content. It was therefore thought advisable to conduct a series of experiments on the effects of various soil moisture contents on the disease and its control. Soil moisture is of extreme importance on the incidence of root rots and stem base rots, and to illustrate this, it should suffice to mention the case of damping-off of seedlings of practically all kinds. The damping-off, due largely to species of Pythium, is largely induced by excessive watering of seedling stock and the consequent flooding of the seed bed and lack of soil aeration. Whether the lack of aeration or the abundance of water is the prime factor in damping-off, or whether the disease is the result of the interplay of a complex of factors is still a moot point, but competent gardeners and nurserymen lay the main stress on the abundance of water.

It is impossible to separate the two factors of soil moisture and soil aeration, but we can state in general terms that they bear an inverse relationship to each other, in that if there is an abundance of water there is a

deficiency in aeration and vice versa.

The seed used in the experiments to be described below was of the original sample of Odessa, and it was disinfected in the usual way at the rate of ½ oz. of New Improved Ceresan per bushel of seed. The soil used was a fairly coarse sand which had a water-holding capacity of about 12% reckoned on an air-dry basis.

In the experiment the sand was dried on a large table for three weeks before use and it was completely dry to the touch at the time of the experiment. It was then weighed out into large flat boxes or 'flats', each flat containing the same amount of sand. The sand in each flat was then dumped in a separate pile on the table and the amount of water each had to receive was added to it, a little at a time and thoroughly mixed until it was all added and the distribution was uniform. The sand was then returned to the flat and the whole weighed and kept at that weight by watering once a day.

The seeds were sown in lines in each flat, 20 seeds being sown per line with 10 lines to the box, 5 of them being sown with untreated seed and the remainder with seed treated with New Improved Ceresan. Two flats were used for each moisture content, that is lots of 200 plants per experimental unit of treated or untreated seed. When the

flats were being watered it was tried to spread the deficient water as uniformly over the surface of the soil as possible using a very fine sprayed watering can, and to avoid temperature changes, water was used from the water tank in the greenhouse whose water was usually fairly near the temperature of the house. The soil contained the following percentages of water, 0, 1, 3, 7 and 10, and the plants were uprooted nine days after sowing and examined for seedling blight. A little water was sprinkled on the top of 0% flats to enable some growth to take place but this was very little and was between .25 and .5%. The results are tabulated in Table IV.

Table IV.

The Effect of varying Soil Moisture Content on the Incidence of Seedling Blight of Barley grown from treated and untreated seed.

Percentage soil water	UNTREATED		TREATED	
	% Emergence	% Disease*	% Emergence	% Disease*
0	84	45.1	92	5.4
1	86	59.8	97	7.2
3	83	59.2	97	2.1
7	83	65.1	93	3.3
10	78	69.3	99	1.0

* Calculated as in Table II.

The results in Table IV indicate a few interesting con-

clusions and can be used to amplify some of the ideas which have been already stated. In examining the results in the control plants there is a fair uniformity in germination, with the exception may be of that at 10% moisture in the soil. In that instance there is a fall in the emergence of some 5% from the minimum of the other readings, and it is thought that this fall is most likely related to the amount of disease noted at the various levels.

The percentages of disease in the untreated plants are significant in that they show a very decided increase from the lower soil water contents to the higher ones. This is rather difficult to account for satisfactorily, but the most obvious explanations are easily dismissed. There must have been as good conditions for the growth of the fungi at the lower water contents as at the higher, because if there was enough water for seed germination to take place, then there was ample for the germination of fungal spores which only require a very thin film of water for this purpose. The chance of secondary infection being facilitated by higher water contents is quite feasible, but for the fact that the uprooting of the plants was carried out before there was an opportunity for a secondary spread of the disease.

The most likely explanation and probably the true one

is that the plants at very low soil moisture contents were much more resistant to the attack of the parasitising fungi than those growing under damper soil conditions. It was noted that barley seedlings grown under what amounted to xerophytic conditions had very tough and wiry stems, not very thick in diameter but much more difficult to break than the more succulent stems of plants grown in moister soil. It is quite conceivable that such a stem would be more difficult to enter and parasitise than the softer succulent ones and so this would afford a possible explanation of the marked increase in the percentage disease in high-moisture-content soils.

There is another factor which may aid in the explanation of this increase in disease and that is the factor of soil aeration and CO_2 content. Lundegardh showed recently that foot rot of wheat, and snow-moulds on rye and grasses were made more severe by an increase in the carbon dioxide concentration, for at 2% CO_2 in the soil the wheat plants were weak, while at that concentration the parasite, Fusarium culmorum (W.G.Sm.) Sacc., was growing very well and actively parasitising the wheat. In the soils therefore with a high moisture content there may have been an increase in the CO_2 concentration as a result of respiration from the roots, and so there was the potentiality for a

greater amount of disease. This higher percentage of disease at the 10% moisture content level could easily account for the fall in emergence at that same content, by assuming that more of the parasitised plants were killed before they reached the surface than at the lower concentrations.

The effect of the varying moisture content of the soil on the action of the disinfectant is not so well marked but the disease percentages fall into two groups, viz. the 5.4 and 7.2% group and that composed of the others with 2.1, 3.3 and 1.0 % disease respectively. It would seem from Table IV that increase in the soil moisture content has a beneficial effect on the action of the disinfectant although it is increasing the chances of disease in the untreated controls. That this effect must be due to better contact with the fungus is obvious as the toxic agent itself does not change, and the most feasible explanation is that the disinfectant is acting in the solution around the seeds. It would follow then that a scarcity of water would not allow all the disinfectant to go into solution or to be readily accessible when the young shoot emerges from the seed. The higher soil-water contents would not have this drawback and there would be an abundance of available moisture to dissolve the therapeutic agent and to spread

it all over the seed. There should be, therefore, a maximum water content of the soil above which the disinfecting solution would begin to be less concentrated, and efficiency would thereby decrease, but at the moisture-contents used this point was not reached.

An analysis of the figures given above would indicate that most of the results could be explained on the basis of the disinfectant acting as a solution, but there is a fairly critical factor which has not been given much consideration, namely the time factor. Most of the seed has been sown very soon after disinfection, allowing practically no time for the New Improved Ceresan to volatilise and obtain access to the fungal spores as a vapour. This evidence then, although it can be used in support of a solubility theory, cannot be used against a gaseous theory of the action of the disinfectant as the latter theory has never had a proper test. Experiments were therefore designed to give both theories a fair trial and they are recorded in the next section of this thesis.

Experiments on the Mode of Action of New Improved Ceresan.

In the experiments which have previously been described an effort has been made to study the effect of environmental factors on the incidence of seedling blight of barley and on the action of New Improved Ceresan. Any conclusions

pertaining to the latter objective were used to try to elucidate the mode of action of the disinfectant and the method whereby it obtained access to the fungal spores and exerted its toxic effect. Such a method however is purely inferential and deductive and is not based on direct experimental evidence. It was decided therefore to try a more direct approach to the problem of the action of the disinfectant.

There are some great technical difficulties to overcome in work of this nature which are almost insuperable. In the first place, once seed has been treated with New Improved Ceresan it is impossible to remove the disinfectant without wetting the seed. An attempt was made to do this using currents of air but it was not a successful method, and as the seeds will not grow on absolutely dry sand, water has to be applied to enable any estimation of the amount of disease to be made. The disadvantage of wetting the seed lies of course in the fact that the solution factor immediately comes into play. This can only be overcome by suspending the seed over some of the disinfectant and allowing the vapour from it to act without any actual contact being made. A method such as this is of course open to the objection that the relative quantities of seed and disinfectant to be used are not known, as it would be very

impractical to try to suspend a bushel of seed over one half ounce of New Improved Ceresan, even if complete penetration of the gas throughout the heap of seed were possible.

In the preliminary set of experiments this 'quantity' factor was not fully appreciated and so the results from one point of view are valueless but they are interesting from another standpoint and so the experiment and the results will be described.

The method used was very simple. Small petri-plates containing 200 seeds were placed inside larger petri-plates which contained a small quantity of the disinfectants, both old and new Ceresan being used. The lid of the larger petri-plate was then closed and the seeds were in a small air-tight chamber which, it was hoped, would become full of the vapour of the disinfectants and if the disinfecting action took place in the vapour phase, the seed ought to develop into clean plants when sown. Unfortunately such a method only demonstrated the difficulty of keeping an extremely fine dust like New Improved Ceresan from floating in the air, for the seed when examined showed that a fine film of both the New and the Old Ceresan had been deposited on them. This could easily have been due to slight movements of the plates during the period of storage or even to convection currents set up in the enclosed space.

In the experiment seeds were stored over New Improved Ceresan, Ceresan, Mercury, Mercuric Chloride (1-1000), and seed disinfected in the standard way with New Improved Ceresan and Old Ceresan was similarly stored as also was a like quantity of untreated seed. The seeds were maintained like this for 13 days then taken out and sown as usual in sterilised soil in sterilised pots. They were allowed to grow for 11 days when they were uprooted and examined for disease. Prior to uprooting it was noted that those seeds stored over the solution of mercuric chloride were very small and slow in emerging, and the numbers emerging were very much lower than in the other treated experiments or even in the untreated controls. The results are tabulated in Table V.

Table V.

The Incidence of Seedling Blight in Barley grown from Seed stored over different Substances for a Period of 13 Days.

Substance	% Emergence	% Disease*
Control,	84.5	60
Treated with N.I.Ceresan,	95.5	1.1
Treated with Old Ceresan,	96	10.2
Stored over N.I.Ceresan,	93	1.0
Stored over Old Ceresan,	93.5	21.8
Stored over Mercury,	72.5	7.
Stored over Mercuric Chloride,	54.5	40.6

* As in Table II.

From the above Table it is obvious that some measure of control has been arrived at in all cases, with the possible exception of the case of seeds stored over Mercuric chloride which although showing only 40% disease still show only 54% emergence, and the reduction in the amount of disease can be more than accounted for by the fall of 30% in the number of plants growing above the soil. The mercuric chloride in this case was in the form of a solution at a concentration of 1:1000 and as this was the only case in which water was present in the system it was thought that the humidity might have had some effects in producing this strange result. Experiments to elucidate this point farther are described in the next section of this thesis.

The results of the treatment with New Improved Ceresan as a directly applied fungicide and as a vapour can be discounted in this case on account of the dust obtaining access to the seed and so the two treatments were practically identical. The same argument also holds good for the old Ceresan experiments but there is an interesting point here, in that whereas in the New Ceresan experiments control was practically the same in both cases, where the old Ceresan was used there was twice as much disease in the seed stored over the vapour as in the seed directly treated. The explanation of this could be very simple, merely the differ-

ence in the efficiency of the two compounds farther exaggerated by the difference in the rate of application. Thus New Improved Ceresan is applied at the rate of ½ oz. per bushel and the old Ceresan at 2 ozs. per bushel, and the difference in rates does not equalise the difference in toxicity. Thus a small quantity of New Improved Ceresan would be much more efficient than the equivalent quantity of old Ceresan, and not only would this be the case but it would only be one quarter of the weight of the old Ceresan. It follows that where they are present in equal quantity, even if they are at an optimum for Old Ceresan, there will still be a decided superiority in the action of the New Improved Ceresan and where they are present in very small amounts, as in the case in point, this difference will be much more obvious. The point of superior volatility could also be used to explain the greater control obtained by storage over New Improved Ceresan, than over the old Ceresan, for if the same amount of dust from both disinfectants settled on the seed, then the difference might be the additional New Improved Ceresan vapour. Which of two explanations is the correct one it is impossible to say for both seem equally likely.

Another interesting feature of these results is the measure of control obtained by the storage over mercury.

In this case mercury vapour must have been the active agent and not only did it decrease the percentage of disease but it also proved toxic to a certain extent to the germinating seeds, causing a reduction in germination of 12% from the untreated controls. This is a piece of experimental evidence on the point that the toxicity of mercury can be expressed even in the vapour phase, both to fungi and to the young plants as there was no opportunity for the mercury to come into actual contact with the seeds.

Why then is the mercury not as toxic as New Improved Ceresan? The answer probably lies in the relative surface area of the two and in their relative volatility with which also is allied the fact that the New Improved Ceresan was in more intimate contact with the seed and so had a better opportunity for disinfecting. The chemistry however of ethyl mercury phosphate is not to be found in any available text or paper and so the problem cannot be finally answered.

These experiments although instructive on many points, had not actually gone far towards solving the specific problem which was under consideration but they had pointed out many of the obstacles in the way, such as the 'quantity' factor and the difficulty of keeping the vapour apart from the dust. It was decided therefore to avoid the quantity factor by comparing the control obtained by the solution

and by the vapour with each other and not with the control obtained using seed treated in the usual way. The same amount of Ceresan would be used in the solution and supplying the vapour and the degree of control could thus be established.

For this purpose 100 pots half full of sand were used, the sand being completely air dry. Forty of the pots were taken into a separate room and a mixture of fine sand containing enough New Improved Ceresan to be seen as a bluish tinge by the naked eye was sifted over the surface of them. They were then allowed to stand for a day to let the dust settle and then they were covered with a layer of sand about one inch thick. After another day another layer of sand was strewn on the surface of each pot and it was now certain that there was actually no disinfectant on the surface of the soil. The other pots were then filled with sand to the same level as this first batch and untreated seed was put on the top of the forty pots containing New Improved Ceresan and on the top of forty of the pots of plain dry sand, twenty seeds being placed on each pot. The other twenty pots had seed disinfected in the usual way and at the customary rate placed on top of them, to give some comparative figures from what is reckoned to be as perfect control as is available, but not for direct comparison with

the vapour-solution figures.

The pots were then left for 17 days in a still place, but with those containing New Improved Ceresan well separated from the others. The drainage hole in the bottom of the pots had been plugged with paper so that if the vapour sank it would collect in the foot of the pot and fill it up until it reached the level of the seeds. Each pot was also covered with a piece of stiff paper which acted as a lid and enclosed the vapour. All the pots including the controls were treated alike. At the end of seventeen days the seeds from twenty of the pots containing the Ceresan sand were changed with the seeds from twenty pots which contained only sand.

There were therefore 20 pots of seed on plain sand which had never been over the New Improved Ceresan, and 20 pots of plain sand containing seed which had been exposed to the vapour of New Improved Ceresan for seventeen days. There were also 20 pots of seed on sand which contained Ceresan which had been exposed to the vapour for seventeen days and 20 pots of seed on similar sand, but which had never been exposed to the vapour. The pots were then irrigated from the bottom so that as the water passed up it would dissolve the Ceresan and carry the solution to the seed on top of the sand. All the pots had been

covered with a layer of sand before this irrigation to provide coverage to keep the seeds moist. The experiment therefore contained the following series:-

(a) 20 pots of 20 seeds each which had been exposed to the vapour of New Improved Ceresan.

(b) 20 pots exposed only to the solution of the disinfectant.

(c) 20 pots exposed to both solution and vapour.

(d) 20 pots exposed to neither solution nor vapour.

(e) 20 pots of seed treated with New Improved Ceresan.

The pots were irrigated from the bottom throughout the entire growing period to prevent the solution from being washed away from the seed, and after 12 days/were the seedlings uprooted and examined for seedling blight. The results are tabulated in Table VI.

Table VI.

Seedling Blight on Barley exposed to different Modes of Action of New Improved Ceresan.

Treatment	% Emergence	% Disease*
Control,	94.25	62.5
Vapour acting,	94.5	22.3
Solution acting,	95.25	19.8
Solution and Vapour acting,	95.	21.2
Dust treatment,	95.75	2.9

* See Table II.

The above table calls for a few comments on account of its comparatively inconclusive nature. The treatment with the dust of New Improved Ceresan reduces the incidence of the disease by the usual very marked percentage but although the other methods also reduce the incidence of the disease they do not do so in such a striking manner. This may be either a question of quantity or of efficiency but it is unlikely to be that of quantity as a very small amount of New Improved Ceresan if applied in the proper way can disinfect a relatively large quantity of seed. The fungicide, too, is active in very low dilutions, so that the idea that there was not enough disinfectant present either as a liquid or as a vapour is extremely unlikely, for there was definitely more than sufficient New Improved Ceresan in the soil to disinfect some hundred times as much seed as was used. That the difference was not due to experimental error was shown by the fact that a similar experiment gave comparable results only in this latter case the control by the vapour and the solution was not even so great as in the present experiment. The only explanation is that there is actually some virtue in the dry dust disinfection, and as has been suggested before this can only lie in a greater intimacy of contact between the fungus and the disinfectant.

The experiment does show that there is a disinfecting

action of the vapour, and also a similar action by the solution, but it is remarkable that the combined disinfectant action of the solution and the vapour is no greater than the individual action of either. This would be very difficult to explain as a matter of quantity, for were quantity the limiting factor it would be expected that the solution and the vapour would have an additive effect, and this is decidedly not so. Another possible explanation might lie in the idea of concentration of both the gas and the solution being too low to kill all the spores but only was effective against the least resistant. The variation in resistance of various physiologic races of the same fungus to toxic chemicals has been demonstrated by Verral, while the occurrence of many physiologic races of Helminthosporium sativum has been conclusively proven by work of Christensen (1). The same arguments however as were used against the idea of concentration before, mitigate against it in this case also, and tend rather to negative it. To explain this lack of summation, recourse must be had once more to the point of intimacy of contact. The results indicate that the same percentage of the parasitic fungi was killed in the case of both vapour and solution. This definite percentage could very easily have been those fungi whose spores were located on the surface of the seed or

a little way inside the space enclosed by the glumes, with the result that only part of the infection was removed. Thus some of the seeds would be clean and disease-free while some of the others, which had spores well protected by the seed coat, would still carry infection, the results of which would show up in the seedling plants.

In a large number of seeds, such as was used in this experiment, the disease remaining on the seed would appear on a definite percentage of the plants and would be responsible for the comparatively high infection figures in the case of those seeds exposed to the vapour and the solution alone. In seeds disinfected with the dust, there would be the necessary intimacy to kill almost all the spores as it acted in the vapour or the solution-phase.

