A DISEASE OF AUCUBA JAPONICA THUNB.

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Introduction.

A disease causing serious damage to shrubs of the Japan laurel (Aucuba japonica Thunb.) has been known for a number of years. As no previous reference to this condition was discoverable in the literature the present investigation was undertaken with the object of describing the features characteristic of the disease and, if possible, of determining the causative agency.

History of the Disease and Geographical Distribution.

Records of this particular blight of Aucuba supplied to the writer by a number of plant pathologists indicate that the incidence of the disease varies greatly in severity from year to year. In 1929, for instance, its occurrence was so widespread and conspicuous that it might justly be regarded as an epiphytotic. Subsequent years brought less virulent attacks and the number of bushes involved was also smaller. During the present year, 1932, the fresh outbreaks observed have been relatively fewer and the symptoms less marked. From the observations of the writer and from information furnished by other observers from different localities in Scotland and England the disease appears to be of wide distribution. The fact that affected bushes are found in both industrial and rural areas and growing under a variety of soil conditions tends to rule out the possiblity of atmospheric pollution by industrial processes or of the composition of the soil/

soil peculiar to given localities being the cause of, or at least a contributory factor in the production of the disease.

Economic Importance.

While this shrub is of slight commercial value, the popularity it enjoys as an ornamental plant and its extensive use in the lay-out of public grounds make the unsightly appearance of the blighted twigs, and frequently of completely killed bushes, if not an indication of serious financial loss, a concern, nevertheless, of considerable importance to the horticulturalist.

Symptomatology.

The more ephemeral form of the disease is characterised by the local die-back of the ultimate branches (Plate II, A (1) and (2)). The necrosis appears to originate in the apical region of the twig, and spreads as an advancing margin towards the base of the affected shoot (Plate II, A (2)), where it is usually terminated by the formation of an absciss layer at the junction of the healthy and diseased limbs of the fork (Plate II, A (1)). The discoloration also travels from the killed apex into the petioles and mid-ribs of the apical leaves, whence it spreads along the lateral veins and through the mesophyll until finally all the leaves are killed, and the shoot presents a typically blighted appearance. After death, the leaves remain attached, becoming black, rigid, and brittle, and, since the waxy cuticle does not appear to be altered by the processes of decay, they also/

also display a highly burnished surface. As the cork barrier at the base of the diseased spur only extends from the epidermis to the woody cylinder, the decayed parts are not separated off immediately, but are broken down subsequently by saprophytic fungi, chiefly, however, by Phomopsis aucubae Trav. Occasionally the necrosis extends beyond the first branch junction, and may sometimes involve an entire branch system, but usually where the first symptoms are apical, the regions attacked are isolated in the manner described above, and the plant recovers.

Evidence of disease has also been noticed on individual leaves towards the apical half. This restricted form of necrosis was observed during the present summer, 1932, on the young apical leaves of vigorous, succulent, unbranched shoots of the current growing season (Plate III, A). The blackening of the tissues tended to follow the veins (Plate III, B), but the encroachment on the mesophyll, marked by a water-soaked margin, proceeded almost as rapidly. In some cases, however, isolated areas of mesophyll were necrosed in advance of this margin (Plate III, B). This condition usually resulted in leaf fall before the leaf was more than half diseased, thus preventing any possibility of the stem apex being reached.

A number of shrubs displaying these symptoms were examined from time to time in order to determine if the root system had suffered any comparable injury. These observations were made on material from soil types contrasting in point of physical/

physical properties and chemical constitution, so that any effects apparent on the roots would not be attributable solely to local environment. On certain of the young succulent roots symptoms of disease were apparent, which might be described as analogous to the twig blight of the branches. The typical root lesions assumed the form of a necrosis, which spread from the apex back into the older regions of the root, and resulted in the dying back of varying lengths of these terminal portions.

There still remains to be described a more systemic aspect of the disease. Shrubs of the type illustrated in Plate I were quite frequently encountered where the symptoms were of a distinct and more general nature. The whole bush is affected simultaneously, and necrosis can be observed at many different points along the same branch system, with irregularly shaped areas of green, and more or less turgid and healthy, tissues intervening. The cause of the trouble in this case appears to be more disseminated, and probably arises at some point low down on the axis, thus interfering rather vitally in the general metabolism of the plant. As a result, growth is arrested; the leaves fail to develop their normal size, and although not showing signs of active disease, their stunted and toneless habit along with the brevity of the internodes, and sometimes even the death of the stem apex, bespeak defective nutrition. Twigs, exhibiting the retarded growth form preceding final necrosis, which are characteristic of the systemic type of the disease, are/

are shown in Plate II, B. In addition, the older parts of the branches on such plants often bear superficial lesions of a corky texture, which form transverse or longitudinal fissures of the epidermis. These excrescences may also occur as tubercular knobs, and in other pustular shapes. Such disfigurement of the otherwise fresh stem must not be confused with the emergence of a normal periderm, for in aucuba the superficial layers remain green and the epidermis persistent during several years' growth. Indeed cases were observed where, in spite of the presence of rugose lesions on the upper parts of the branch, the epidermis farther down the stem was still glossy and uninterrupted except for the occurrence of normal lenticellar tissue. It seems that in many places, the lenticels may be the centre from which these cankers radiate, but this does not prove to be true generally as a consideration of the breaking up of the leaf scars by these cracks will immediately show.