The general conclusion to be drawn from the above experiments is that the seed disinfectant can act in the gaseous and in the liquid phase, but that under the experimental conditions and with the techniques used, neither of the two was as efficient as the dust applied in the prescribed manner and at the recognised rate. When disinfected seed is used, the action of the disinfectant will be dependent on the time lapse between disinfection and sowing, for if there is a long interval a large part of the disinfective action could take place as a gas, but immediately on sowing further disinfection will take place with the disinfectant acting as a solution.

Experiments on Storage of Barley Seed.

In Table V it is to be seen that the seed stored over a solution of mercuric chloride showed a very low percentage emergence as well as a lower disease content, and it was stated in the text at that point that this effect would be discussed later, along with some experiments bearing on it. It was thought at that time that it was the method of storage over water which was the prime factor in the peculiar result and a series of experiments dealing with the factor of moisture were set up.

Previously in this work on seed disinfection, some experiments, especially those dealing with storage of seed at various temperatures, had been ruined by water having been splashed on the seed by other users of the temperature chambers. In one such experiment the wet seed was sown out in the usual way and in the two cases where the seed had become moist, germination had been reduced from 97% in the dry to 64% in the damp seed, although at the time of storage there had not been enough water present to cause germination.

A similar state of affairs is associated with a condition which for want of a better name is known as 'sick' seed. It has been noted that 'sickness' in seed, usually of wheat and barley, occurs generally after a wet harvest

when the grain has been thrashed and stored while it still contained a high percentage of moisture. Such grain after storage, unless properly aerated, always has a distinctly musty smell and in sowing invariably germinates very badly, independently of seed treatment prior to sowing or not. One other effect of 'sickness', in wheat especially, is that when such grain is milled, the quality of the resultant flour is of an inferior grade, and products made from it suffer from what is technically called 'off-flavours'.

In the seed which had been stored and allowed to get damp, it was observed that there were various moulds beginning to develop and some of the seeds were noted to have that slimy touch associated with the presence of bacteria, whose presence was verified by microscopic examination. It was this seed which germinated so poorly.

A somewhat similar phenomenon has been noted by various workers on vernalisation or iarovisation of cereals. In this process there are a number of stages of which at least three are generally recognised, viz. a photostage, followed by a thermostage, and according to Krevoi and Kiricenko a farther photostage. In the technique of this process of vernalisation seed is moistened until germination is just commencing, then no more water is added to it. The seed is then exposed to a certain duration of illumination, during

which the photostage is completed. Subsequent to this the seed is exposed to a specific temperature, varying with the seed, for a definite period, after which it may be sown or else the third stage may be induced if necessary.

During the exposure to temperature, thermo-stage, the damp seed is sometimes overgrown with moulds and such seed germinates very poorly. Usually the thermostage is carried out at temperatures too low for fungi to grow, but in some plants the temperature used permits of a certain amount of spore germination and of fungal growth. This lack of germination in a number of instances only related by the presence of water in the system, led to an investigation of the role of the water and the fungi in the non-emergence of the seedlings and incidentally the use of New Improved Ceresan as a curative or a palliative measure.

The heavily infected barley seed, Odessa, was used, half of a sample was disinfected with New Improved Ceresan, and the other half used in its natural state. Each of these samples was again halved and one of the halves was stored in a moist chamber and one in a dry chamber in each case. Thus there was disinfected seed stored in dry and moist conditions, and natural seed stored similarly. The seed was left like this for 21 days when it was removed for the planting, but previous to the actual sowing the

natural seed stored in the damp atmosphere was halved again and one of the halves disinfected. The following series therefore comprised the experiment:

(a) Untreated seed from a dry atmosphere.
(b) Untreated seed from a moist atmosphere.
(c) Treated seed from a dry atmosphere.
(d) Treated seed from a moist atmosphere.
(e) Seed which had been treated after storage in a moist atmosphere.

The seed was then sown in pots of sterile soil, 20 seeds per pot, 20 pots per method of storage, i.e. the unit was 400 plants. It was noted that the untreated seed stored in the moist air had a prolific fungal growth on it, while the treated seed stored in the same way had no growth at all. The plants were allowed to grow for 15 days, and were then uprooted and examined for foot rot and seedling blight. The results are contained in Table VII.

Table VII.

The Effect of Method of Storage of treated and and untreated Barley Seed on the Incidence of Seedling Blight, and on Emergence.

Treatment	Time of Treatment	Storage	% Emergence	% Disease*
None	-	Dry	78	64
None	-	Moist	54	52
New Imp.Ceres.	Before storage	Dry	88	0
do.	do.	Moist	83	2
do.	After storage	Moist	61	2

* as in Table II.

The results illustrate very well a number of interesting facts which are very indicative of the line of approach to the 'sick' seed problem. The most obvious feature of the results is the marked decrease in germination subsequent to the storage of untreated seed over water. That this effect is general and will take place even if the seed is disinfected before sowing can be seen also. There is an interesting feature in that treated seed stored over water does not show this remarkable fall in germination and this point would seem to indicate that the presence of fungi or bacteria is correlated with the lack of germinative power. The fall in disease percentage in the seed stored over water has no especial significance and is most likely due to most of the infected plants having been killed off before emergence so that only the strongest and cleanest seeds have produced plants.

It would seem that since the 'sickness' of the seed is associated with fungi and bacteria, that in the presence of moisture the spores would germinate and affect the seed or the embryo in such a way as to prevent germination. In treated seed however the vapour of New Improved Ceresan or the solution of it in the film of condensed moisture either killed these spores as they germinated or else prevented their germination. The obvious method by which fungi or

bacteria would decrease the germinative power of seed would be by actual invasion of the growing points of the young plant, but an examination of the results seems to indicate that this is not the case.

In the first instance, seed disinfection will not cure actual infection or disease lesions. The misuse of the term disinfection is responsible for confusion, for the action of New Improved Ceresan is one of disinfestation, that is the removal or killing of spores which infest the seed and which would ultimately infect the seed were it to start growing. The term 'seed disinfection' however is so widely used that it would only tend to confuse still further were new terms to be introduced here. Since seed disinfection does not heal lesions already present or cure infection, it would be expected that the seedlings from seed which had been stored over water would show a much higher percentage of disease lesions even if the seed had been subsequently disinfected, for this would not remove the disease which had developed during the storage period. This however is not the case, for seedlings grown from seed stored in a moist atmosphere and then disinfected, show only as much disease - 2% - as those treated before moist storage.

This could be explained by postulating that all the

seeds which had become parasitised during moist storage had been killed and so could not germinate at all. This explanation however seems very unlikely as 100% mortality is extremely rare with even the most virulent diseases and such being the case some of the parasitised seeds would have been able to emerge and so would have increased the percentage disease in seedlings from those seeds treated after storage. There is no indication of this in the results. There is an alternative suggestion which has experimental evidence to support it, and is therefore much the more reasonable.

This alternative suggestion is that the fungi grew on the damp seed and were able to live saprophytically on the bran layer and the loose fragments of husk and chaff on the seed. In the process of growing they liberated staling products or in some way caused toxins to be formed which were fatal to the growth of the young seedling so that it never emerged. It was noted in this experiment that a large number of these plants which did emerge were very short and thick, bearing distorted and much thickened leaves. This, it is believed, is also due to the formation of toxins which in this instance were not sufficiently concentrated to cause the death of the seedling but merely impeded its growth to a very marked degree.

On the basis of this explanation all the figures in Table VII become quite comprehensible, the fall in emergence in these seeds disinfected after damp storage is explained, and on the assumption of the killing by toxins, one would expect an equal amount of disease in all cases where the seed was treated which is what is found in the experiment.

To test this hypothesis of the toxicity of the bye-products of the physiology of fungi growing on the seed coat, another experiment was performed using the filtrate directly. A quantity of barley, about 50 gms., was moistened with water and incubated at 25°C.. A prolific growth of fungi took place on the grain and after 2 weeks' growth, the whole mass of fungi and barley was washed until 100 ccs. of washings had been collected. As a control an equal quantity of dry barley, had been stored in the incubator and it was washed in the same way. These washings were then sterilised by passing them through a Chamberland Pasteur filter and some clean barley seed, variety Peatland, soaked in each of them for a day and a night.

There was therefore clean barley seed soaked for approximately 36 hours in washings of fungi and barley and some soaked in washings of barley alone. B oth lots of seed were then rinsed in water and sown with an ordinary sample of Peatland as a check. Four hundred seeds of each

formed the experimental unit. The results are contained in Table VIII.

Table VIII.

A Comparison of Washings from Barley on which Fungi had grown and from Barley alone, on the emergence of Peatland Barley.

Source of Washings	% Emergence	% Disease
None	94	3.0
Barley	88	2.7
Barley & Fungi	63	3.0

The conclusion from the above results is quite in support of the hypothesis of toxic substances formed by fungi on the seed coat of the barley seed, for where the fungi were allowed to grow, germination fell by 31%, while the fall in the washings from barley itself is almost negligible. This work was done by the writer in January, 1937, and in the April issue of the Ohio Agricultural Experiment Station Bi-monthly Bulletin R.C.Thomas (25) has a short article (pp.43-45) in which he describes some experiments he performed on wheat seed. Thomas grew fungi isolated from wheat seed on wheat bran and showed that under his conditions substances toxic to wheat grain were present in the filtrate from his cultures. Species of Aspergillus were the most potent in the production of this toxic prin-

ciple and he showed that 43-day-old cultures were extremely toxic causing a reduction of 78% in germination but that 6-day-old cultures were quite ineffective.

This production of toxins by moulds on damp wheat and barley is another reason why seed should be very thoroughly dried and good aeration ensured before any quantity of grain is stored. Similar toxins have been found to have been harmful to pigs to which mouldy grain has been fed, and so the 'off-tastes' in flour might well be of a similar nature.

Discussion.

Disinfection of seed before sowing is one of the wisest forms of crop insurance which a farmer could take. Not only is the chance of disease considerably reduced, but a better yield is practically ensured and seed taken from such a resultant crop is clean and less likely to cause trouble in the future. A really good seed disinfectant should possess a number of very important qualities such as efficiency in action, duration of effect, ability to act with equal vigour over a wide range of environmental conditions and to protect many crops. It must also possess the more practical virtues of being cheap, easy to apply, and if possible should not be toxic to stock or human beings who may eat treated seed or else be employed

in the disinfection process.

New Improved Ceresan or ethyl mercury phosphate, which is the disinfectant with which the foregoing study was made, possesses all these characteristics and therefore is ideally suited to its purpose. It must not be implied however that there are no other disinfectant dusts with similar powers, because practically all of the proprietary compounds on the market at present for the specific purpose of disinfection, are equally effective. The choice of New Improved Ceresan for the work undertaken above was made solely for the reason that it is probably the most widely used of dust disinfectants and is as representative a type as could be chosen.

In one way maybe this choice was not a wise one, for ethyl mercury phosphate is so efficient in its fungicidal action that major differences in treatment with it might still result in complete control of the disease and so give no indication of the factors affecting the action of the disinfectant. This point however cannot be avoided and it is thought that the results described above justify the choice, apart altogether from the uselessness of conducting a series of experiments on one of the other less efficient compounds which is no longer in use and whose manufacture has ceased.

In the control of a seed-borne disease, such as seedling blight of barley, the use of New Improved Ceresan results in a number of benefits which are somewhat incidental to the killing of the Helminthosporium spp. and the Fusarium spp. actually responsible for the disease. There is a greater emergence of plants from treated seed than from untreated seed, and not only are there more plants but all of these are stronger and healthier than the corresponding plants from seed sown as it naturally occurs. A further major benefit accrues from the action of the disinfectant on soil organisms, as a small volume of soil surrounding the seed is also sterilised by the fungicide and in the case of seed which has to lie in the soil for a few weeks before it emerges, the growth of saprophytic fungi on the seed coat is prevented and there is therefore no liberation of the deleterious toxins discussed in the last section of this paper.

Even in seed carrying neither spores of Helminthosporium nor of Fusarium, seed disinfection is therefore a wise precaution for the microflora of the seed and soil, even if only saprophytic members are present, can have a deleterious effect on the growth and germination of seeds. It should be apparent therefore that prolonged use of seed disinfectant over a period of many years would in time

almost eliminate the seed-borne diseases and so remove one of the many hazards to farming. Complete elimination of these is however impossible as the seedling blight when present as leaf lesions can attack and parasitise the wild grasses in hedge-rows around the edges of the field, and from these infections, spores can be spread to the seed of the crop being grown, producing the disease in the seedling stage the following spring. It should be almost axiomatic for the farmer to treat his seed as the cost is so little and the benefit to the crop is so marked. One bushel of seed can be treated at a cost of about 2½d which is a total cost of 1/- per acre, a very small investment for the resultant increases in yield, and the reduction of the number of possible hazards.

The disease under consideration is very typical of a large number of seed-borne diseases such as Leaf Stripe of Oats and Barley, Covered Smut of Wheat and Oats and the various root rots and seedling blights on other cereals and grasses, all of which can be controlled by seed-disinfection. All of these are affected to a greater or lesser extent by the climatic and edaphic conditions under which the plants are grown but these variations due to temperature, moisture etc. are specific for the disease and the host concerned and so generalisations cannot be

made to include all the diseases, from the observations and data relating to the seedling blight of barley.

In the incidence of the seedling blight, soil temperature and moisture play an important role, the amount of the disease being chiefly determined by these factors. However, these factors do not eliminate the disease but merely lessen or increase the amount of it, as the fungi concerned have a parasitising action over a wide range of conditions. Thus although the maximum amount of disease occurs around 30°C., there is no indication of a temperature at which there is absolutely no disease, but rather there is a lower limit to the disease which in the experiments described was between 35% and 40% at 13°-18°C.. The action of New Improved Ceresan is equally effective at all these temperatures so that it is a universal means of control.

The seed disinfectant has been said to act as a gas and as a solution but from the results described above, it would seem to be able to act in either phase, although the major part of the action in seed treated and sown within a short space of time is undoubtedly in the liquid or solution phase. A point which emerges from the results is that there is some value in the extreme fineness of the division of the dust, coupled with the greater intimacy

of contact and better coverage which the fine dust ensures. This point has been noted also by Cunningham (5) with respect to sulphur dusts and he has correlated the efficiency of the dust and the size of the particles inversely, that is as the particles decrease in size the efficiency increases.

The study of seed disinfection is however still very new and there remain many problems still to be solved before a logical approach can be made towards the manufacture of the ideal seed disinfectant. Many of these are concerned with the action of the disinfectant itself and others relate to the disease process in the plant and the ecological conditions governing this process. These must be fully understood and integrated in relation to each other before science can say with conviction that it can control plant diseases.

Summary.

An outline is given of the history of seed disinfection and of the relevant literature.

Seedling blight of barley is described as being caused by species of Fusarium and Helminthosporium and the symptoms are stated to be brown discolorations of the stem base and roots causing complete rotting in severe cases.

Leaf lesions may also be present.

The effect of ethyl mercury phosphate (New Improved Ceresan) in reducing the fungus population of the seed is described and the control of the disease by this disinfectant demonstrated.

Soil temperature is important in the occurrence of seedling blight, there being a maximum disease at 30°C.. The efficiency of the disinfectant is not affected by the temperature of storage or the soil temperature.

Soil moisture increases the occurrence of the disease as it itself increases, but it also increases the efficiency of the fungicide up to a maximum above which this efficiency may decline.

It is shown that the New Improved Ceresan can act as a gas or as a liquid but that direct application of the dust to the seed gives the best control. This is thought to be a question of intimacy of contact.

Besides controlling Fusarium spp. and Helminthosporium spp., New Improved Ceresan is shown to be extremely effective in reducing the growth of saprophytic fungi on the seed which liberate toxins and so prevent germination.

A brief discussion of the benefits of seed treatment is given and it is decided that in practice the disinfective action will take place in the soil solution.

Fig. 1. Seedling Blight of Barley.

The two plants on the extreme left show leaf lesions and rotting of the stem base due to H. sativum, the four centre plants illustrate Fusarium attack becoming less severe in the right hand plants, and the two plants on the extreme right are healthy.

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APPENDIX A

Literature Review (Cont'd.)

Attempts have been made, ever since this disease was recognised, to discover some effective method of control, and since Helminthosporium sativum has been recognised as a root-rotting organism for many years, it has been paid particular attention. Ravn (18) in 1900 recommended the hot-water treatment previously devised by Jensen in 1887 (5) and stated that it was a fairly satisfactory means of control of the Helminthosporium disease. Such a method is, however, dangerous and cumbersome, and it is practically unused now except for the control of such diseases as loose smut of wheat and barley where the infection is very deep seated and cannot be reached by the surface disinfectants.

In 1917, however, L. J. Stakman (20) described a Helminthosporium disease of wheat and stated that this fungus has very often a species of Fusarium associated with it and that the fungi caused a typical root rot. She demonstrated that the disease was mainly seed-borne and obtained a reduction in the amount of Helminthosporium by 'long-time soaking in formaldehyde'. The fungi isolated by her were also capable of infecting barley, and the disease of wheat is obviously the counterpart of the barley disease here described.

There was no doubt in the minds of many workers that a

part of the infection of crops of wheat and barley which suffered from these seedling blights came from the soil. The earliest work of Bolley (1) in 1912 recognised this 'soil sickness' and accordingly a rotation of crops was advocated, and as late as 1923 and 1925 McKinney (12, 13) was suggesting the same remedy. Henry (3) also advocated rotation as a means of control of foot rots stating that 'the rotation of unrelated crops probably offers the best substitute for soil sterilisation,' but noted that the application of fertilisers, including lime, caused a noticeable decrease in the amount of foot rot.

About this period, the new organic mercurial compounds were being tested widely as control measures for many diseases and it was only natural that they should be tried against barley blight. Christensen was one of the first in this field in 1926, and he writes in relation to Helminthosporium sativum (2) 'the amount of seedling blight can be reduced by treating the seed with certain mercuric compounds provided it is sown in comparatively clean soil.' Leukel, Dickson, and Johnson (9, 10, 11,) in a series of papers on the effect of seed treatment on certain barley seed-borne diseases obtain very good results with the organic mercury compounds, especially when the fungus was Helminthosporium gramineum, which causes barley stripe. R. S. Kirby (6) in a very extensive bulletin from Cornell University deals with all the

major cereal diseases and their control, and in the section on barley diseases he advocates the use of the organic mercury compounds in solution.

The use of these chemicals in the form of dusts was becoming more popular and Lambert, Rodenhiser, and Flor demonstrated their efficacy against the covered smuts of the small-grained cereals. Simmonds (19), also tested the degree of control obtained by the use of a large number of these disinfectants when acting on a foot rot of oats, caused by Fusarium culmorum, which was also shown to be pathogenic on barley, and showed that the amount of disease was much reduced by the use of the organic mercurial dusts. In New Zealand, Neill (17) demonstrated the increase in stand obtained by the use of New Improved Ceresan when applied to infected seed of either wheat, barley or oats, and Koehler in 1935 and '36 (7, 8) was able to secure practically complete control of all the seed-borne diseases of cereals, excepting the loose smuts of barley and wheat, by treating the seed with the same fungicide.

Machacek and Greaney (15) working with 'black-point' disease of cereals, which is a disease very similar to seedling blight in its effects on the young plants, obtained very marked control of the disease by the use of New Improved Ceresan and Leytosan, and in a series of experiments in 1936 found that treatment of 'black-pointed' seed with these compounds, resulted

in an increase in the crop amounting to seven bushels per acre. The same two authors in 1935 (14) obtained similar results in the use of the organic mercurial dusts in the control of Fusarium culmorum and Helminthosporium sativum causing root rots of cereals. The results of Mitra and Bose (16), also demonstrate the measure of control obtained by the use of New Improved Ceresan on diseases of barley, especially H. sativum, H. teres and H. gramineum. They found, however, that at Pusa, India, there was a certain degree of infection from the soil and advocated resistant varieties for complete control.

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A P P E N D I X B

Seedling Blight of Barley (Cont'd.)

(a) Causal organisms.

As well as those fungi previously described as parasitic on barley seedlings, there are many others of greater or lesser importance. Probably the first to be described was Fusarium culmorum (W.G.Sm.) Sacc. which was described in England by Smith (22) in 1884 as causing a root rot of wheat and was named by him Fusisporium culmorum but this was changed by Saccardo (16) to its present form. F. culmorum is parasitic on all the cereals as are the majority of the fungi causing root rots and this fungus has been recorded by Simmonds (21) as causing much damage on oats also.

Other members of the genus Fusarium have been described as being root rotting in their action such as F. nivale Ces. which causes much loss on the Continent of Europe according to Schaffnit (18) and Ihssen (8). In the United States, a considerable amount of work has been done by Atanasoff (1, 2) who lists the following species as being important in the root rot problem:- F. herbarum (Corda) Fries, F. arcuosporum Sherb., F. scirpi Lamb, and Fautr., F. solani (Mart.) Ap. and Wr., F. arthrosporiodes Sherb. and F. redolens Wr..,

Many species of Helminthosporium have also been ascribed important roles in this disease besides the well-known H. sativum P.K.B.. Johnson (9) has described H. gramineum Rabh. as an active parasite, but it is very likely, according to Drechsler (5), that the fungus described as H. gramineum was really H. sativum. Ravn (14) claims a high degree of pathogenicity for H. teres and Henry (7) finds a certain amount of disease resulting from infection with a new species of Helminthosporium, namely H. pedicellatum Henry. He also finds two other Helminthosporia which differ in their conidial measurements from H. sativum to be parasitic and he called them Helminthosporium 'N' and Helminthosporium 'M'. He does not, however, disregard the possibility that these new forms may be mutants from an original H. sativum parent. Henry (7) also describes F. moniliforme Sheldon as being an active parasite and Selby has reported a similar state for F. roseum.

(b) Source of Infection of Seedlings.

There are two obvious sources of infection of the seedling plants, namely, the seed itself and the soil and their relative importance will depend on the ability of the fungus to overwinter on the seed and to withstand the vagaries of winter in the soil or on infected stubble. Each, therefore, must be considered.

So far as soil is concerned the evidence is fairly conclusive that the majority of the root-rotting fungi can

overwinter in some form or other in the soil. Sheldon (20) states that F. moniliforme 'probably overwinters in the soil', and this probability was proven fact by a series of experments of Henry (7) who, using both spores and mycelial cultures was able to re-obtain the fungus after exposure in soil to the winter of Minnesota. Christensen (3) found that mycelium of H. sativum would overwinter in Minnesota but stated that the spores did not. Henry (7), however, obtained 64% germination in overwintered spores of H. sativum and even so high as 96% in his type Helminthosporium 'M'. Simmonds (21) also was able to isolate F. culmorum from stubble which had been overwintered at Saskatoon, Canada.

It is possible, therefore, that the soil may be a factor in the development of seedling blight since it may be a source of infection, but it cannot be said to be a very important factor except in very close-cropped land where only a single crop is grown for a number of years. In ordinary farming practise where rotation is the rule, the antibiotic activity of the other constituents of the soil microflora will tend to eradicate the less common fungus. This has been confirmed by O'Brien and Dennis (12). Another argument against the soil being a major source of infection is the success of seed disinfection, for, were the disease coming from unclean soil, the sowing of fungus-free seed would not

alter the ultimate amount of disease materially. It is, however, the case that seed disinfection will almost control the disease completely, therefore the main source of infection for the seedling must be the seed itself.

That the seed of wheat, barley, and oats carries the fungi responsible for the foot rotting diseases is well known as a result of the work of Christensen and Stakman (4), Evans (6), Machacek and Greaney (10, 11,) etc. As none of the fungi concerned are systemic in the host it follows, therefore, that the infection of the seed must come from an external source such as the air or else be transmitted by insects. Extensive examinations of the spore content of the air have been made by Stakman et al. (23) in relation to cereal rusts and they repeatedly found spores of H. sativum in their spore traps. Similar work has been done by the U.S. Department of Agriculture, and Machacek and Greaney (11) have been able to correlate the Alternaria and Helminthosporium content of the air with the infection of wheat seed by the respective fungi.

The actual time of infection of the seed varies and it would seem from an analysis of the results of the various writers that it could occur at almost any stage of growth. Peyronel (13) states that infection of seed with H. sativum takes place after fertilisation of the ovule has occurred, while Scott and Sallans (19) are of the opinion that infection

can occur any time after the blossoming period. Rosella (15) finds infection taking place even earlier than either of these two authors and reports that H. sativum has been found on barley heads even while they were still enclosed by the leaf sheaths. Henry (7) obtained the maximum infection at the 'milk' stage in the development of the seed while Sallans (17) with Alternaria believes the crucial period to be after the 'soft dough' stage.

It is most likely that infection can take place at all these times and actually does in nature, for one often finds very plump and well filled heads infected to a very high degree with H. sativum, which would indicate that the infection occurred after the grain was fully ripe or at least too late to affect its development materially. On the other hand barley and wheat seeds infected with seedling blight organisms are often small and light, due, no doubt, to early infection by the fungi and consequent damage to the seed.

Where early infection has occurred one can demonstrate the presence of fungus mycelium in the embryo, but with late infection of the seed the fungi live on the decaying styles and stigmas and rapidly pass into a sclerotial or a spore resting stage in which they can exist for many months. Once, however, the seed begins to germinate and the young tender tissues emerge, they are very easily parasitised by the fungi with the resultant foot and root rots.

(c) Symptoms and Etiology of the Disease.

The disease symptoms can be divided into two classes, namely, a pre-emergence blight and a root and foot rotting phase. Where the infection of the seed has taken place early in its development the fungal mycelium is within the embryo itself, and whenever the embryo goes into active growth the fungus spreads throughout it and by killing the growing point prevents the emergence of the seedling. This is the pre-emergence blight phase and this tends to reduce the number of established plants in the field and hence the crop.

The root and foot rotting types of the disease arise from seed which has been affected later in its development and in which the fungus is present in the form of resting spores or mycelian sclerotia underneath the glumes. In this case, the result of the disease rather takes the form of a race as to whether the seedling can outgrow the fungus by forming tissue more quickly than the fungus can destroy it, or whether the fungus can so hinder the growth of the seedling so that it is finally killed.

In the case of the seedlings which do emerge and are infected with the root-rotting organisms, definite symptoms can be noted on the above ground parts of the plant. As a rule infected seedlings tend to be smaller than the non-infected ones, and they also show a tendency to develop an excessive number of tillers, resulting in a smaller, more bushy type of growth than that of the ordinary plant. The

basal leaves in infected plants either turn brown and die very quickly or else develop a deep green colour and have a waxy appearance on the surface. They often show a curling of the leaf and also various distortions of the lamina in the form of exceptionally broad blades. Where the parasite is one of the species of Helminthosporium there are often leaf spots develop which are typical, with dark purple edges and a brown centre. See fig. 1.

Infected roots may be either primary or secondary in origin but are always brittle and break away from the parent plant very easily. According to Weniger (24) infection of the young roots and shoot takes place before the plant even emerges from the seed.

The effects of Fusarium species are rather different from those of Helminthosporium so far as the roots are concerned, but in both cases a small appresorium attaches the germinating spore to the root surface and allows the entry of the infection hypha.

In the case of Fusarium graminearum, and F. moniliforme, (7), the fungus tends to aggregate in the stele of the root where it is intracellular. It spreads from there to the cortex where it may be both inter- and intracellular in its location and where it causes the general rot. The walls of infected cells become light brown in colour and very often the cells are filled with a light brown mass of disintegrated

cell contents which impart the typical light colour to the Fusarium rot. The fungal hyphae spread easily up and down the root which results in the general rotting of the roots and the death of the plants. F. culmorum, on the other hand, is rather more like Helminthosporium according to Simmonds (21) for it is very rarely found in the stele being almost entirely confined to the cortex.

As already described the action of Helminthosporium is more localised and it tends to cause the appearance of spots or streaks on the infected parts. The hyphae of this species are confined to the cortex and seem unable to penetrate the endodermis which acts as a barrier to their progress. Infected cells are filled with the dark brown hyphae of the fungus, and these hyphae along with the darker colour of the decaying cells make a darker brown spot than that of the Fusaria.

Helminthosporium spp. are, however, not confined to the root or stem base but cause leaf spots and stripes, which symptoms are not to be found in attacks by the Fusaria alone, although in very damp weather the attack of Fusarium may extend quite a little way up the stem.

Diseased Material used in the Experiments

The seed, as described in the text, came from a farm, near Morris, Minnesota and formed part of a sample submitted

to the University Seed Testing Bureau for examination. It was found to be very highly infected with the root rotting fungi by that Bureau, which finding was amply corroborated by the results described in the body of the thesis. The average emergence of the seed was about 80% as compared with 90% in treated seed and of the 80% emergence of untreated seed, an average of 55-60% were diseased to a greater or lesser degree.

Isolations made by plating out the seed (see Sect. 'Plating out Experiments') revealed the presence of pathogenic fungi which were responsible for the disease. Of one hundred Odessa seeds 60 of them were infected with Helminthosporium and 5 with Fusarium. The Helminthosporium was practically all H. sativum, at least 90% being of that one species, the other 10% were divided between H. gramineum and H. teres with the former predominating. The Fusarium was mainly F. graminearum, although F. avenaceum was obtained from a few of the plated seeds.

Examination of the seed revealed the presence of conidia of both Helminthosporium and Fusarium between the glumes and underneath the husk, especially towards the micropylar end of the seed. The most common evidence of Helminthosporium was the presence of the coarse brown mycelium which often quite discoloured the seed and the dark almost plate-like sclerotial masses of hyphae of Helminthosporium which were

probably the result of the conidia germinating in the Autumn and going into the scleratiol dormant stage at the onset of winter and the drying of the seed. Alternaria spores were very commonly observed in the same places but they are only very slightly pathogenic, if at all.

The distribution of these fungi on the seed was very largely confined to the half remote from the embryo, the so-called 'upper' half. This was first observed in preliminary planting experiments where half seeds were often sown along with the whole ones, and on emerging the resultant seedlings, although weak from lack of food, were quite healthy and showed very little evidence of the root or foot rot. Microscopic examination and an experiment in which halved seeds were plated separately confirmed this finding, for the upper end of the seed and in particular that region where the stigma had been was the most heavily infected with fungi.

That these spores and mycelial sclerotia were viable is obvious, of course, from the plating experiments and from the planting experiments, for, in the one case the surface of the seed was sterile and any fungi developing must come from under the seed coat, and in the other case, the soil and container were sterile and so the infection was due to the seed.

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A P P E N D I X C

New Improved Ceresan, its constitutions and its effects.

As has been previously stated, there is a great deal of secrecy surrounding the constitution of the organic mercurial disinfectants, and this is by no means obviated by the difficulty in preparing similar compounds. According to the patent laws of Great Britain the composition of the dust must be displayed on the cover of the container in which it is sold, but in most cases this amounts to practically nothing as there is no description furnished of the mode of preparation. Other difficulties seem to be that different dusts such as New Improved Ceresan, New Improved Semesan, and New Improved Semesan, Jr., for example, have the same chemical description but are not equally efficacious.

Attempts to evaluate these dusts on the basis of mercury content have been unsuccessful, and even the chemotherapeutic index of Binz and Bausch has not shown itself a reliable guide. The attempts made to evaluate the dusts on the basis of chemical composition have also been of little use in the understanding of their action, for dusts with widely different compositions have given equal degrees of control, and there seems to be no property or reaction-type which will link them together.

As described in the thesis, New Improved Ceresan, contains as an active agent ethyl mercury phosphate which has the

formula $C_2H_5.Hg,H_2PO_4$. There are also a number of other constituents of the dust, the more important being 77% of silica, 12% organic matter, Magnesium, Iron, and Aluminium oxides 9.5%, and Lime .6% The ethyl mercury phosphate is adsorbed on silica dust in a very fine state of division, as it has been shown that surface area is a very important factor in determining the efficiency of the dust. The point of surface area is not very clear as to its importance, for not only is the active agent soluble in water (60%) but it is also volatile and one cannot judge which of these factors is the more important. Where the sulphur fungicides are concerned a similar feature has been observed in that fineness of precipitate is essential for efficient action and this has been related to the surface from which the particles of sulphur vapour are given off in which state it is supposed to enter the spore. The analogy to New Improved Ceresan is, of course, obvious.

The solubility of ethyl mercury phosphate in water, about 60%, is not so important as it might seem on first appearance, for the presence of fungus spores may alter the liquid around them, or may even act on the mercury ions themselves causing a change in character. McCallan and Wilcoxon have claimed that spores of various fungi cause changes in sulphur with which they come in contact which changes may result in the death of the spores themselves.

Mercury ions themselves, however, are toxic of spores so that there is really no need to postulate any mechanism to change their form.

It is believed that the mercury ions owe their toxic action to the coagulation of the protoplasm caused by them. This reaction is in the nature of a 'salting-out' and has an electrolytic cause. The ethyl mercuric phosphate has then the potentiality of gaining access to the spore either as a vapour or in solution and in either case fineness of division not only ensures that a high proportion of the dust will reach the active state, but also increases the chances of the dust's penetrating to all parts of the seed and covering the spores very completely.

Such information as is at present available therefore, is not of much use in understanding the mode of action of the dust itself, but there is a possibility that the continued use of such dusts will cause an increased interest in their behaviour, and so result in the solution of the problem.

The Degeneration of Metropolitan Bent, - Agrostis stolonifera.

Contents

The Degeneration of Metropolitan Bent, - Agrostis stolonifera.

Introduction.

In recent years the study of disease of turf has been given fresh impetus by the establishment of the Board of Greenkeeping Research in England and by the founding of the Greens Section of the United States Golf Association in the United States. These institutions are however, still in their infancy and although the amount of work done by them has been enormous, still one feels that the surface of the problem has only been scratched and there yet remains much basic work to be done before one can say that much is known about the Scientific aspects of greenkeeping.

Diseases of turf have been recognised for many years. Old woodcuts depict players on very patchy greens, which would suggest a wide prevalence of disease even at that distant date. The modern interest in turf diseases began in 1914 when F. W. Taylor described a disease of turf in Philadelphia, which was shown a year later to be due to Rhizoctonia solani, Kuhn (9). Since that time, interest in this type of disease has grown, which is not surprising when one considers the large financial interests directly

involved.

Many diseases of turf are now known to be caused by fungi, but the cause of many others is as yet unknown; hence their control is purely empirical and pragmatic (7). There is also a wide range in the type of grass used on golf greens, each keeper having selected the the strain which seemed best suited to its immediate environment, the majority of these strains arising as bud sports from the clonely propagated mother lawn. This diversity of strains leads to a lack of unity in cultural practise, which naturally increases the opportunity for the occurrence of disease.

The disease of Metropolitan Bent which will be described has not been reported hitherto, and, so far as the writer's experience goes, is limited to Minnesota and the neighbouring states. However, it may not have been recognised in other regions or possibly confused with the more common turf disorders.

Symptoms and General Description.

The disease is confined to that variety of Agrostis stolonifera known as Metropolitan Bent, where its occurrence leads to the death of plant. An affected green has a general browning and death of the grass over large areas. (Fig. 1). Typical Brown Patch (Rhizoctonia solani) is characterised by the localisation of the

infection to certain well defined areas which may subsequently fuse, but in the case of the disease being described, the death of the grass takes place generally, although it may be more rapid on different parts of the same green.

Leaf tips of affected plants have a greyish-brown discolouration, followed by a withering and progressive destruction of the whole leaf blade. The roots of affected plants are very short and brittle, breaking easily even with the most careful handling. As the disease progresses, the green gradually becomes denuded until none or very little of the original grass is left. Where the Metropolitan Bent has died out, it usually is replaced by Poa annua so that the whole green becomes mottled, with Poa growing upright, the remaining prostrate green bent, and the brown dead patches.