The condition of the root system of such plants bears out further the systemic nature of the affection, for besides the terminal necroses, already noted for the more local phase of the disease, we find lateral superficial lesions in the older parts of the roots analogous to the corky scabs observed on the branches. A careful distinction, however, must be drawn between such isolated superficial areas of phellogenic activity and the scars left on the main roots by the dying back of laterals to the point where they first ruptured the cortex.

In/

In what might be termed this chronic form of the disease, the necrosis of the individual organs, and parts thereof, may be comparatively slower, but in every observed case of this nature the plant was doomed from the commencement, and it appeared only a matter of time, perhaps as long as several seasons, before it should ultimately succumb.

Pathological Histology.

As a preliminary step in the attempt to determine the cause of disease, typical portions of the lesions produced on the several organs affected were sectioned, and, after staining by a technique appropriate to the particular object in view, subjected to critical examination. For the sake of simplicity, the observations on the different organs will be dealt with seriatim.

Stem. As it was considered that the line of demarcation between the healthy and diseased portions of the stem might prove the more instructive with regard to the cause of necrosis, longitudinal sections of necrotic junctions similar to that shown in Plate II, A (2) were cut at a thickness of 4μ. In spite of the variety of fungal and bacterial staining methods employed in the preparation of these sections, no evidence of parasitic invasion was ever obtained. Slightly in advance of the disintegrating tissues, characterised in the pith by a collapse and subsequent lamellation of the cells, masses of intracellular material were found, which at first sight suggested the appearance of bacterial clumps/

clumps (Plate II, D). On resolution under a higher power, however, these inclusions (Plate II, E) were seen to consist of an amorphous matrix, containing granules of irregular form, which never showed a retention of the stain stronger than the rather faint affinity displayed by the entire intracellular mass. These inclusions must not be confused with the groups of sand crystals (finely amorphous Calcium oxalate) which occurred in varying amounts in the tissues of normal stems of Aucuba sectioned for comparison.

Leaf. The type of leaf lesion selected for examination was that where the necrosis had apparently originated in the region of the leaf apex, and was spreading towards the base (Plate III, A and B). From certain specimens, it was obvious that the commencement of the blighted condition could not be related to the marginal serratures nor to the leaf apex, since in many cases all of these points were still unaffected when the mid-rib and surrounding lamina were already in necrosis. Transverse sections of the lamina cut 3μ thick and so as to include the margins of killed and living areas of the mesophyll were examined first under the low power (Plate III, C). The collapse of the dead cells has caused a marked reduction in the thickness of the lamina: the tissues have fallen in towards the adaxial surface. The lower epidermis defining the lesion has not been attacked, while the upper epidermis is disintegrating for a considerable distance on either side of the conspicuously diseased area. The diseased/

diseased cells have become occluded with a dense black material which renders indistinguishable cell walls and lumina, and presents a picture of uniform opacity. At the point where the discoloration appears to be in the act of spreading into healthy mesophyll, examination under higher magnification (Plate III, D) shows an ill-defined margin where the black opaque cells are adjoined with others in which a lighter brown structureless mass is present: still further from the seat of necrosis, the cells contain a light brown, gelatinous-like material, which does not stain and carries no significant particles. Longitudinal sections from the mid-ribs of similar diseased leaves show histological features comparable with those just described. In this position, the vascular channels appear to favour a rapid spread of necrosis (Plate III, E) and the tissues towards the adaxial surface are the first to be affected as the disease advances. More highly magnified, the tissues show the same black material filling the tracheids (Plate III, F). The outline of the cell walls, however, is not always followed exactly, for interruptions, mainly of circular or elliptical form, are frequently to be noticed in these otherwise opaque columns of dark brown cell content. A transition to a more diffuse type of cell inclusion occurs again at the margin of disease, but here, as previously, there was nothing present that might suggest an aetiological explanation. Transverse and longitudinal sections of the mid-rib and lamina of normal leaves were cut for comparison.