The survival of a few individual plants is a consistent and noteworthy feature. These are of a lighter green colour so that their appearance makes a spot of green here and there on the brown areas, to which the name "freckle" by which the disease is known to the greenkeepers, is due.

Ecology of the Disease.

The time of appearance of the symptoms varies,

severe attacks having been seen both in spring and fall. However, it most commonly appears in spring or early summer and in a short time (2-3 weeks) large areas of the green are apparently completely burned up. The writer made his first observations in the late fall (1st. November) and even then the destruction was well nigh complete on diseased greens.

The question of both soil and air drainage was examined with respect to this degeneration, as it had been previously shown by Monteith (6) that pockets of still air increased the incidence of brown patch. This avenue of approach gave no information anent the occurrence of this present disease, as it was just as prevalent and destructive in hollows as on exposed high plateaux or on slopes. Monteith (6) also showed that surrounding the green with trees decreased the air drainage, but although the degeneration was very marked on some very well sheltered greens still equally destructive epiphytotics could be seen on greens which were fully exposed to winds from all directions.

The type of subsoil on which this degeneration could be found varied from a light loam through soils rich in humus to heavy clays which would indicate that the physical texture at any rate of the soil had no

effect on the incidence or destructiveness. It would follow that the drainage or amount of moisture in the soil could also be said to be non-important or at least relatively unimportant.

One factor was very constant on all the examined diseased greens and that was the extreme density of the turf mat. Metropolitan Bent stolonises very freely and a high percentage of these stolons take root and develop new plants, thus building up a dense and relatively impermeable surface. So dense is this mat that in many cases aeration is impaired to such an extent that grass clippings and dead roots are not completely destroyed, and a top soil is built up in some cases two or three inches thick which is composed of nothing but plant debris. This is probably due to a lack of oxygen which is necessary for the bacteria to destroy the organic debris with the formation of humus and the liberation of minerals and carbon dioxide.

Attempts to Isolate a Causal Organism.

Pieces of diseased roots, leaves, and stems were surface sterilised in mercuric chloride or silver nitrate. They were washed in sterile distilled water and plated on potato dextrose agar, and on Richard's agar. Other pieces were simply washed in sterile distilled water

and from the resulting fungal and bacterial colonies isolations were made. A large number of organisms were thus obtained and used to inoculate healthy plants in the greenhouse.

The inoculum was increased on sterile oat hulls or potato agar until a sufficient quantity of inoculum could be obtained for the purpose. Inoculation was then made by smearing the organism on the leaves immediately after clipping, by sprinkling a spore and mycelium suspension on the plant and on the soil, and by inserting the fungus growing on oat hulls among the roots. This was done for some twenty different isolates but in no case was there evidence of pathogenicity.

On incubation in a damp chamber the diseased tips developed a bloom of fine grey mycelium but on isolation and inoculation this proved to be non-pathogenic and was probably a saprophyte on the decayed leaves. None of the bacteria gave positive results.

Examination of soil samples failed to reveal the presence of any grub or insect which might be the cause of the disease, and attempts to transmit the disease by rubbing healthy plants with diseased leaves also failed. This accumulation of negative evidence seemed to indicate that the degeneration was non-parasitic in origin.

Control Measures.

When the disease was first noted in the field, it was naturally assumed to be similar to Brown Patch and Snow Mould in having a fungus origin; therefore attempts were made to control it by means of chemicals, especially mercurials. These efforts, using mercurous and mercuric chloride, were unsuccessful. Similar experiments in the laboratory, using the mercury compounds and copper sulphate, also yielded negative results. The latter when applied in too great concentrations caused injury as has been previously reported (1). These efforts to control the disease by chemicals furnish additional evidence that the disease is not caused by fungi, and, although by no means conclusive, is nevertheless cumulative.

Since the disease was not checked by chemicals it was thought that drainage and water availability might be of some importance. Tests were made in the greenhouse, as they had also been made by greenkeepers in the field, and no combination of watering schedules had any effect towards affecting the severity of the disease. Excessive watering on the contrary led to the appearance of a chlorosis along the edges and at the tips of the leaves, but the symptoms thus manifested had no resemblance to those of the disease under investigation.

To improve the aeration of the greens they were very heavily spiked or opened up with an iron fork. The surface was then raked severely to break up the very dense surface mat and so allow the free circulation of air around the infected plants. A slight beneficial effect was obtained in this way, but it was neither permanent nor great and was due in all probability to the actual benefit of spiking on the grass itself and not to the alteration of the environment to one less suitable for fungus development. Thus although the grass grew better the disease symptoms were still present and the improvement was only a slight transitory alleviation and by no means a method of control.

The application of fertilisers and top dressing to the greens also brought on a little benefit, but this also was temporary, and had no lasting effect, being once more a direct action on the plant and not on environmental effect on the relation between a fungus and the grass.

Subsequent Investigations.

Various greenkeepers had noted that there was sometimes a recovery of the diseased areas in which the grass came back and a new, but rather poor, turf was formed. This turf was of a slightly lighter green and probably resulted from the spread of the disease-escaping plants previously mentioned. In the pots in the greenhouse it

was noted that, after six months, a number of the diseased turfs had recovered and were growing vigourously.

This recovery grass had, as had already been noted, a lighter green colour than the original Metropolitan Bent and a more bunchy appearance caused by more stems arising from the one point on the stolon (Fig. 2.). There was also a very decided difference in the root systems of the two types, that on Metropolitan being only half as long as the escape-plant. In the light of the recent findings of O'Brien and Dennis (8), this difference in the length of the root system acquired a special significance and the investigations were extended.

A comparison was made between the root system of Metropolitan Bent which could be attacked by the disease and a variety known as Woodhill which was resistant to the disease. In plants from pots in the greenhouse after six months growth, the root system of Woodhill Bent was more than twice as long and many times more extensive than that of healthy Metropolitan grown under the same conditions. The average length of the ten longest Woodhill roots was 17.7 centimetres while for Metropolitan it was only 8.6 cms., and the difference in number, volume, and surface area can be seen in Fig. 3. There was another very striking difference and that was the number of dead roots on the

Metropolitan turf which could easily be washed away, but which under natural conditions would accumulate as a dense, relatively impermeable surface layer.

Since these plants had been grown under very artificial conditions in the greenhouse, it was thought advisable to see if this held good in the fields. Turfs of 9 inches square and about 18 inches deep were cut in the greens and brought into the laboratory. They were then left to soak overnight and the soil washed off. When the whole turf had been treated thus and the roots could be separated easily, square pieces of equal size were taken from the centre of the original turfs and the root length examined. This latter selection was to obviate the chance of roots having been cut by the spade when the turf was dug out.

The turf thus obtained can be seen in Fig. 4, where it is evident that the conditions seen in the greenhouse pots hold good for the fields also. The roots of Woodhill once more were about twice as long and much greater in surface area than those of the Metropolitan Bent. The difference in the respective root lengths thus seems to be consistent.

It was thought that it might be interesting to compare the "escape-grass" with healthy Metropolitan, and here it was found that the escape-plants were the same

type as the grass in the recovered turf, with the longer root systems and bunchy growth-form.

Some experiments were made on the effect of fertilisers on the diseased plants. Applications of $(NH_4)_2 SO_4$, KH_2PO_3, and H_3BO_3 were made and the plants watched over a period of six weeks. In no case was there any sign of recovery, and the death of these experimental plants together with heavy falls of snow, vitiated the chance of obtaining new material.

Discussion.

The conditions under which golf greens are kept are ideal for the development of disease epidemics. A large number of plants are grown under very artificial conditions; they are forced to grow vegetatively by artificial manures, they are watered and cut regularly, so that their growth is succulent, in which condition they readily fall prey to any invading organism.

In the case of the degeneration described above, no casual organism could be isolated from diseased plants. This would indicate that the disease is non-parasitic in origin and this conclusion is supported by a great deal of circumstantial evidence. In the first place, neither mercurials nor copper sulphate had any effect in checking the incidence of the disease, and as these chemicals, especially the mercury chlorides, are extremely active

fungicides, some weight must be given to this finding. A second factor was the fact that the disease could appear over a very wide range of climatic, topographic and edaphic conditions. In the usual fungal diseases there are definite factors which influence the attack e.g. Dahl demonstrated that low temperatures were necessary for the snow mould, Fusarium nivale (3) while Erwin (4) and Coons (2) showed that the presence of epidemics of late blight of potato was dependent on the interaction of temperature and moisture. Since this degeneration is so very varied in its appearance, there is therefore rather conclusive evidence that it is of non-parasitic origin.

There are many factors which will cause the onset of non-parasitic diseases, but it has been shown above that moisture and soil aeration do not appreciably affect the disease, so it is concluded that these factors can be eliminated.

In a recent paper, O'Brien and Dennis (8) showed that the difference between susceptible and resistent varieties of swedes to boron deficiency was related to root length. They state: "It appears that the resistent varieties possess a much greater root system than do non-resistent varieties". It is evident therefore that there are very good reasons for arguing that the degeneration is due to

to a deficiency of some minor element or elements in the soil, as the analogy between the swedes and the Metropolitan and Woodhill Bents is very obvious.

The extreme density of the turf of Metropolitan Bent coupled with the very short root system would tend to make it more prone to deficiency diseases than a plant of the Woodhill type. The roots are so small and short that they derive their mineral nutriment from a comparitively small area of soil, and the point is further emphasised by the thickness and density of the turf by reason of which only a small part of the root system will penetrate to the soil. This being the case, the major portion of the root will be growing in the debris of grass clippings and roots, which will not be subject to the normal mineralising action of the bacteria, as this action is an oxidation process (10) and the circulation of oxygen in such a dense turf is very poor.

This poor circulation of oxygen can be seen in another way. The root of Metropolitan Bent, if examined in situ, can be seen often to turn up at the tip. It is thought that this negatively geotropic response is in reality a positively aerotropic one and is due to the lack of oxygen mentioned above. The benefit of the "spiking" in loosening the surface of the green would then

be explained on this basis.

Applications of artificial fertilisers might replace a little of the deficient elements, but Hurst (5) has shown the extreme purity of the modern artificial products in which there are barely traces of the minor elements. It is concluded therefore that this degeneration of Metropolitan is in the nature of a deficiency disease whose exact nature is not yet fully understood.

Summary.

The symptoms of a new disease of Metropolitan Bent are described as, death of the plants over large areas of the green, with the death of a leaf beginning at the tip. Typical of the disease is the escape of a few plants of a lighter green in the diseased areas.

The disease can not be controlled by mercurials, aeration, or alteration of the water schedule.

The susceptible variety is characterised by very dense turf formation and extremely short roots, while the escaping plants and resistent varieties have much longer roots.

From an analysis of the evidence the disease is thought to be due to the deficiency of one of the minor elements in the plant.

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Fig. 1. Typical diseased area. The dark scattered patches are Poa annua invading the dead turf, while the normal turf covering can be seen in the lower right hand corner.

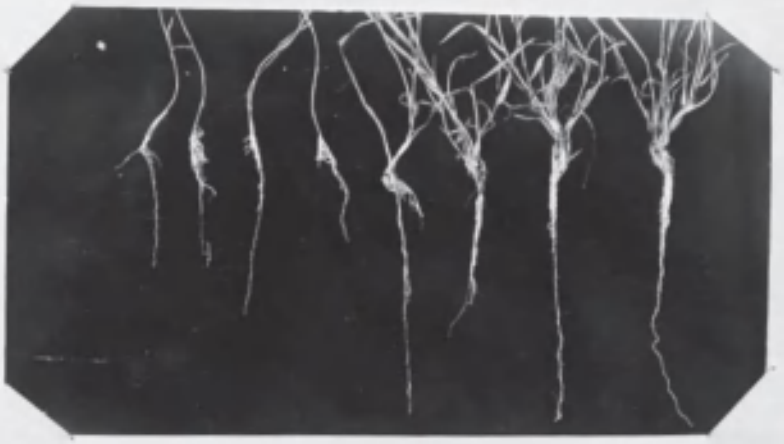

Fig. 2. "Escape" grass - right - as compared with healthy Metropolitan Bent - left. The bunchy appearance of the escape grass and the much longer root system are apparent.

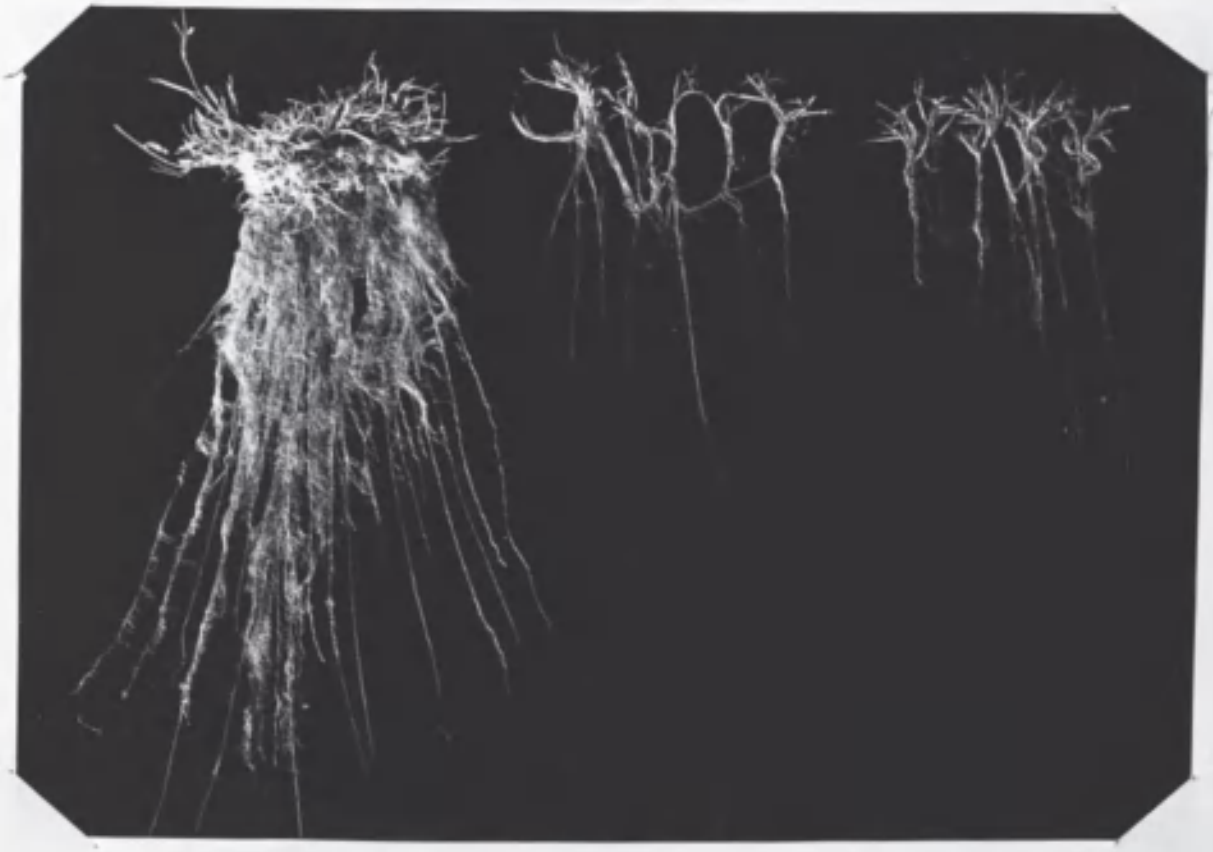

Fig. 3. Disease resistent Woodhill Bent - left - as compared with healthy Metropolitan Bent - right. These plants were grown in pots for six months before the photographs were taken, and exaggerate the normal difference in root development.

Fig. 4. Healthy Woodhill Bent - right - and healthy Metropolitan - left, obtained from turf cut from the University Golf course, St. Paul, Minn.

A Study of the Interaction of Two Fruit-Rotting Fungi. Oospora citri-aurantii and Penicillium digitatum.

Contents.

A Study of the Interaction of Two Fruit-Rotting Fungi, Oospora citri-aurantii and Penicillium digitatum.

Introduction.

While the importance of the pure culture technic of growing fungi cannot be over-estimated, it still leaves a number of very basic problems unsolved. One of these is that by its very nature the pure culture method tends to deviate from natural conditions as the technics improve, for whereas nature very rarely works with one organism, man starts not only from a single organism but even from a single cell of such an organism.

It is of the utmost importance, therefore, to try to approximate more closely to natural conditions and to discover if by using artificial technics we may not be missing points of the utmost interest and importance in the understanding of the biology of fungi.

The work to be described below began as a direct result of reading a paper by Savastano and Fawcett (25) who emphasized the importance of using mixed cultures of fungi and demonstrated the practical significance of such a procedure. Since a very voluminous literature has accumulated on the antibiotic interaction of fungi, it was decided to study another phase of the problem and attempt to elucidate some

of the principles of the synergistic or mutually beneficial action of fungi. This subject has never been treated at any length in summary papers, and so it seems advisable to review the literature to date and gain some perspective of the general situation.

Literature Review.

The references to stimulatory action of one fungus on another are contained mainly as incidental facts in papers dealing with other subjects. Harder (14), however, in 1911 studied the interaction of Ascomycetes and Basidiomycetes in mixed culture and, although dealing extensively with the antibiotic effects, he mentions a case of synergism. Coniophora cerebella (Pers.) Schroet. was inhibited in its growth by Penicillium glaucum Link for a while. Later, however, this inhibition was overcome and C.cerebella overgrew the Penicillium at a rate greater than it would grow alone in pure culture.

Fawcett (11) studied the gummosis of citrus trees caused by Pythiacystis citrophthora S. and S. and showed that the severity of the disease was markedly increased when species of Fusarium were present in the infected parts. Ruggieri (24) made a similar observation on the effect of Fusarium on Phytophthora parasitica Dastur, although it is not unlikely that they were describing the same disease. A

similar effect has been recorded by Brommelhuss (5) when Ophiobolus graminis Sacc. caused greater damage to wheat if inoculated with Alternaria sp. than if inoculated alone.