Root/

Root. Roots showing typical die-back from the apex were sectioned longitudinally at the necrotic junction. The primary invasion in the case of the root appeared to be confined to two more or less definite regions. These were firstly the exodermal layer and a few layers of the outer cortex, and secondly the stele, especially in the cambium and phloem regions. The rest of the cortex and the pith was free from traces of disease. (Plate IV, A and C). The peripheral necrosis (Plate IV, A) is marked by the presence in the diseased cells of characteristic granular contents, the appearance of which is more clearly indicated under the higher magnification of Plate IV, B. The cells in the necrosed stelar region possess similar pathological contents (Plate IV, C and D). Comparing detailed portions of the disease tissue in the stele (Plate IV, D) with diseased areas from the superficial layers (Plate IV, B), it was seen that the pathological picture was practically identical in the two cases. It is also important to note that the configurations assumed by the contents of cells in necrosis were essentially similar whether they were situated in diseased stem, leaf or root. Since in diseased roots, as in affected leaves and stems, no signs of a specific pathogen were observed, it seems all the more necessary to emphasise the occurrence of morbid characters common to these several organs. Of course, it does not follow that the same agency is operative in the production of similar intracellular figures in the different diseased regions mentioned, but/

but in the absence of other aetiological indication the stressing of this histological similarity is tentatively justifiable. It may be pointed out that these cell bodies from different parts of the diseased plant resembled one another also in their failure to react to mordanting and differential staining, and in their retaining a native deep brown coloration.

General Observations on the Diseased Tissues.

To complete this account, certain more general considerations deserve mention. In the first place, there was no production of cavities in the diseased tissues by a separation of the cell walls, and the walls themselves were never observed to be penetrated: the cell inclusions were confined to individual cells, and where there was an alignment of similar elements, they were segmented off by the intervening intact cell walls. Secondly, intercellular material of any definite or significant morphology was never detected, and finally it was noticed that, although similar inclusions could be identified in different diseased organs, at any one site there appeared quite a diversity of types. For example, there were reticulate formations (Plate IV, D), granular (Plate IV, B and Plate II, E), and opaque, sometimes with characterisitc and symmetrical perforations, (Plate III, F and Plate IV, D).

Isolations from Typical Lesions.

In an attempt to establish the cause of disease, numerous platings/

platings of the necrosed tissue from stem, leaf and root were made on nutrient gelatin and agar. The material employed for this purpose was collected from diseased shrubs in a number of different localities (including the Botanic Gardens, The University, and Kelvingrove Park, Glasgow; and also Bearsden and Falkirk) so as to give a fair representation from the point of view of the disease distribution. The isolations were repeated at different periods throughout the year. Pieces of tissue were excised with sterile scalpel from the junction of the diseased and healthy parts of the stem, root or leaf after thorough sterilisation of the surface, first with absolute alcohol, to remove, in the case of stem and leaf, superficial dirt from the still intact and glossy epidermis, followed by corrosive sublimate (1 part in 1,000 of distilled water) which was finally washed off with sterile distilled water. The tissue fragments were then either transferred directly to tubes of melted nutrient gelatin and plated out or were first macerated in sterile distilled water, and the tubes inoculated with loopfuls of the suspended material. Even more satisfactory results were achieved by cutting oblique sections with a sterile razor from conveniently situated lesions on the stem, or by slitting diseased roots lengthwise after preliminary sterilisation of the surface, and implanting these sections or semicylindrical root portions, as the case might be, immediately on to the solid surface of an agar or gelatin poured plate. From these plates, incubated at

room/

room temperature or in the incubator at 20-21°C., there was one type of bacterial growth consistently isolated in pure culture. A typical isolation is shown in Plate V, A, where the stem sections are encircled by a halo of pure growth of the organism. Further comparison of the organisms obtained from diseased stem, leaf and root established the identity of the bacterium as the same for cultures isolated from material differing in point of locality, of season, and of the diseased organ from which it was obtained. In case it might be objected that this organism was a superficial contamination normally epiphytic or even that it might possibly be a commensal of aucuba, the precaution was taken of plating, as controls, sections of healthy tissues taken from a number of points on different shrubs and previously subjected to the same conditions as to superficial sterilisation, etc., as the diseased parts used in isolation inocula. No growth was obtained from such sections of healthy tissue. Since, therefore, the surface asepsis of the diseased organs can be deemed complete, and this would most certainly be true in the case of necrosed twigs where the epidermis is glossy and unbroken by the disease, we are entitled to conclude that the organism is present internally. What has been said holds only for those areas of diseased tissue which are within a moderate distance of the zone of active necrosis, usually from two to eight inches from the junction of healthy and diseased parts, but varying according to the degree of maturity of the tissue affected, and,

related/

related to this or depending on other factors, the rate of progress of the disease. In parts of the stem which had been dead of the disease for a considerable time, in addition to the bacterium, the fungus Phomopsis aucubae Trav. was persistently isolated, but the regions from which it was isolated indicated that its presence was consequent on the killing of the branches by the black necrosis already described. No fungal growth was obtained from cultures of badly diseased roots, but on leaves dead of the blight for some time, the pycnidia and extruded spore tendrils of Phomopsis were frequently observed, especially if the diseased leafy twigs had been kept for a day or so in a closed vessel under moist conditions. In all such cases, however, the observations indicated that the necrosis was the primary cause of death, and was antecedent by a considerable extent both temporally and spatially to the secondary fungal invasion.

A third type of organism which often appeared during isolations from necrosed tissue, more completely disorganised than the initial stages from which the bacterium was obtained in pure culture, was a gram positive yeast, ellipsoidal in form, usually with a nipple-like termination at one pole and frequently showing budding although chains were not observed: the cells occurring singly or at most showing late stages in abstriction of the daughter units from one end of the parent cell. With isolations in gelatin it was practically impossible to distinguish between the yeast colonies and the bacterial colonies on account of the rapid liquefaction of the medium and the similarity/

similarity of growth form assumed by both types of organism. On agar, however, the surface growth of the yeast was dull and dry with a smooth entire margin and a tendency to become raised and rounded, an appearance which was at once distinguishable from the moist, shiny and more spreading growth of the bacterial colonies. From the rougher wrinkled nature of the surface in the older parts of diseased twigs it was considered probable that the preliminary sterilisation of the surface had not been completely effective and that the yeast was of the nature of an external contamination from the atmosphere. This view was held to be the more correct as the presence of organisms of this size in the more central tissues could hardly fail to be noticed during histological examination.

With regard to the saprophytism of Phomopsis aucubae Trav. it may be desirable to complete at this point the description of the observations on the relation of this fungus to the disease under investigation. As recounted above no fungal mycelium was at any time observed in the tissues in the process of undergoing necrosis and there is no record in the present work of a fungus having been isolated from such tissue. But there is a type of die-back in which there is no encroaching margin of necrosis and where this fungus appears to be the sole infection. The symptoms of this local dying-off of the smaller branches must be carefully compared with, and distinguished from, those of the blight disease we are studying. It was observed that certain twigs on otherwise healthy bushes were dying off from natural/

natural causes. This was probably due to a shading out of overcrowded branches badly situated with regard to light penetration. The very first symptom exhibited by these twigs was a slight lightening of the green of the stem, accompanied by a similar yellowing of the mesophyll of the leaf blades, the vascular reticula of which stood out by contrast intensely green. The most important feature, however, was the physiological isolation of the twig at this early stage by the formation of the abscission zone which girdled the base of the twig at its junction with the parent shoot and cut across cortex and extraxylar vascular tissues. It would appear, then, that such branches , from nutritional causes, are capable of being virtually separated off at exactly the same point as blighted twigs react under the stimulus of disease. An example of an uninfected naturally dying-off twig of this type separated away at the absciss is shown in Plate II, C (1). Following the history of twigs of this description we find that the moribund tissues provide suitable pabulum for the saprophytic fungus whose inroads are not long delayed. The final result is a brown rot (Plate II, C (2)) which causes the leaves to become thin, light-brown, dry and papery, a condition and appearance which are probably referable to the original depletion of the plastic food materials before decay set in. The minute erumpent pycnidia of Phomopsis aucubae Trav. are ultimately seen on the light parchment-like epidermis of the dried-out twig, but although pure cultures of the fungus may be obtained from such a source it

is apparent in the light of the foregoing observations that the fungus is not the cause of the die-back and is essentially saprophytic. At all events recognising the differences in the symptoms characteristic of natural die-back and of die-back due to blight the two types are not likely to be confused nor will we be easily misled into assigning as the cause of either the entrance of Phomopsis, or indeed of any other fungus, as a primary invader.

Inoculation Experiments.

In view of the constant appearance of the same species of bacterium in isolations from typical lesions of stem, leaf and root a number of artificial inoculations were made in order to see if infection could be effected and the symptoms characteristic of the disease reproduced. For this purpose about 25 cuttings from healthy bushes of aucuba were set up in the greenhouse in flasks containing sterile culture solutions and grown till they showed abundant root production and the addition of fresh foliage. Potted cuttings, including some green (non-variegated) varieties, were also used for inoculation. Another quicker but more temporary method of preparing selected vigorous shoots for experiment was by enclosing them in sterilised boiling tubes containing cotton wool pads and distilled water at the bottom and plugged with cotton wool to prevent drying out of the inocula. Finally, in a number of outdoor experiments/