The stimulatory action of fungi in increasing perithecial production has been noted by McCormick (18) and Heald and Pool (16). The former studied Thielavia basicola Zopf. and found that the presence of other fungi, such as Cladosporium fulvum Cke. and Aspergillus umbrosus B. and S., stimulated perithecial production, while the latter, using Melanospora pampeana Speg. showed a similar effect in cultures mixed with Fusarium moniliforme Sheld. or F.culmorum (W.G. Sm.) Sacc.

Bacterial synergism has been the subject of much study, especially with regard to the soil microflora (27), but recently a very interesting example of phytopathogenic synergism has been shown. Burkholder and Guterman (7) in examining a bacterial leaf spot of Hedera helix found a non-pathogenic bacterium present with the real pathogen, and the former had the power of increasing the pathogenicity of the true causal agent. This work was extended by White and McCulloch (28), who isolated ten bacteria from diseased leaves. They found that one was synergistic to the pathogen while the others gave purely negative results.

Waksman (27) has shown that one organism in the presence of a second may be forced to do something which it normally

would not do. Thus, species of Actinomyces would not grow on corn stalks if inoculated alone, but if accompanied by species of Rhizopus or Aspergillus niger Van Tiegh, they grew very well and caused an active decomposition of the straw. Similar to this was the work of Rege (23) on the decomposition of cellulose by fungi where an association of Acrimoniella, Aspergillus sp., and Coprinus sp. could cause a much more rapid decomposition than the separate fungi acting alone.

The growth of fungi in mixed culture has been studied by Zeller and Schmitz (30) who demonstrated that the major effect of fungi was their antibiotic action, but cite the instance of Pleurotus sapidus Kalchbr. growing over Aspergillus glaucus Link at an accelerated rate, when compared with its growth rate in pure culture. Seedling blight of wheat has been examined in this connection by Geach (13) who showed that Urocystis tritici Körn would cause 2 per cent blights, Helminthosporium sativum P.K.B. 8 per cent blight, while the two acting together caused 54 per cent blight.

The work of Savastano and Fawcett (25) has been one of the most extensive pieces of research on the interaction of organisms, and it was particularly good in demonstrating how environmental conditions would influence the components and interaction of a mixture of fruit-rotting fungi. In their work the stimulatory effect of fungi on each other

was described, and they note the instance of Penicillium digitatum Sacc. and Oospora citri-aurantii (Ferraris) Sacc. where acting together they caused a rot greater than the sum of the rots caused by their individual action. Diplodia natalensis Evans seemed to have a stimulatory effect on all the fungi with which it was associated, this being especially marked when mixed with Colletotrichum gleosporioides Penz.

In his study on mixed cultures, Porter (22) divided the interaction of fungi into five classes, but in none of his classes did he include any stimulatory phenomena. The synergistic effect, however, is not so marked in fungi growing from separate inocula side by side, and thus it is very easy to miss. Similarly, Machacek (19) in his work on the association of phytopathogens obtained no evidence of stimulatory action, although both of the above papers are very excellent in their report on the antibiotic effects of fungi.

Synergism and the stimulatory action of fungi on each other is very little understood; therefore it was proposed to obtain fungi which had a synergistic effect with respect to their pathogenicity and to see if similar effects could be obtained in culture under controlled conditions. It was then proposed to attempt to find the cause of this synergism and, if possible, to arrive at some explanation of the phenomenon.

The problem was divided into two parts: namely, the effect of the interaction of the fungi on the colony type under various conditions and the effect of the mixture on the growth rate of the colonies. Thus, although the growth in culture cannot be said to be analogous to that under natural conditions, yet one may obtain information which may help to elucidate the problems as they occur in nature.

Material and Methods.

It was decided that cultural conditions might approximate more closely to those prevailing in a more stable environment than in an actively growing plant. Fungi causing fruit rots were selected therefore as being the most suitable for this study; and, since the synergistic effect of Penicillium digitatum and Oospora citri-aurantii had been commented on by Savastano and Fawcett (25), the major part of the work was done with them.

Cultures of both these organisms were obtained from Dr. H.S. Fawcett. They were grown on Cook's agar in petri plates, each of which contained 20 cc. of medium, and inoculation was made from young cultures in all cases, the pieces of inoculum being of equal size (about 2 mm. square). The mixed colony was obtained by placing the two pieces of inoculum together in the centre of the plate, or else on top of the other, as preliminary experiments had shown that there

was no difference in either method.

The diameter of the resultant colony was measured in two directions and the mean taken as the true diameter, measurement beginning the second day after inoculation and being made daily until the end of the experiment. The experiments were done in quadruplicate or sextuplicate in all cases. Filtrates were obtained by growing the fungi in modified Richard's medium (i) and filtering off the mycelial mat. This mycelial mat was dried to constant weight at 80°C. and weighed to determine growth in liquid medium, the filter papers having previously been dried to constant weight.

In inoculating lemons the standard technic was used, that is, the fruit was washed and then surface sterilized in mercuric chloride. A plug of rind and a little pulp was then removed under sterile conditions, the inoculum placed in the hole thus formed, and the plug replaced. The area around the wounded surface and the wound itself were sealed with paraffin wax and the fruit stored in a moist chamber to encourage the development of rot.

Unless otherwise stated, all the work was carried out laboratory temperatures. Nevertheless, so far as possible, temperature was maintained at 75°F. No comparisons are made, however, between results obtained at different times, only replicates in the same experimental lot being compared.

(i) In Richard's solution with KH_2PO_4 instead of K_2HPO_4

Colony Types of Pure and Mixed Culture.

The colony type varied within fairly wide limits over the range of media used, this being true not only for the pure cultures but also for the mixed. Since descriptions of growth form on a large number of media would be unnecessary, the more significant types will be tabulated with the intention of indicating the results on which the conclusions are based.

On Cook's medium, Penicillium digitatum forms a very heavily sporulating green colony with a white mycelial border. It is virtually a non-staling type, as it covers the plate if allowed to grow over a sufficient period. Oospora citri-aurantii, on the other hand, forms a flat slimy white colony with mycelial wefts closely appressed to the agar. On some media, e.g. potato-dextrose agar, it forms hair-like aerial projections composed of large numbers of hyphae joined together, but in pure culture on Cook's medium these are not visible.

The colony growing from a mixed inoculum is typically Oosporaceous in appearance, with a few modifications which appear after a long period of growth. The white appearance of the Oospera develops a greenish tinge, and on microscopic examination one can see conidia of Penicillium growing in the centre. This growth of Penicillium causes a growth-form reaction in the Oospora which forms these aerial hyphae

which are normally not formed on this medium-(Plate 1). The pure Oospora colony stales before the Penicillium, and this is apparent in the mixed colony whose edge may keep on growing as pure Penicillium, with no signs of Oospora, even on microscopic examination.

Typical of the effect of medium on the growth form of the mixture is that of variation in the carbon-nitrogen ratio. Table 1 summarizes this effect and is based on the examination of six plates in each of two experiments, with an equal number of checks in each case. There was some variation between plates, but so far as possible that is taken into account and does not affect the general statements made in the table.

It is obvious that a high carbon-nitrogen ratio favours the growth of Penicillium in pure culture, while the normal Oospora type is modified to a white aerial form. At the other extreme, an extremely low carbon-nitrogen ratio is detrimental to the growth of the Penicillium, resulting in small, dense colonies lacking their normal colour. Oospora, however, although not growing in a normal way, maintains the size and vigour of the colony. These changes are reflected in the growth of the mixed colonies.

When the ratio of carbon to nitrogen is 8:1, the mixed colony begins its growth as Oosporaceous in nature, but very soon Penicillium appears around the edges and seems to check

Table 1. Effect of the carbon-nitrogen ratio on the growth type of Oospora citri-aurantii and Penicillium digitatum in pure and in mixed culture.(a)

Carbon-nitrogen ratio expressed in grams per litre.	TYPE OF COLONY.		
	Penicillium digitatum	Oospora citri-aurantii	P.digitatum + O.citri-aurantii
$\frac{80}{10}$	Densely sporulating colony bordered by a zone of fine white mycelium.	Very white aerial colonies, largely superficial.	When young the colonies resemble Oospora, but as they get older, by the formation of "fan-tails"(b) a ring of Penicillium is formed which stops the further growth of the Oospora.
$\frac{40}{10}$	Sporulating centre with a more diffuse outer zone.	Flat white colonies not so aerial as previous one, sporulating in the centre and a little smaller.	Young colonies are like Oospora but soon fan-tails appear which are Penicillium and curve back around the colony but may not surround it.
$\frac{10}{10}$	Colonies are greenish with no diffuse edge.	The colonies are flat and a little slimy. About same size as above.	For first few days the colonies resemble Oospora, but the appearance of "fan-tails" initiates marginal growth of Penicillium which however does not nearly surround the Oospora part.
$\frac{10}{40}$	Small dense brownish colonies with no sporulation.	Very dense white colonies with a well defined edge. Larger than the above.	The mixed colonies resemble Oospora all the time with the same dense whiteness.
$\frac{10}{80}$	Very small dense brownish colonies.	Extremely white colonies.	In the mixed colonies which resemble Oospora very closely, no evidence of Penic. can be detected.

(a) Summary of experiments done in duplicate with six replicates per experiment.

(b) "Fan-tails": for explanation see text.

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the growth of Oospora, for the rest of the colony becomes like Penicillium. At the junction of the Oospora and the Penicillium can be seen a zone where the aerial hairs of the former are being developed, and along this line there also is a brownish discoloration of the agar. The Penicillium around the edges has been observed to grow back over the Oospora culture and eventually covers a large area of the colony.

The origin of the Penicillium around the edge of the colony is due to what is being called "fan-tails". At some point along the edge of the mixed colony, before the Penicillium has appeared, there is to be seen a fan-shaped wedge of clear hyphae growing out into the medium ahead of the rest of the colony. This fan-tail evidently arises at any point on the circumference of the colony and is possibly due to the checking of the growth of the Oospora, for in a day this clear hyphal aggregate evinces itself as Penicillium and begins to curve around the Oospora colony, until it may completely surround it.

In some cases this fan-tail can be traced to the centre of the colony where it has its origin, but in other cases it arises just as a spot of Penicillium at the edge of the colony, which eventually will surround the whole.

As the carbon-nitrogen ratio gets less, it can be seen that the Penicillium is becoming smaller while the Oospora retains its size and vigorous growth. There is also in the Penicillium a loss of the fine zone of myeelium which surrounded it in the higher carbon-nitrogen ratio colonies. This loss of vigour in the Penicillium is seen in the mixed colony where the fan-tails appear but never surround the Oospora colony as in the previous case. This is apparent at a ratio of 1:1.

In the extremely low carbon-nitrogen ratios the Penicillium colony is very small while that of the Oospora is fairly large; thus in the mixed colony there is absolutely no sign of any Penicillium, nor are there any modifications of the mixed colony which would indicate that the Penicillium was growing along with the pure culture of the Oospora, even under the microscope.

Since the above and a number of preliminary experiments had shown the importance of the carbon-nitrogen ratio, it was decided to investigate the effect of the actual amounts of carbon and nitrogen in the form of dextrose and peptone. The summary results for dextrose can be seen in Table 2 and for peptone in Table 3, but they will not be discussed separately, as they illustrate the same principle, each serving to strengthen the other.

It can be seen very easily that the two fungi are antithetic in their nutritive requirements. The Penicillium

will hardly grow at all if there is not a sufficient quantity of carbohydrate in the medium, while the Oospora, although affected by changes in the amount of carbon, does not seem to be so dependent on the presence of a great deal of carbon. The reverse is true, however, for nitrogen (Table 3), for in this case it is the Oospora which has the absolute necessity for nitrogen to get much growth, whereas the Penicillium can grow reasonably well in its absence.

This variation in nutritive requirements can be seen very well displayed in the mixed culture, for where the Penicillium grows badly in pure culture its influence is hardly seen or felt in the mixed culture, and vice versa. The case of the Oospora is slightly different, because it dominates the association for a longer or shorter period, irrespective of whether it is growing badly or well. This, however, raises the question of stimulation which will be considered in a later part of this paper.

Experiments were also made on the effects of temperature on the growth and interaction of the organisms, but they only serve to illustrate farther the points made above, although there are a few things worth mentioning. In the first place, Oospora will grow over a wider range of temperature than Penicillium, i.e. from 10°C. to 36°C., at which extreme temperatures the latter makes no growth. At 28°C. and 24½°C. the two organisms grow at practically the same

Table 2. Summary of the effects of the absolute quantity of carbohydrate on the growth and interaction of Penicillium digitatum and Oospora citri-auarantii.

Amount of dextrose in grams per liter	Type of Colony		
	Penicillium digitatum	Oospora citri-aurantii	P. digitarum and O. citri-aurantii
20.	Green colonies with very heavy sporulation.	Flat white slimy colonies.	Young colonies resemble Oospora but by a development of "fan-tails" the original Oospora is surrounded by Penicillium. Development of Oospora hairs.
10.	Colonies similar to the above but a little smaller.	Thinner colonies of same size as above.	The usual Oospora beginning is surrounded by Penicillium, and a little brownish colour is developed.
7.5	Colonies develop a brownish discolouration and are smaller than before.	Thin flat colonies, same size as above.	There is a longer period of Oospora dominance in these colonies, but can still see the Penicillium at edges. Brownish colour developed.
5.	A very marked brownish discolouration of still smaller colonies.	Colonies similar to above.	The Oospora is becoming more dominant, but the Penicillium can still be detected.
2.5	Smaller poorer colonies.	Thin colonies but of the same size as the above.	There can still be seen Penicillium in the mixed colonies, but it is scarce and hardly apparent. The Oospora may out-

(Table 2, contd.)

Amount of dextrose in grams per liter	Type of colony		
	Penicillium digitatum	Oospora-citri-aurantii	P. digitatum and O. citri-aurantii.
			grow the Penicillium and appear at the edges.
0.	Very poor small non-sporulating colonies. Definite zonation.	Thin colonies very little reduction in size.	There is no sign of Penicillium and can see only Oespora.

Table 3. Summary of the effects of the absolute quantity of nitrogen on the growth and interaction of Penicillium and Oospora citri-aurantii.

Amount of Peptone in grams per liter	Type of Colony		
	Penicillium digitatum	Cospora citri-aurantii	P. digitatum and O. citri-aurantii
10.	Large green colonies, much sporulation.	Flat white slimy colonies.	Initially the colonies are like Oospora but as they age get the appearance of Penicillium which dominates the association.
7.5	Colonies like above but smaller.	Little smaller and thinner colonies.	When young the colonies are like Oospora but they soon develop fan-tails and this is followed by the domination by Penicillium.
5.	Colonies are smaller but still like the above.	Flat thin smaller colonies.	Oospera is still dominant at the beginning but Penicillium soon dominates from fan-tails. Hairs of Oospora are developed in the mixed culture.
2.5	Colonies are a little more serial and not quite so green.	Colonies are very irregular in outline and can see the individual hyaline hyphae in strands.	At first growth is normal Cospora but soon it is overgrown by Penicillium both by fan-tails and from development from the centre.

(Table 3, contd.)

Amount of Peptone in grams per liter.	Type of Colony		
	Penicillium digitatum	Oospora-citri-aurantii.	P. digitatum and O. citri-aurantii.
0.	Colonies are white and fluffy and very thin.	Practically no growth save for a few hyphae.	Begins as ordinary Oospora but very soon Penicillium appears in the centre and covers the colonies.

rate, but the Oospora is quicker to start growth, so that the colonies consist of an outer growing zone of Oospora and a central zone of Penicillium and Oospora which also is increasing in size at the same rate as the outer edge. The Oospora, however, being a more rapidly staled fungus, stops growth first, so that the Penicillium in the centre keeps on growing and expanding until it almost covers the Oospora and the whole colony.

Growth Rate of Pure and Mixed Cultures.

A. In Liquid Medium.

The medium used was Richard's modified solution, and the methods have already been described whereby the dry weight of the fungus was determined. This experiment was done twice, and each weight is the average of six weights of mycelium of the same age (see Table 4.). The growth rate was not the same in both experiments, but the course followed by the curves was the same, and the difference is attributable to the effect of temperature.

The typical curves obtained for the growth of the two organisms and the mixture can be seen in fig.1, but there are a few points of interest which deserve attention being drawn to them. The cultures in the flasks while very young showed a slightly greater growth in the mixed cultures than in any of the pure ones, but by the time enough mycelium had

grown to be able to weigh, this initial stimulation had disappeared.

From the curves it will be seen that the Penicillium makes the greatest total growth of them all, as indicated by the highest point of the graph, the mixed colony comes next, and the Oospora grows very little at all. There is, however, a farther feature to be noticed in that the maximum growth of the Penicillium colony is reached around the forty-fourth day, while in the mixed colony the peak is not reached until the sixty-sixth day. There seems therefore to be some quality in the mixture which tends to keep it growing for a longer period of time than the Penicillium. This however may be a question of exhaustion of food by the rapidly growing fungus, but there is another aspect which must be noted. This is the fact that the presence of the Oospora slows down the autolysing process, for in the mixed culture there is an autolysis of .139 gms., while in the pure culture of Penicillium this is an autolysis of .26 gms. altogether and of .171 gms. in the same period of time, even after the initial rapid fall.

This difference in the rate of autolysis can also be seen by direct observation. The culture of Penicillium begins to be very friable as it gets older, and instead of the mycelial mat clinging together it breaks up. In the mixed colony, on the other hand, the hyphal mat remains as

Table 4. Weight of mycelium, in milligrams, of pure and mixed cultures of Oospora citri-aurantii and Penicillium digitatum grown in liquid Richard's medium.(a)

Age of colonies in days	Weight of mycelium in milligrams		
	Penicillium digitatum	Oospora citri-aurantii	P. digitatum + O. citri-aurantii
21	186	26	175
30	274	46	239
44	382	55	319
66	296	35	351
99	158	35	220
117	125	32	222

(a) Each weight in the average of six replicates.