experiments, healthy bushes and rooted cuttings in the University gardens were utilised. The inoculations were performed first in the spring and later at different periods throughout the growing season. Suspensions for inoculation were prepared in sterile distilled water or beef extract broth from vigorous 2-3 days old sub-cultures on agar slants of single colonies of the organism freshly isolated from the diseased tissues obtained from time to time from different localities. It was considered that, in the event of the organism proving pathogenic, infection was most likely to be secured by introducing the inoculum into the fresh, succulent, more immature tissues towards the growing point. Accordingly the parts inoculated were situated in stem and leaves of the current season's growth and more specifically the following points were chosen, viz.: the apex and marginal serratures of the leaf, the mid rib region, the mesophyll of the mid-lamina, and the petiole: and in the stem the growing point, the first few internodes and at the lenticels where these had appeared towards the base of the new growth. The inoculations in the stem were made principally by hypodermic injection with the needle inserted to different depths of cortex, pith, etc., according to the particular experiment. With inoculations in the open the needle wounds were sealed over with vaseline to prevent evaporation of the inoculum and when possible the operations were carried out when atmospheric conditions were humid. In the case of the greenhouse cuttings the wounds

were covered with moistened cotton wool and the flasks or pots placed in shallow dishes containing water and the plants covered with bell-jars. Inoculations of the leaves were made by puncturing or scarifying the tissues with a sterile needle through a suspension of the bacteria placed on the surface or the suspension in some cases was simply spread over the surface of the upper or lower epidermis without pricking the leaf. The stigmata and nectaries of the female flowers were similarly inoculated by laying on a suspension of the organism and without mechanically injuring the floral parts. The making of oblique incisions on stems and petioles followed by inoculation and subsequent binding up was also resorted to in an endeavour to induce the organism to "take on". The roots of several of the twigs in water culture were also punctured under suspensions of the organism. All the inoculation abrasions were repeated on controls using sterile water blanks and sterile broth in place of the usual suspensions. From about 150 inoculations of the types described above no positive results were obtained although in many cases the observations were extended to 12 weeks after inoculation. Similar negative results were obtained in a series of experiments in which inoculation was accomplished by a method of continued infiltration. Apical portions of shoots, including in some cases inflorescence axes, were enclosed in glass cylinders which were sealed at the bottom around the twigs to form a water-tight joint. The cylinders were filled with a suspension of the organism and the stem apex or leaves then pierced with a sterile needle or scalped slits cut in/

in the stem. This procedure, however, was no more productive of pathological symptoms in the inoculated shoots than the methods previously adopted.

But while inoculation failed to demonstrate any aetiological relation between the organism and the lesions from which it was so consistently isolated in pure culture yet the constant association suggested that the bacterium might with profit be studied more completely. Since no micro-organism is listed by Elliott (3) as being pathogenic for aucuba or as occurring saprophytically on this plant and since further, no exactly corresponding description could be traced in Bergey (1) a fairly detailed characterisation of the organism is now given.

THE ORGANISM ASSOCIATED WITH AUCUBA NECROSIS.

Morphology.

The aucuba organism is a short, usually slightly curved, rod with rounded ends, occurring singly or in pairs frequently arranged to form an obtuse angle. Smears from 3 days' old cultures on beef infusion agar stained with carbol fuchsine give rods varying in length from .90μ to 1.70μ and in diameter from .45μ to .60μ and with an average measurement of .60μ by 1.20μ. In broth culture the corresponding measurements are 1.70μ to 3.40μ and .6μ to .85μ and an average size of .65μ by 2.6μ. No capsules are produced by the organism on agar or in broth when films are stained by Hiss's method. Examined in wet preparations the/

the organism exhibits a darting or "vibrioid" type of motility and stained by Kirkpatrick's method (4) is shown to possess 1 to 4 lophotrichate flagella (Plate V, E. and F.). No endospores have been observed.

Staining Reactions.

The organism is definitely gram negative and is not acid-fast. It stains well with carbol fuchsine but not so intensely with the aniline dyes. Stained with Loeffler's flagellum stain and with the metachromatic Carbol Thionin refractive granules were sometimes observed at one or both poles of the bacillary body: these granules did not stain as glycogen with iodine.

CULTURAL CHARACTERS*

Beef Infusion - agar slant.

In tubes held at 20°C. the growth along the strokes is moderate to strong, whitish to light gray in colour, slightly convex, translucent at the margin becoming opaque towards the centre; surface smooth, glistening; margin smooth, with a tendency to flattened undulations; of viscid consistency. A few tiny pits in the surface were frequently observed with the aid of a lens. In old cultures (several months) spiked branched crystals were formed expanding from the surface growth into the medium.

Beef Infusion - agar plates.

On poured plates surface colonies can be detected with the naked eye on the second day and by the sixth day have reached

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* NOTE. Unless otherwise stated the organism was cultivated at 20° C.

a diameter of 4 mms.; the maximum diameter attained after 10 days was 11 mms. The surface colonies are at first circular and homogeneous by transmitted light (Plate 5, B); they are slightly raised, whitish grey in colour with moist glistening surface and smooth entire margin; later (8 days onwards) they appear flatter and by reflected light a number of faint slightly raised concentric rings and a few slightly depressed radiate markings are observable; the margin remains entire but is now possessed of major undulations. The buried colonies are small (less than 1 mm. after 6 days) lenticular and brown with an opaque centre and a more translucent regular margin which becomes granular as the colony ages without increase in size.