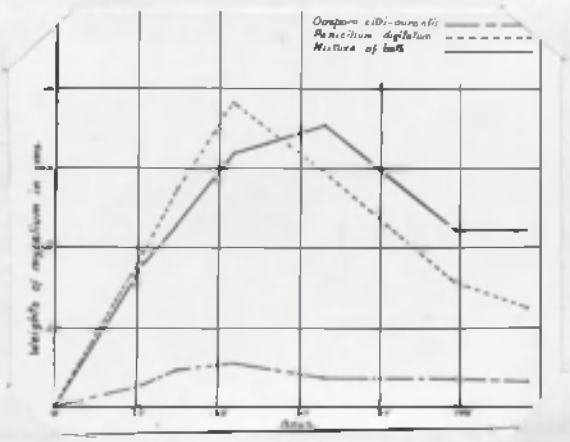

Fig. 1. Growth of Oospora citri-aurantii and Penicillium digitatum in liquid Richard's solution. Note longer period of growth in the mixed culture.

such until the end of the experiment, and there is not the same fragmentation that there is in the other case.

The solution as made up had a hydrogen ion concentration of 6.65, and the growth of the fungi altered this. Where the Oospora was grown alone, the pH was 5.52, with Penicillium alone it was 6.80; and where they were grown in mixed culture it was 6.45. This lowering of the pH in the mixed culture must have been due to the growth of the Oospora, but even so one could not see any Oospora in the final cultures, and so its presence could only be deduced.

From this experiment one can only conclude that the presence of another fungus in a liquid medium may have the effect of slowing down, but lengthening the duration of, the growth period and also of causing a decrease in the amount and rate of autolysis in the mixed as compared to the pure cultures.

B. On Solid Media.

The growth of the separate fungi and of the mixture of the two can be seen in fig.2, where growth is on the ordinary Cook's medium. From that graph it will be seen that Oospora starts growing much faster than Penicillium, but that the mixed colony is growing just a little faster than the other two. This state is maintained for a few days when the staling Oospora stops increasing in size but the Penicillium keeps on growing and soon grows larger than

it. The mixed colony parallels the Oospora very closely until it stops growing, then when the Penicillium is forming "fan-tails" it begins to pick up in growth again, although it may not equal the pure Penicillium. This latter inequality is probably a slight antagonistic effect of the Oospora on the Penicillium which is also evident in the filtrate experiments and on the liquid medium.

The point to which it is proposed to attach some significance is the initial stimulation which can be noted in the mixed colony. The ubiquity of this phenomenon can be seen in fig.3 where the growth of the fungi in pure and mixed cultures is graphed against the carbon-nitrogen ratio, the readings being taken on the second day after inoculation. This can also be seen in Table 5. At this stage the mixed culture is purely Oospora, and no Penicillium can be obtained from the edge of the colony, thus it would seem that the presence of the Penicillium caused a stimulation of the growth of the former. The increase in growth of Oospora towards the end of the graph is due to the fact that growth there is purely superficial.

It is only reasonable to argue that since the Oospora in all the above cases is growing rather well, then there will be less marked stimulation than under conditions not so suitable for the growth of Oospora where any stimulation would be more easily noted. This is illustrated very well in fig.4

Table 5. Diameter in centimeters of colonies of Oospora citri-aurantii and Penicillium digitatum in pure and mixed culture on media of different carbon-nitrogen ratio content after two days.(a)

Carbon-nitrogen ratio	Diameter of Colony in centimeters		
	Penicillium digitatum	Oospora citri-aurantii	P. digitatum + O. citri-aurantii
8/1	1.5	1.6	1.9
6/1	1.5	1.6	1.7
4/1	1.5	1.4	1.5
2/1	1.0	1.4	1.5
1/1	1.0	1.4	1.4
1/2	.7	1.5	1.5
1/4	.7	1.5	1.5
1/6	.7	1.6	1.6
1/8	.7	1.6	1.6

(a) This experiment was done twice with four replications each time. The data are the average of one such experiment.

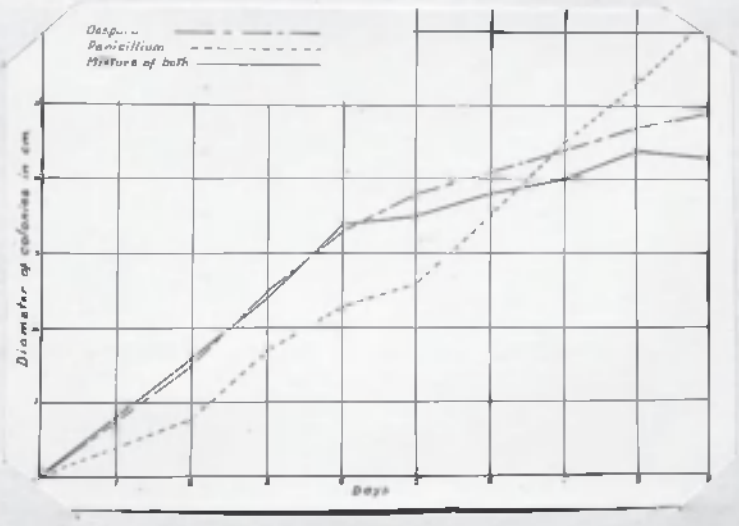

Fig. 2. Growth of Oospora citri-aurantii and Penicillium digitatum in pure and mixed culture.

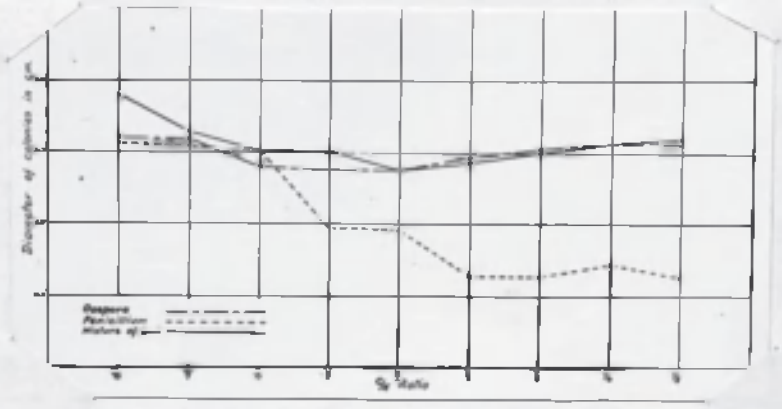

Figure 3. Growth of Penicillium digitatum and Oospora citri-aurantii in pure and mixed culture graphed against the carbon-nitrogen ratio.

where the growth of pure Oospora colonies and mixed colonies at the end of two days is plotted against the amount of nitrogen in the form of peptone in the medium. Data for this graph (Fig.4) are furnished in Table 6.

It can be seen that the size of the Oospora colonies grows increasingly smaller as the nitrogen content of the medium decreases, and while this is true also of the mixed colony the decrease is not nearly so rapid or complete. Thus at zero amounts of nitrogen in the medium the pure colonies of Oospora would not grow at all save for a few very thin short strands, while when the Penicillium was also present, although it had not started to grow, the Oosporaceous part of the colony grew very well. In mixed colonies of 1.38 cm. in diameter, Oospora could be isolated from the edge in pure culture before the pure Oospora colony had even started to grow.

The course of growth of pure and mixed cultures on a nitrogen free medium can be seen in fig.5 and table 7. In this case the initial stimulation was still evident at the end of nine days when the experiment was terminated, and the mixed colony was definitely Oosporaceous in type up to the fifth day when a little Penicillium could be seen in the centre before beginning to surround the culture, which it finally did.

In all the cases observed the Oospora has been the fungus which has received the stimulation and there is some evidence to indicate that there may be an antibiotic action by it on the Penicillium. This phase is, however, made more difficult by reason of the faster initial growth rate of the Oospora which might mask the growth of very young Penicillium. The size of the mixed colony is therefore patterned on that of the fungus which is growing best and if conditions are good for Oospora and bad for Penicillium, the growth form of the mixed colony is that of the Oospora. If, on the other hand, the Oospora is inhibited or slow in its growth, then the Penicillium comes out in the form of fan-tails and the growth pattern thereafter follows that of Penicillium.

The stimulation of Oospora by being grown in association with Penicillium led to the question as to whether filtrates of the fungi would exert the same effect. The filtrates were therefore saved from the liquid medium experiments and used to test this out. Part of the filtrate was sterilized by autoclaving and part by the Chamberlain filter. It was then incorporated in the agar and the fungi grown on it. This was done both for media containing nitrogen and for some without nitrogen. The filtrate was from cultures ninety-nine days old.

In an initial experiment using 12 per cent of the filtrate of the fungi in the medium the results were as in Table 8.

Table 6. Growth of Oospora citri-aurantii alone and with Penicillium digitatum after two days in media of varying peptone content.(a)

Grams of peptone per litre.	DIAMETER OF COLONY IN CENTIMETRES	
	Oospora. citri-aurantii	C.citri-aurantii plus P.digitatum.
10.0	1.9	1.9
7.5	1.9	1.9
5.0	1.5	1.9
2.5	.8	1.6
0.	.4	1.4

(a) At two days only the Oospora had started to grow, thus the additional growth in the mixed culture was due to some stimulation of the Oospora by the Penicillium.

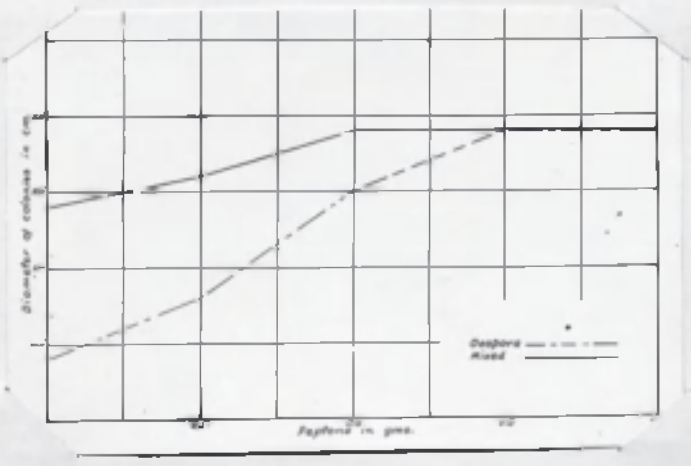

Figure 4. Growth of Oospora citri-aurantii in pure culture and mixed with Penicillium digitatum graphed against the amount of peptone in the medium.

Table 7. Growth of Oospora citri-aurantii and Penicillium digitatum in pure and mixed culture on a nitrogen-free medium.

Age of colony in days.	DIAMETER OF COLONY IN CENTIMETRES.		
	Penicillium digitatum	Oospora citri-aurantii.	P.digitatum plus C.citri-aurantii
2	.5	.5	1.4
3	.9	.6	1.9
4	1.3	.7	2.5
5.	1.7	.7	2.8(a)
6	2.0	.9	3.1
7	2.5	.9	3.4
8	2.9	1.0	3.6
9	3.2	1.2	3.8

(a) At this stage could see Penicillium in the mixed culture.

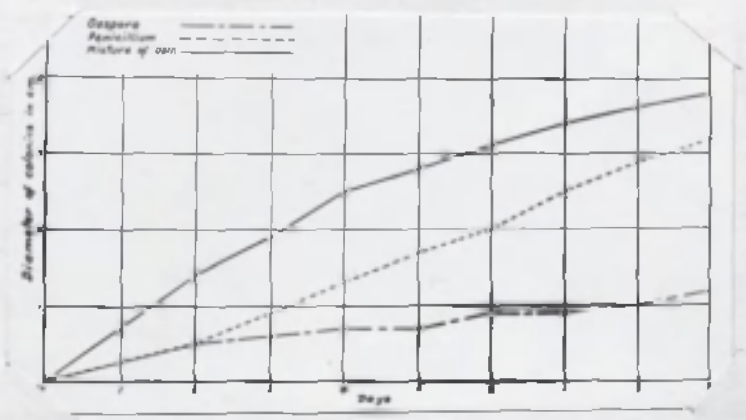

Figure 5. Growth of Oospora citri-aurantii and Penicillium digitatum in pure and mixed culture on a nitrogen free medium.

It can be seen that there is no marked stimulation, in fact the effects of the filtrate on Oospora citri-aurantii is to diminish the size of the colonies to a very marked degree. This, however, might have been due to the concentration of the filtrate used, for Boysen-Jensen (4) has shown that even growth-promoting substances may decrease the size of the colonies of fungi if used in high concentrations. An experiment was made, therefore, using a range of concentrations, and its results can be seen in Table 9.

From the table above it can be seen that the filtrate of the Oospora has no stimulating effect on either of the fungi at any of the concentrations used. In fact it seemed to exert a depressing effect on the growth of the Penicillium at the higher concentrations i.e. 1:250. This effect, however, is not especially marked and is just beyond the range of experimental error.

The filtrate from the Penicillium on the other hand has a very marked effect and this is especially noticeable where there is no nitrogen in the medium, under which conditions the Oospora normally makes very little or no growth. In this case, however, the presence of the filtrate permits the growth of the inoculum to 2.7 cm. in diameter which is relatively speaking a large colony (Plate 2). There is a similar stimulatory effect on the Oospora grown on the complete medium, but here it is not so striking. The Penicillium

Table 8. Effect of 12 per cent filtrate of Oospora citri-aurantii and Penicillium digitatum on their growth on Cook's medium after seven days.

Fungus	DIAMETER OF COLONY IN CENTIMETRES.				
	Without filtrate	Filtrate of Oospora		Filtrate of Penic.	
		Autoclaved	Filtered	Autoclaved	Filtered
Oospora citri-aurantii.	6.5	4.9	4.8	4.5	4.5
Penicillium digitatum.	4.6	4.6	4.7	4.4	4.6

Table 9. Effect of concentration of filtrate on the growth of Oospora citri-aurantii and Penicillium digitatum after seven days.

Source of filtrate	Concentration of filtrate	DIAMETER OF COLONIES IN CENTIMETRES.			
		O.citri-aurantii		P.digitatum.	
		Medium - nitrogen	Medium + nitrogen	Medium - nitrogen	Medium + nitrogen.
Oospora	1:250	No growth	3.8	2.9	5.7
do	1:2500	do	3.8	2.7	6.1
do.	1:25000	do.	3.7	2.7	6.1
do	1:250000	do	3.7	2.6	6.3
Penicillium.	1:250	2.7	4.3	2.5	6.0
do	1:2500	1.0	3.8	2.6	5.9
do	1:250000	.6	3.8	2.4	5.6
do	1:250000	No growth	3.6	2.6	5.8
No filtrate.		do	3.8	2.8	

filtrate, however, decreases the growth of the colonies of Penicilium but this again is not especially marked.

It might be argued that the stimulation thus obtained was due to the addition of nitrogen to the nitrogen-deficient medium, but the likelihood of this is very slight, due to the fact that an equal, or even larger, quantity of nitrogen was added in the filtrate from the Oospora colonies without causing any stimulation at all. There is also a further point mitigating against this claim, and that is that the stimulation could be obtained on media where there was an ample supply of nitrogen and of the other food materials also.

These experiments suggested that some substance was formed by the Penicillium during its growth which had the effect of stimulating or of promoting the growth of Oospora. This was also shown by the fact that if the Penicillium inoculum were placed on top of the Oospora there was a marked stimulation, whereas, if another piece of Oospora was put on top of the first, there was no stimulation. This stimulatory substance evidently was formed as a result of the growth of the Penicillium and diffused into the agar, since the growth stimulating effect on the Oospora could be seen before the Penicillium had started to grow.

The experiment of Savastano and Fawcett (25) was repeated, using lemons as the substrate. There was a sub-

stantially greater rot from the mixed inoculum than from either of the two acting singly (Plate 3). Attempts to increase the effect of the Oospora met with confusing results, owing to the fact that if the filtrate were added along with the inoculum, the latter was "drowned out" owing to the slow absorption of the filtrate by the lemons. Adding the filtrate later than the Oospora inoculum led to a great deal of contamination, as the part of the fruit on which the Oospora was growing was the most rotten and could not be handled easily without exposing it to much external contamination.

There were, however, indications that the filtrate from the Penicillium might accelerate the rot in the lemon, although much work will need to be done before it can be stated with certainty.

A short study was made of the effect of the filtrate of Penicillium on the growth of various other fungi on a nitrogen free medium. The filtrate was used in the same concentration as had been used in the case of the Oospora, namely 1:250. The results indicate that while the filtrate is not specific in its action, the number of fungi on which it has an effect is limited.

Stimulation of a limited type could be seen in cultures of Pyronema sp. and of Helminthosporium gramineum (Rabh.) Erik., while H.sativum P.K.B. was inhibited a little as also

were a number of physiologic races of Ustilago zeae (Beckm.) Ung. and Gibberella saubinetti (Mont.) Sacc.

Discussion and Conclusions.

Colony Type in Mixed Culture.

The type of colony resulting from an inoculum of a mixture of two fungi follows a definite pattern and is indicative of the type of growth made by the fungi individually on the medium. Thus, to phrase it generally, in an association or mixture of two fungi the more obvious macroscopic appearance will be determined by that fungus which is best suited to growth on the particular medium concerned. This is illustrated by reference to Table 1 where at high carbon-nitrogen ratios the mixed culture was practically all Penicillium, whereas at low ratios it was completely Oospora.

This dominance by the best growing organism is to be expected, but there is another factor which must be considered, namely, the rate of growth as opposed to the total quantity. In all the cases here, Oospora started growing much faster than the Penicillium; therefore, very young, mixed colonies always appeared as if dominated by Oospora. If the growth after this initial advantage were the same for both organisms, the condition would prevail as at 24½°C., where there was practically a Penicillium colony within an Oospora colony; but if one of the components stopped growing first,

as Oospora at the high carbon-nitrogen ratios, then the other could keep on growing and spreading, by fan-tails perhaps, and so would ultimately dominate the association.

In a mixture of organisms there are also modifications of growth not to be seen in the pure culture. This can be exemplified by the appearance of the Oospora hairs in mixed culture, especially at the point of juncture with Penicillium while none were present in the pure cultures. The odour of the mixed cultures was quite distinct from that of either of the fungi alone, and various modifications of colour could also be seen.

There is a definite reaction between the two fungi, Oospora citri-aurantii and Penicillium digitatum, which is evinced not only in the type of mixed colony produced by them, but also in the rate of growth of this colony. Under the experimental conditions there was always an initial stimulation of the Oospora by the Penicillium, the extent and duration of which was dependent on cultural factors.

The maximum stimulation can be seen under conditions which are very unfavourable for the growth of Oospora, which is only to be expected, as there naturally would be but little visible evidence of such a stimulation under good growing conditions. There are indications that the presence of the Oospora is detrimental to the growth of the Penicillium, and that it has other modifying effects, such as

changing the amount and rate of autolysis of mixed cultures.

Liquid media in very small quantities in which the fungi had grown had a similar stimulatory effect, but at high concentrations the filtrate of either fungus had a depressing effect on the growth of the other, while at low concentrations, 1:250, the filtrate of the Penicillium could stimulate growth in the Oospora colonies when they would not even grow in its absence. This filtrate, however, was not ubiquitous in its action as it had a depressing effect on the growth of some other fungi on which it was tried. The increase in rot in fruit caused by a mixture of the two fungi might be due to this stimulative effect of Penicillium on the growth of the Oospora, but, to date, the filtrate experiments are inconclusive.

The growth of any mixture of organisms depends on the effect of a number of factors, such as the individual growth of the fungi concerned, their interaction as regards nutrition, staling products, etc., and the factors of temperature, moisture or acidity which might modify the growth of one without affecting the other. Generally speaking, under a given set of conditions and on a certain medium, the fungus which is best suited to this habitat will dominate the association. That this is generally true can be seen by an examination of table 1 where under conditions where Penicillium grows well it dominates the mixture, while under con-

ditions favourable for the growth of Oospora, this fungus will dominate.

This, however, is not always true, for the questions of rate of growth, especially in the very young colonies, and of stimulation serve to confuse the issue and make the general truth false in a few cases. Obviously, a fungus which starts growth very quickly will appear dominant over a more slowly growing one, irrespective of their final amounts of growth, although this dominance may be only temporary. Then again, a fungus, if growing poorly on a medium, may be stimulated to growth by another, or by some by-product of another, and so it will occupy a much greater proportion of a mixed colony than it otherwise would have. This is exemplified in the case of the Oospora on a nitrogen deficient medium, where it will not grow alone but will do so if Penicillium is present.

This stimulation is a very interesting feature in organisms which are often parasitic in nature together, and so a brief consideration of the nature of such a stimulation might be instructive and indicate lines of thought.

Nature of the Stimulation of Oospora citri-aurantii by Penicillium digitatum.

There have been many instances of the stimulation of one fungus by another, and often by extracts of another.

This, however, has usually been observed as stimulation of production of reproductive structures, with regard to which Wilson (29), Hawker (15), McCormick (18), and Heald and Pool (16) have excellent papers. The type of stimulation caused by Penicillium in this case is of a different nature, for whereas Penicillium increases the growth rate, the initiation of reproductive structures is usually dependent on a checking of the growth rate. The authors cited above, however, made no determinations of the rate of growth so their results cannot be given as much consideration here as might be desirable. There is a point, however, in that production of reproductive bodies might have been accelerated by more rapid exhaustion of the medium consequent on more fapid growth, in which case their results would be comparable.

They are all agreed, however, that the stimulation is caused by some substance or substances secreted into the medium by one of the fungi associated with the stimulated one. Asthana and Hawker (1) in another paper suggest, however, that the stimulatory substance is produced by the fungus on which it acts, but is produced more abundantly if another fungus is present. But this merely pushes the question back a little, as the nature of the substance stimulating production of the reproductive-stimulatory-substance is still unknown. In fact it would seem to make things less clear rather than more clear.

Heald and Pool (16) claim that the stimulatory substance observed by them was thermostable, while McCormick (18) claims that the extracts with which he worked lost their activity on heating. Wilson (29) says that the extract of Penicillium used in his experiments diminished in activity if autoclaved, so it is very probable that a number of different substances exert stimulatory effects. In the extract used in the work being described here, the stimulatory substance was thermostable unless subjected to prolonged severe autoclaving.

The beneficial effect of certain accessory food factors on plant growth was observed by Bottomley in 1914 (2), and he showed that these were organic in nature (2 and 3). That certain accessory foods were necessary for fungi was shown by Buston and Praminik (8) for Nematospora gossypii and they related this factor to the "bios" substances (9). The stimulatory substance formed by Penicillium in the case now under consideration may possibly be of a bios nature and since Mockeridge (20) has demonstrated production of bios by microorganisms the theory has some support.

The relation of bios to vitamines is not very clear but they are closely related chemically and possibly are of the same nature. The action of excessive concentrations of vitamines on fungi is to cause inhibition, Funk and Freedman (12) showing an effective maximum and then a sharp decline in

effect. Schelling (26) concluded that Vitamine B acts on fungi like the addition of minute quantities of a toxin and such is the action of the stimulatory substance formed by Penicillium. An additional similarity can be seen by the work of Lepeschkin (17), who showed that the action of vitamines causes a stimulation mainly at the beginning of growth; and this initial stimulus has been apparent throughout this work.

The production of growth hormones, mostly indole-acetic acid or heteroauxin by fungi has been demonstrated by many workers but the work of Boysen-Jensen (4), Bunning (6), and Nielsen (21) showed that the action of heteroauxin was to decrease the amount of mycelium formed and not to increase it, as has been found here. Although the stimulatory substance may be a hormone, the evidence indicates the contrary.

The significance of the production of stimulatory substances by components of an association cannot be over emphasized especially when soil - or seed-borne fungi are concerned. It has been shown by Christensen and Stakman (10) that barley seed carries an extensive fungus flora, some fungi of which are pathogenic. Their interaction, however, is as yet unknown. Thus what might be a mildly pathogenic fungus doing little damage, when in the presence of another fungus might become an extremely virulent parasite causing severe and widespread epidemics.

Summary.

The interaction of Oospora citri-aurantii (Ferraris) Sacc. and Penicillium digitatum Sacc. has been studied in culture, since they have a synergistic effect on lemon fruits.

It has been shown that the better growing fungus will in the majority of cases be dominant in the association, imparting the characteristic growth rate and growth form.

The appearance of the fungi is modified by the presence of the other in the same culture.

An initial stimulation of the Oospora by the Penicillium has been a constantly observed phenomenon throughout the work.

This stimulation can be obtained from liquid media in which the Penicillium has grown, and is caused by a thermostable substance.

In high concentrations (12 per cent) this substance is inhibitory in action but at lower concentrations (4 per cent) it causes stimulation of the Oospora.

The substance is thermostable, but prolonged and severe autoclaving decreases its efficiency.

It is thought that the substance may be of "bios" or vitamine nature.

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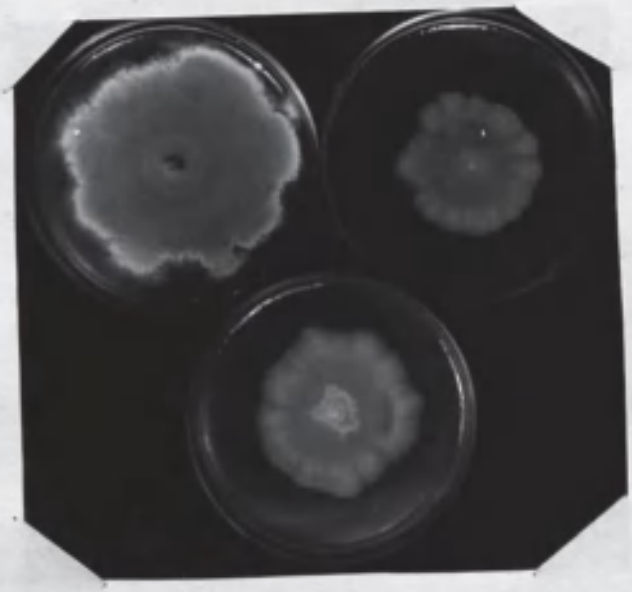

Plate 1.

Growth on Cook's medium.

Top left - *Penicillium digitatum*.

Top right - *Oospora citri-aurantii*.

Bottom centre - Mixed culture.

Note similarity to Oospora and the white centre caused by the hair-like filaments of Oospora.

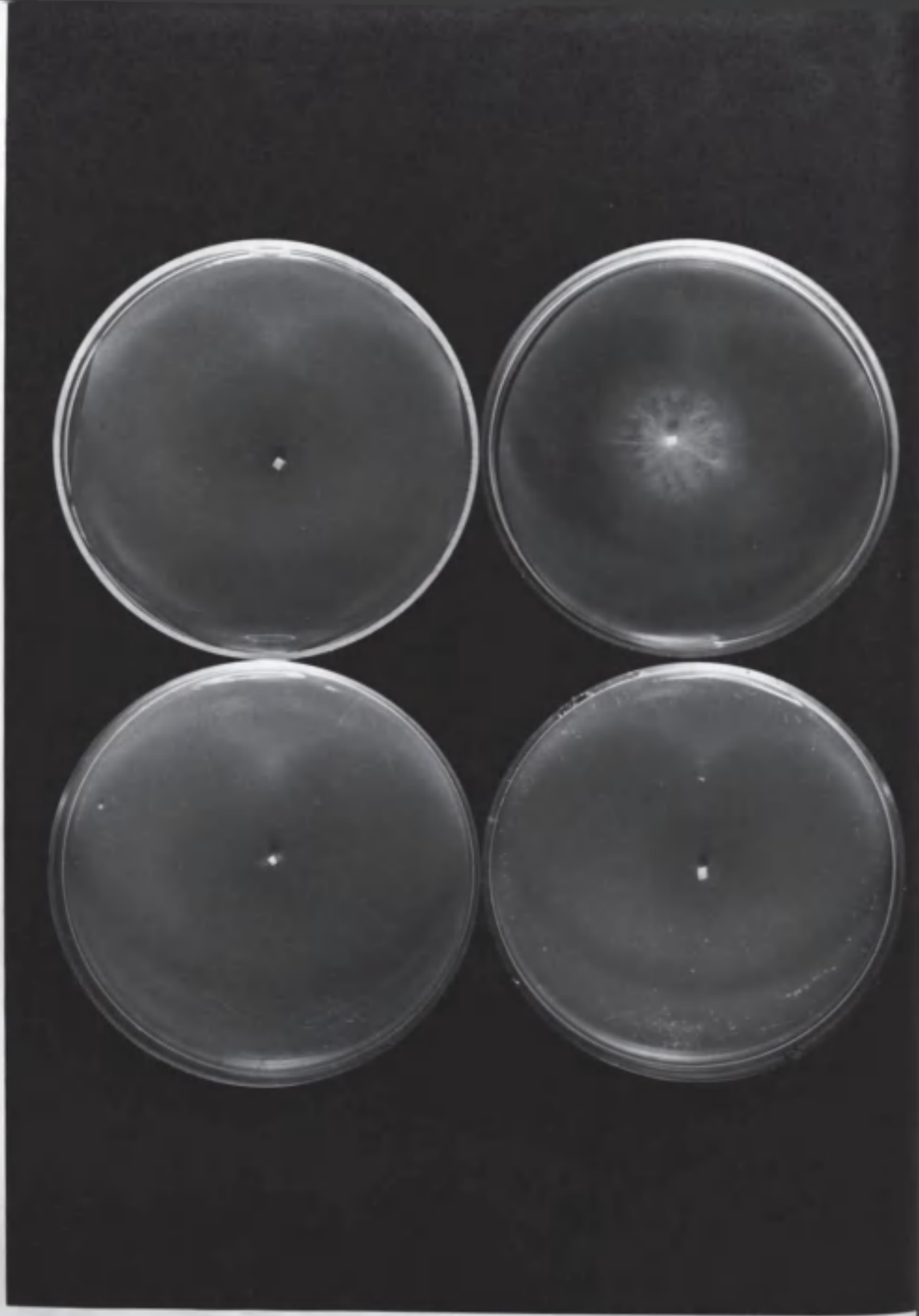

Plate 2.

Growth of Oospora citri-aurantii on Cook's medium without peptone and filtrate.

Top left	- Check i.e.	- Nitrogen
Top right	- Oospora	- Nitrogen plus .4 per cent filtrate of Penicillium.
Bottom right	- Oospora	- Nitrogen plus .04 per cent filtrate of Penicillium.
Bottom left	- Oospora	- Nitrogen plus .004 per cent filtrate of Penicillium.

Note amount of growth with .4 per cent filtrate and decreasing amounts with ,04 and .004 per cent.

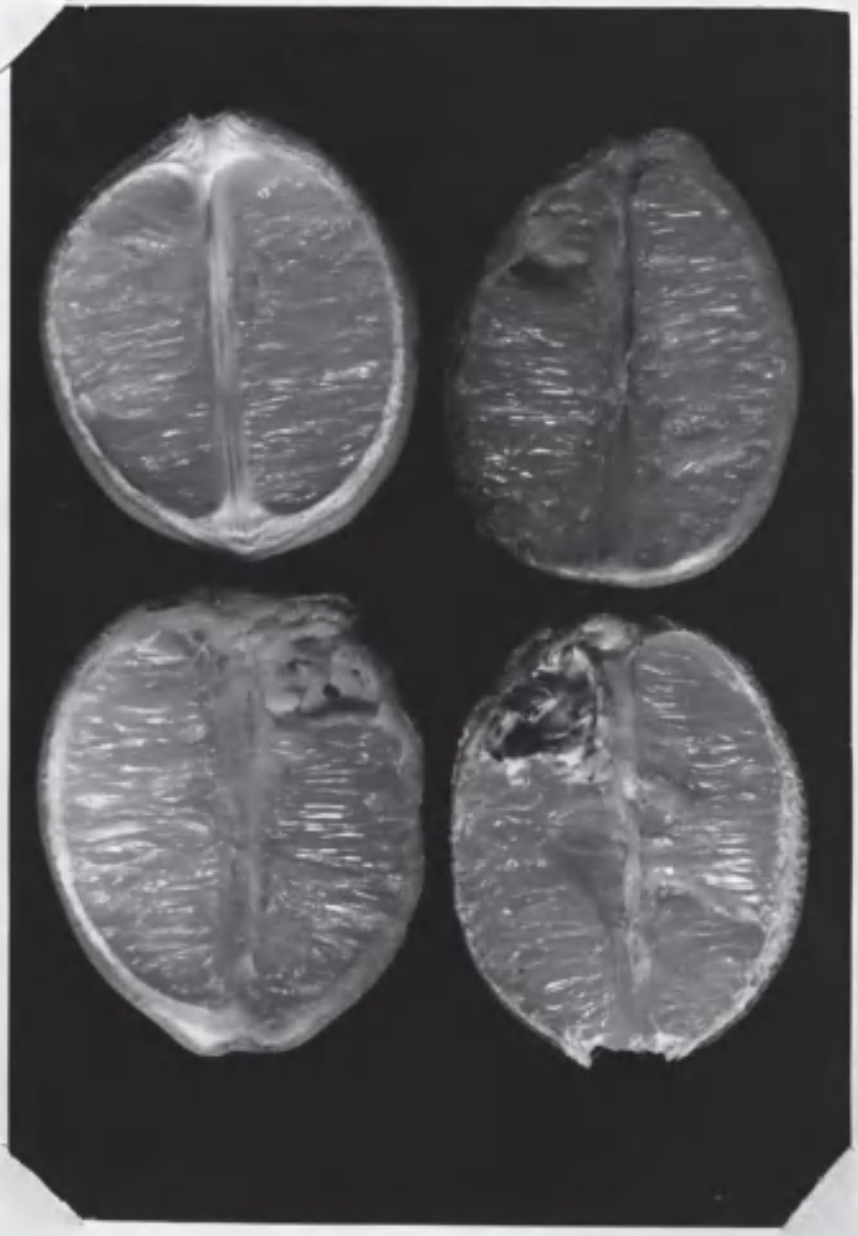

Plate 3.

Rot of lemons produced by inoculation with Penicillium digitatum. Oospora citri-aurantii alone, and in combination (after 11 days).

Top left - Check
Top right - Both fungi.
Bottom left - Oospora.
Bottom right - Penicillium.

Note increased rot in the mixed inoculation.

A Study of the Transfer of fixed Nitrogen from the Nodule to the Plant in the Garden Pea. Pisum sativum L.

C o n t e n t s.

A Study of the Transfer of fixed Nitrogen from the Nodule to the Plant in the Garden Pea, Pisum sativum L.

Introduction.

The use of clover and similar leguminous plants for enrichment of the soil has been practised since the times of the Romans, and the reason for this was shown by Gilbert and Lowes in Britain and by Schultz and Leipitz in Germany to be the ability of such plants to fix and use atmospheric nitrogen. This faculty is due to the root nodules on the plants which are outgrowths initiated by and containing bacteria and it is these bacteria which are the active agents in the fixation of the atmospheric nitrogen.

This fixation and use of atmospheric nitrogen is probably unique in the plant kingdom although similar claims have been made with respect to mycorrhiza fungi by Rayner and others. These claims are however far from substantiated and so the biotic fixation of nitrogen is limited to the root-nodule, B.radicicola. The fixation of nitrogen by the bacteria is however not the same thing as the transfer of the fixed nitrogen from the bacteria to the host cells and thence to the rest of the plant-body. It is this process of transfer which the experiment being described was designed to study.

Literature Review.