Beef Extract (Lemco) agar slants and plates.

On this medium the form of the growths obtained was similar to that on beef infusion agar.

Beef Extract (Lemco) broth.

The medium is clouded, most densely towards the surface, after 24 hours. By the second day rim and pellicle formation commenced. The lacey pellicle, though connected with the rim, was easily detached by the slightest agitation and even in undisturbed tubes older cultures showed abundant sediment derived from the dejection of the surface skin.

Potato Slopes.

The growth on potato is decidely brown from the commencement and is fairly abundant though not covering the entire surface of/

of the potato. Growth tends to be somewhat heaped along the strokes; the consistency is viscid; the surface is glistening, smooth and the margin entire but thrown into arcs. Abundant precipitate occurs at the bottom of the water which is otherwise clear. The substance of the potato is discoloured throughout to a brown which is less intense than the pigmented growth.

PHYSIOLOGICAL PROPERTIES.

Chromogenesis.

When grown on beef infusion media the organism produces a light green water soluble extracellular pigment diffusing into the medium of agar slants which it impregnates completely by the third day. With subsequent growth or ageing of the culture pigmentation is not intensified. No colour is formed from media prepared with beef extract (Lemco).

Liquefaction of Gelatin.

When the organism is isolated from gelatin plates originally and shake cultures inoculated from a single colony, on gelatin, of the original isolation then typically liquefying colonies are apparent in the dilution plates by the second day. On the sixth day the surface colonies, now 4-5 mms. in diameter, are situated at the centre of a circular area of liquefaction (Plate V, C). The colony at this stage, when photographed by oblique transmitted light, consists of a dense white centre with zoogloeal threads spreading into the liquefied halo but to the actual/

actual margin of liquefaction bacterial growth does not appear to have penetrated. The buried colonies are minute (about .5 mm. after 6 days at 20°C) and brownish opaque with a somewhat diffuse discontinuous margin, the position of the colonies being indicated by depressions at the surface of the medium (Plate V, C).

Gelatin Stabs.

In stab cultures inoculated immediately from gelatin isolations liquefaction is rapid; at first napiform it later becomes saccate and proceeds to completion in 14 days and sometimes in an even less period.

Loss of Power of Liquefaction.

On account of the diagnostic and taxonomic importance attached to this property the following account of alteration in the organism's behaviour in this respect consequent on its prolonged cultivation on agar may be considered relevant. It was observed that poured gelatin plates inoculated with a culture which had been held on agar through successive transfers gave raised, white, opaque, non-liquefying colonies (Plate V, D) of an anomalous (for gelatin) type. In order to determine whether a non-liquefying variant had been segregated or whether the change could be explained on physiological grounds alone the following experiment was undertaken. A culture of the organism, which may for convenience be designated A, obtained from the liquefied material of a gelatin stab was plated out on gelatin and/

and a typical colony (all the colonies were of the liquefying type) was used to inoculate an agar slope on January 8, 1932. After repeated sub-culturing on agar poured plates were made from a recent transfer (of March 21) on March 24. After two days numerous colonies of the raised circular opaque type were visible on dilutions I and II, but the medium showed no signs of incipient liquefaction. The same procedure was repeated with a parallel culture B obtained from a raised non-liquefying colony on gelatin dilution culture seeded from an isolation which had been grown previously on agar for many generations. The inoculum from colony B was first sub-cultured on agar slope on January 8. After successive transfers it was finally plated out on gelatin on March 24 from a sub-culture of March 21. By March 26 typical raised colonies had appeared which in growth form and absence of liquefaction were indistinguishable from those obtained simultaneously and under comparable environmental conditions from the culture described under A above. Several days later, however, it was observed that both A and B colonies were sinking slightly into depressions caused by the slow liquefaction of the medium. A final observation on April 12 showed that the gelatin on all A and B plates had been completely liquefied. It is concluded, therefore, that the organism's power to cause liquefaction is, if not permanently destroyed, at least lost temporarily and modified to such an extent by growth on agar as to render diagnosis on this basis uncertain unless the cultural history of an isolation can be specified exactly./

exactly. Morphologically and physiologically, therefore, except in respect of gelatin, the rapidly liquefying and slowly liquefying forms of the organism are identical.