There have been many summaries of the research done on bacterial symbiosis, of which that by Fred, Baldwin and McCoy is the most complete (4), and that of Wilson (13) the most recent. Neither of these two summaries had much to say on the question of the transfer of the fixed nitrogen and in the opinion of such older workers as Hiltner, Frank and Nobbe the liberation of nitrogen for the use of the plant was accounted for by enzymatic digestion of the bacteria. Thornton (9 and 10) and Dangeard (3) however both note that in cytological examinations of nodules, signs of enzymatic digestion are apparent only in old nodules whereas the transfer of nitrogen from bacteria to the plant can be demonstrated even in very young plants. This finding prompted Dangeard to postulate a passive excretion of nitrogen by the bacteria into the cytoplasm of the host from the very initiation of the symbiosis.

Recent papers by Bond, (1, 2) and Wilson and Umbreit (15) are concerned directly with this problem and will be discussed here. From a quantitative study of the amounts of nitrogen in the nodules and in the plants of soya beans, Bond has shown that from the very first appearance of the nodules a high percentage of the nitrogen fixed by the bacteria (about 80%) is transferred to the plant. These results were obtained by taking samples of the crop at intervals, removing

the nodules from the plants and estimating the amount of nitrogen in the nodules and the plants separately. Thus in successive samplings the difference between the total nitrogen in both plants and nodules is the amount fixed, while the difference between the amounts in the plants is the quantity of nitrogen transferred. Thus the ratio transfer:fixation can be calculated. From his results and in view of the constantly high rate of transfer found even from the initiation of infection, Bond (1) is of the opinion that the transfer mechanism is one of excretion of nitrogenous compounds by the bacteria and that the fixation may be in the nature of a respiratory process. The nitrogenous compounds would therefore be products of this respiratory process excreted into the host cytoplasm.

The paper by Wilson and Umbreit (15) while taking no exception to Bond's data, states that his conclusions are not justified. They present similar data from a large number of experiments on soya beans but say that since the data represent the two ends of a reaction it is not permissable to postulate a mechanism for their explanation. In an experiment with inoculated plants supplied with nitrogen in the culture solution they showed that graphs similar to those of Bond could be obtained with regard to the absorption of nitrogen from the soil and its distribution in the plant. Thus, it is stated,

that since the nodule and plant are a symbiotic unit, the distribution of nitrogen in inoculated plants is purely a normal process as a steady rate of transfer from the nodule to the plant would be expected and the emphasis placed on the constant transfer-rate by Bond are out of all proportion to its importance. Wilson and Umbreit also show that at the very beginning of fixation a considerable quantity of nitrogen is retained in the nodule and they think that this may be necessary for the formation of the nodule tissues.

The experiment about to be described was similar to Bond's original soya bean experiments and was designed to examine his figures using another leguminous plant namely the garden pea, Pisum sativum L.

Method.

The plants were grown in washed, nitrogen-free silver sand in earthenware jars each of which contained 8.5 lbs. of sand having no accessto an external nitrogen supply. The sand in the control or uninoculated pots was sterilised. Throughout the experiment the sand in the jars was maintained at a 12% moisture content estimated on the dry weight of the sand, this level being renewed each day with distilled water and once per fortnight with nitrogen-free culture solution.

Seeds of Pisum sativum, variety 'Little Marvel' were

used only those seeds whose weight lay between .235 and .25 gm. being sown for the experiment. The sand in the pots was made up to the requisite water-content and 12 seeds put to soak on top of each pot covered with damp filter-paper. After 5 days the seeds were sufficiently swollen, the radicle being visible, and they were sown at a depth of 1½ inches at the rate of 10 seeds per pot.

On top of each seed as it was sown, 1 c.c. of a suspension of root nodule bacteria was sprinkled, and after the seeds had been covered, 10 c.c. of the inoculum were sprinkled on the surface of the sand. This inoculum was prepared by crushing 2.5 gm. of washed nodules from garden peas in sterile water and making up to 400 ccs. It was then allowed to settle and decanted to remove the supernatant liquid which was used as the inoculum.

In the case of the sterile controls the seeds were dipped in absolute alcohol and flamed twice to accomplish surface sterilisation, while the sand and the pots had been autoclaved at 2 atmospheres pressure for 8 hours. The sterilised seeds were then allowed to soak and were sown as above only in this case the inoculum was not added.

After two weeks the number of seedlings was reduced to 7 per pot, all the diseased or weakly plants being eliminated, and almost a month after sowing, the cotyledons and testae were removed to prevent loss of nitrogen through

rotting. In a few cases rotting was fairly advanced and so a slight loss was unavoidable. The cotyledons and testae were stored in bottles, numbered to correspond with the pot from which they came, containing a little formalin to prevent decay, and any leaves which fell off during the growing season were also stored in these bottles to reduce the chance of loss of nitrogen.

In sampling, 4-6 pots were selected at random and tipped on a sheet of paper, then the plants, root fragments, etc., were carefully collected. The sand was allowed to dry and seived for the smaller root fragments which were collected and washed free of sand before adding to the bulk of the plant material. In the meantime the plants were washed and brushed clean of sand and the nodules for each pot carefully picked off the roots. The plants and the nodules were then pounded separately to a uniform pulp and transferred to evaporating dishes where they were dried to constant weight.

Once constant weight was reached the ground plant was transferred very quickly to a dry bottle with ground glass stopper, which was sealed with wax and then stored over calcium chloride in sealed tins. The nodules were similarly treated, the need for absolutely anhydrous conditions being necessary for the estimation of the nitrogen in the plants and nodules.

The unit throughout the experiment was the pot, i.e.

7 plants all of which were pounded together with their leaves, testae, etc., and whose nodules were treated similarly. The loss in pounding and transferring was quite insignificant, being less than 1 per cent.

The nitrogen estimations were carried out in triplicate using Ranker's modification of the salicylic acid Kjeldhal process (7) on representative samples of the pulverised plants and nodules, and the total amount of nitrogen in the plants and in the nodules was obtained with reference to the total dry weight of each before storage. To prevent any of the material from sticking on the neck of the flask during combustion, the sample of plant or nodule material was weighed out in boats made of nitrogen-free cigarette paper and the whole was slid down the neck of the flask into the bulb.

The controls were similarly treated only in this case there were no nodules to be picked off, but the plants were dried and weighed and the nitrogen estimated on representative samples as above.

Results.

The crop was grown rather late in the season, the seeds being put to soak on the 31st July 1934. Sowing was performed on the 4th August and the cotyledons and testae removed on the 1st September, the number of plants per pot having been reduced to 7 on the 17th of August. As men-

tioned previously, there had been some rotting of the cotyledons before they were removed so a slight loss of nitrogen was unavoidable. The plants received their first watering with culture solution on the 5th of September, all previous waterings having been done with distilled water.

The first sampling was on the 3rd September, 4 pots each containing 7 plants being chosen at random. Further samplings of 4 pots were made on the 12th and 24th of September, and later samplings of 5 pots on the 4th and 22nd of October, with a final sampling of 6 pots on the 5th of November. At this last sampling the plants were very senile and in consequence a number of the nodules on the roots were rotten and easily squashed, with the result that in the separation of the nodules from the plant there was a loss of nitrogen.

In the first two samplings, the nodules were very small and their weight even per pot of 7 plants was practically insignificant. The nodules therefore from two pots were bulked and the estimation of nitrogen done jointly for the two pots from which the nodules came. The other two pots in the sampling of four were treated similarly.

The results of the nitrogen estimations on the nodulated crop over the entire growing period are to be seen in Table 1. The unit used is the pot of 7 plants and the data for quantities of nitrogen are given in terms of that unit

Table 1. The Apparent Fixation of Nitrogen[1] and its Transfer from Nodules to Plants during successive periods of development. The data refer to a unit of 1 pot which is the average of 4-6 pots.

Period of growth (days from sowing.)	N-content of plants less nodules per pot in mgms.	N-content of nodules per pot in mgms.	Total nitrogen per pot[2] in mgms.	Apparent fixation of nitrogen.[3] in mgms.	Transfer from nodules to plants.[4] in mgms.	Transfer/Fixation 100/1
1-30	75.22	10.95	86.17	17.06[5]	6.11[6]	35.8%
30-39	92.78	13.79	106.57	20.4	17.56	86.0%
39-51	131.7	15.80	147.50	40.93	38.92	95.1%
51-61	144.0	16.91	160.91	13.41	12.3	91.7%
61-79	145.9	14.61	160.51	-	1.9	
79-93	138.1	11.39	149.49	-	-	

1. According to Virtanen (11) there is a loss of nitrogen by the nodules to the soil and so the 'fixation' here is only apparent fixation as no allowance is made for this loss. The work of Virtanen will be discussed later in this paper.

2. Sum of columns 2 and 3.

3. The fixation is obtained by subtracting the N-content of plants and nodules per pot from the corresponding figure at the following sampling.

4. The transfer is calculated by subtracting the nitrogen content of the plants, less nodules, per pot, from the corresponding figure at the following sampling.

5. This figure is obtained by subtracting the N-content of 7 seeds (69.11 mgms.) from the total nitrogen at the time of sampling.

6. Calculated from difference between nitrogen fixed and that in the nodules.

During the period intervening between the 51st and the 61st days after sowing, the plants came into flower and on a few of them pods began to form, while at the next sampling i.e. on the 79th day after sowing, there were pods on practically all the plants.

The uninoculated controls were sampled three times and the nitrogen estimated in the plants. In no case during the entire sampling period of the 48-74th days was there any significant increase in the amount of nitrogen in the plants over that in the seeds, the average in the seeds being 69.1 mgm. per 7 seeds and 70.5 mgms. in the plants. It can be safely assumed that the increase in nitrogen content in inoculated plants is due to the fixatory powers of the bacteria.

From Table 1. there are three obvious phases in the activity of fixation and rate of transfer. During the first thirty days of growth the rate of apparent fixation is low and only 35.8% of the nitrogen fixed is transferred. There is greater efficiency in fixation after the 30th day until the 51st day when it is at a maximum, falling rapidly thereafter. The percentage of transferred nitrogen however rises even after the fixation has dropped and from the 39th -61st days is very high being about 90% while there is transfer of nitrogen from the nodules to the plant when there is no fixation at all which furnishes a value of infinity for

the transfer:fixation ratio.

The loss of nitrogen in the final analysis of total nitrogen in the plants and nodules is to be accounted for by the rotting of the nodules and of the fine roots, making their recovery and inclusion in the sample quite impossible.

Discussion.

The work of Virtanen and von Hausen (11, 12) modifies the results as expressed in this paper to a considerable degree for they showed that there was often a marked excretion of nitrogen from nodulated leguminous plants into the soil. This work has been confirmed by Bond (2) for the pea, but not for the soya bean and broad bean, and Wilson (14) also reports on his inability to verify the presence of excretion in a number of leguminous plants. Bond (1) has shown in his paper on soya beans that although the excretion factor lessens the accuracy of his figures for percentage transfer of fixed nitrogen, the difference between this ratio as calculated by him, and the ratio as it would have to be re-calculated if the leakage into the soil were known, is such that the latter would approach much closer to 100% transfer than his ratios actually show.

The same argument holds in the case of the work being described. The percentage obtained from calculation of $\frac{\text{Transfer}}{\text{Apparent fixation}} \times \frac{100}{1}$ is less than that obtained from

$$\frac{\text{Transfer to plant plus excretion to sand (x)}}{\text{Apparent fixation plus excretion to sand (x)}} \times \frac{100}{1}$$

and so Virtamen's results do not negativate the results as obtained here, but merely set a minimum value for the ratio. In experiments with peas Bond found 7 mgms. of nitrogen were excreted for 60 mgms. fixed by the plant, i.e. there was about 12% excretion, and by adding this figure, which is a maximum, to the results obtained by the present writer, the efficiency of transfer becomes even more accentuated.

One of the significant features of this experiment is the low efficiency of fixation and the low rate of transfer during the early stages of growth. The low rate of fixation is almost certainly due to the lack of time necessary for the multiplication of the bacteria in the plant tissues. The low percentage transfer in the initial stages agrees with the results of Wilson and Umbreit rather than with those of Bond. Virtanen has shown that the maximum excretion of nitrogen takes place from the very young nodules in the pea and this might be used to explain the low transfer ratio. The maximum excretion figures however are much too low to account for the difference between the transfer rates in the first and second samplings and so another explanation seems necessary. The best hypothesis for this low transfer rate is that of Wilson and Umbreit who believe that the nitrogen fixed by the bacteria is used in the formation of the nodule itself and so is not available for transfer to the

plant body as a whole. Bond on the other hand finds the transfer of nitrogen proceeding at a high rate even at the earliest sampling and there is only a slight indication of the lag in transfer in his figures.

Following this initial phase of low transfer rate, there ensues a period which continues as long as fixation is being carried out, during which 90% of the amount of nitrogen fixed is regularly transported to the plant. This period is only terminated by the increasing inefficiency of the bacteria reducing fixation to zero, there being then no nitrogen left to transfer. The final figure in column 6 which produces a value of infinity in the transfer:fixation ratio may be quite insignificant and of no real value, or it may be an indication of the nitrogen freed from the bacteria by enzymic digestion after fixation has stopped, and the plant is in the post-flowering stage. The amount of fixation reaches a maximum at the time of flowering, in this case about the 39-51st day, and falls sharply thereafter. This may be accounted for by an exhaustion of the plant nutrients consequent on the flowering act, or maybe by a change in the nature of the bacteria and their passing into an inactive state.

The point however which it is essential to stress is that nitrogen is made available to the plant by the bacteria from the very instigation of symbiosis. This nitrogen may be exported to distant parts of the plant or it may be used at

the locus of liberation for the formation of the nodule itself, it is still available to the plant long before there has been any enzymic digestion of bacterial protein. It is reasonable therefore to argue that, independently of how the bacteria provide this nitrogen and the mechanism of its fixation, it must be excreted from the bacterial cell throughout the period of activity of this cell and before it is acted on by plant enzymes. The argument of Wilson and Umbreit (15) that a similar distribution of nitrogen takes place in uninoculated plants with a supply of external nitrogen, only strengthens the excretion hypothesis for in their case there is a constant supply of nitrogen supplied chemically and in the case of the symbiotic bacteria there is a similar supply of nitrogen derived biotically. Their argument, which implies that Bond's figures and those of the writer would seem to indicate a constant supply of nitrogen is precisely what it is desired to show, for this can only be achieved by a continuous excretion of nitrogen from the bacteria.

Actually the continuous transfer of nitrogen from the bacterium to the host cytoplasm is what is being discussed and, it is thought, proved, while the re-distribution of this nitrogen throughout the whole host plant should take place in a manner similar to that of any other supply of nitrogen. Such a constant supply of nitrogen could be accounted for by a continuous digestion of bacteria also, but the work of

Thornton (9, 10) and Dangeard (3) gives no indication of such a process.

Another mechanism could be postulated, namely that the bacteria do not themselves fix nitrogen but provide a stimulus which enables the cells of the plant roots to do so. This hypothesis would be compatible with the results published above as there would then be no need for an excretion theory to account for the constant supply of nitrogen to the plant. This theory of the plant being the active agent in the fixation of nitrogen would also explain the lack of results of workers who have tried to demonstrate bacterial fixation of atmospheric nitrogen in culture, of which there is still no definite proof. Such a mechanism might be in the nature of an antitoxin reaction to eliminate damage which might be done to the plant by toxic by-products of the growth of the bacteria. It could also be argued that there is no reason why the presence of the bacteria should not enable the plant to fix nitrogen rather than the presence of the plant enable the bacteria to perform this process.

There have been claims to the effect that uninoculated plants and especially germinating seeds could fix nitrogen made by such workers as Lipman and Taylor (5), but they have never been finally substantiated. Until the ability of the bacteria to fix nitrogen apart from the plant has been conclusively proven, the idea of the plant being the active

agent in the fixation cannot be disregarded.

From the evidence which has been gathered, and assuming that the bacteria are the fixatory agents it seems that the excretion hypothesis is the best explanation of the facts. The objection of Wilson and Umbreit to this assumption of an excretion mechanism on the grounds that a mechanism is being postulated from the beginning and the end products is valid to a certain degree, but what is being elucidated here is a mechanism of redistribution, in that the nitrogen fixed by the bacteria is released either by some external force such as enzymic action, or by an intrinsic potentiality of the bacterium itself. The actual mechanisms of release from the bacterium do not enter the question at all, the point being that so far as our knowledge goes, the facts are best accounted for by the theory of the excretion (either passive or active) of nitrogenous compounds from the bacterium. There may be external forces acting on the bacteria to cause this release, of which we are not cognisant, but as knowledge is limited by the facts at our disposal we cannot take such things into account, and until the existence of such forces is demonstrated they must be disregarded.

Summary.

A short review is given of the literature relating to the passage of nitrogen from the nodules to the plant.

The experiment is designed to estimate the quantity of nitrogen in the plant alone and in the nodules alone, and from the sum of the two to discover the rate of fixation and from the increases in the nitrogen in the plant, the rate of transfer of the nitrogen from the nodules to the plant.

In the garden pea, Pisum sativum, it is shown that a low initial rate of transfer, 30 per cent of fixation, is followed by a constantly high rate namely 90 per cent until the death of the plant. The low rate at first is thought to be due to the retention of the liberated nitrogen to form nodular tissue, and could the bacteria be separated from the nodules it is thought that the actual transfer from bacterium to host cytoplasm might be at the 90 per cent level all the time.

The constantly high rate of transfer throughout the major part of the life of the plant is best explained as an excretion of nitrogenous compounds by the bacteria as there is no evidence of any enzymic digestion to account for this constancy of supply.

Another hypothesis which would explain all the known facts is that the plant itself is enabled to fix the nitrogen by the bacteria, rather than the bacteria by the plant. If this were true there would be no need to postulate an excretion mechanism, as the distribution of nitrogen would then be as in normal plants supplied with nitrogen, which is what Wilson

and Umbreit showed to be the case in both inoculated and uninoculated plants.

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