Gelatin tubes stabbed from cultures isolated on agar and subsequently grown on that medium give entirely different characters from those described previously under gelatin stabs, where the organism was isolated on and inoculated from gelatin. Liquefaction is slow to commence (about the 4th day) and forms at first just a shallow depression where the surface was punctured and into which the growth sinks. The liquefaction then becomes stratiform and slowly descends the tube, 4 mms. after 6 days in a typical case, 13 mms. after 18 days, 25 mms. after 38 days and after 9 weeks the gelatin was less than half liquefied. On the surface of the solid gelatin below the liquefied zone there was a copious flocculent deposit in a conical heap; rim and reticulate pellicle were formed, the latter being easily precipitable; the liquefied portion of the gelatin was clear and, with beef infusion medium, showed green pigmentation which, however, did not diffuse into the solid gelatin in manner similar to growth on agar.

Fermentation of Sugars and Alcohols.

The following carbohydrates were prepared in concentrations of one per cent with peptone water as basic medium: Glucose, Saccharose, Lactose, Raffinose, Mannite and Dulcite. The fermentable solutions were set up with Durham's fermentation tubes and Andrade's indicator. Inoculated from a young

agar/

agar slant culture and observed every 24 hours up to 4 days and then at intervals of 6 days up till the second month the tubes at no time showed any indication of the production of acid or gas from the test substances although in every case the solutions showed evidence of growth with rim and pellicle formation and a moderate amount of sediment. There was no turbidity at the closed ends of the fermentation tubes.

Litmus Milk.

In litmus milk the litmus is reduced without previous change of reaction. The milk at first forms a finely flocculent coagulum and is later completely peptonised. In one example no change was observed till the sixth day when a rim and sediment had formed and the litmus showed reduction by zones with a clear serum layer commencing at the top. After 22 days a thick creamy layer was present to a depth of 11 mms. from the surface; this was followed by a reduced serum zone of 13 mms., then 13 mms. of incompletely reduced litmus succeeded by a uniform zone extending to the bottom of the tube which contained an abundant precipitate. After 40 days the litmus was completely reduced and showed no later tendency for the colour to return: the milk was completely digested and the whey clear.

Hydrolysis of Starch.

Although the organism had no markedly disintegrative effect on potato cylinders after 30 days' growth the starch of

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a beef extract broth culture containing one per cent soluble starch was completely hydrolysed after 19 days when tested with iodine - potassium iodide solution and compared with uninoculated control tube.

Reduction of Nitrates.

Nitrates are not reduced.

Production of Ammonia.

A trace of ammonia is produced from beef extract (Lemco) broth (containing peptone) as detected by inserting into the tubes strips of filter paper previously dipped in Nessler's solution.

Production of Hydrogen Sulphide.

Hydrogen sulphide is not produced. There was no blackening following stab inoculations of lead acetate agar.

Voges and Proskauer Reaction.

Acetyl-methyl-carbinol is not produced.

Production of Indole.

Indole is not produced. Peptone water cultures 2 days' and 24 days' old were tested with Ehrlich's rosindol reagent and a saturated watery solution of potassium persulphate: the sodium nitrite-sulphuric acid method was also applied: both sets of tests gave negative results.

Toleration of Sodium Chloride.

Grown in beef extract broth containing sodium chloride/

chloride in concentrations of one, two and three per cent and examined after 3 days the organism shows a definite uniform clouding with rim and pellicle formation in the 1% NaCl medium. In 2% NaCl broth turbidity was uniform only for the uppermost 7 mms.; below the strands of growth spread down into the medium and finally disappeared, leaving the lower half of the tube unclouded: no rim or pellicle was formed in this case although a few floccules floated at the surface. With 3 per cent NaCl broth there was no turbidity. The same three cultures, examined after 20 days, showed growth and the occurrence of sediment in all the tubes, but a comparison of the amount of the deposit formed by the organism in the three cases showed that it was inversely proportional to the concentration of NaCl in the respective cultures. Therefore, while growth is not entirely inhibited by a concentration of 3 per cent, the organism is regarded as being sensitive to the presence of sodium chloride.

Relation to Free Oxygen.

The organism is an obligatory aërobe. Agar slant tubes, inoculated and placed in a Buchner's tube, the contained atmosphere of which is deprived of its oxygen by a mixture of pyrogallic acid and caustic soda, show no growth however long anaërobic conditions are maintained. (These anaërobic cultures were retained and examined for signs of growth up to 11 months) No growth occurs along the track of the needle in deep agar and gelatin stab inoculations sealed over immediately after inoculation/

inoculation with 10 c.cs. of the melted medium. Ordinary stab cultures in deep agar tubes show no growth along the track of the inoculating wire except for the first 2 cms. below the surface where there was a slight, granular, discontinuous non-spreading growth. The surface of the medium around the stab, however, was completely covered with a thick, smooth, glistening whitish grey growth with adherent rim. The first examination of one such stab culture was made 6 days after inoculation and later after 18 days, but there was no observed increase in the amount or change in the appearance of the scanty sub-surface growth. As noted above there was no clouding in the closed ends of fermentation tubes (Kuhn's and Durham's).

Relation to Reaction of Medium.

The occurrence of growth on both decidedly acid and decidedly alkaline agar media indicates for the organism a fairly wide range of accommodation to the concentration of hydrogen ions: e.g. abundant growth is obtained on media neutral to phenol phthalein (pH about 8.4). The organism cannot be regarded, therefore, as very sensitive to considerable alterations in the pH of the medium.

Temperature Relations.

The optimum temperature for growth lies between 20° and 25°C. Growth occurs at 37° but it is very thin, flat and serous and at this temperature the organism produces no green pigment on beef infusion media. The maximum temperature is about 40°C and the thermal death point in the region of 50°C.

Longevity on Artificial Media.

Sub-cultures from liquefied gelatin show that the organism is fairly long-lived (4 to 6 months) on this medium. Cultures on agar slopes, however, appear to be much shorter lived as a specific example will indicate. A sub-culture of August 23rd 1932 when used to inoculate an agar slant on September 24th gave no growth at room temperature but a loop-ful of the original culture suspended in beef broth gave a slight turbidity. On placing loopfuls of the broth sub-culture on an agar slant typical growth was obtained. The foregoing experience demonstrates the fact that in this instance very few of the bacteria remained viable after a month's cultivation on beef agar.

TECHNICAL DESCRIPTION OF THE AUCUBA ORGANISM.

A short, slightly curved, rod with rounded ends, .90 to 1.70 μ long by .45 to .60 μ in diameter, occurring singly or in pairs and motile by one to four polar flagella. It is non-encapsulated, non-sporiferous; Gram-negative and non-acid-fast; forms circular, smooth, glistening, homogeneous, viscid, whitish to light grey, 4 to 6 mm. colonies on beef agar (after 5 days) clouds beef broth with formation of rim, pellicle and sediment; liquefies gelatin; peptonises litmus milk slowly but completely with reduction of the litmus; diastatic action moderate; has no fermentative action on glucose/

glucose, saccharose, lactose, raffinose, mannite and dulcite; non-nitrate reducing; does not produce hydrogen sulphide, indole or acetyl-methyl-carbinol; ammonia production doubtful; growth retarded by sodium chloride; forms a green diffusible pigment from beef infusion but not from beef extract media. It gives a brown non-spreading growth on potato with discoloration of the substrate. It is an obligatory aërobe with an optimum temperature between 20° and 25°C, a thermal death point about 50°C and a maximum of 40°C. It was isolated from root stem, and leaf lesions of the Japan laurel.

ACTION OF BACTERIAL TOXINS AND ENZYMES ON HEALTHY AUCUBA TISSUE.

In order to discover if any toxins or enzymes, capable of exerting a lethal or disintegrative effect on the normal tissues of aucuba, were secreted by the organism under the controlled conditions of artificial fluid culture the following procedure was adopted. A freshly prepared sterile decoction of healthy aucuba twigs was inoculated from a recent subculture of the organism on agar and incubated for eight days at 20°C. The culture was then diluted with five times its bulk of alcohol and the precipitated proteins etc. allowed to settle for 24 hours. The contents of the flask were then filtered through a double paper in an ordinary Buchner. The precipitate was finally washed on the filter with absolute alcohol and then transferred to the incubator at 37°C to dry.

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It was then carefully collected and placed in a moderate amount of distilled water to digest for several hours. The resulting solution was passed through a Berkefeld V filter and subsequently distributed in a series of sterile plugged tubes each containing 10 c.cs. of the filtrate. The contents of several of the tubes were boiled and on cooling were poured carefully into a number of small sterile petri-dishes. Other similar petri-dishes were arranged to contain (1) unboiled extract, (2) sterile distilled water to act as checks. Thin sections were then cut with sterile razor from fresh, succulent surface sterilised stems of healthy aucuba plants and several placed in each of the different liquids contained in the petri-dishes. Several blanks of each category were held as controls. The tissues from the boiled and unboiled extracts were examined microscopically after 24 hours and compared with the appearance of sections from the distilled water controls but there was no disintegrative or morbid change apparent in any part of the sections . Observations were again made after a farther period of two days and later again on the 9th day but no alteration in the cell walls, contraction or discoloration of the protoplasts was perceptible in the sections subjected to the action either of the boiled or of the untreated extract. As the control blanks showed that asepsis had been maintained it was concluded, that under these conditions of experiment, the organism exhibited no production of extracellular enzymes or toxins. Cultures in peptone-beef-extract broth similarly treated also showed an inability on the part of the extracts

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to effect lytic or lethal changes in the tissues of aucuba.

Inoculations with Bacterium-free Filtrate.

The failure to induce symptoms of disease by inoculation of healthy aucuba organs with pure cultures of the bacterium isolated from typical lesions and also the absence of any fungal pathogen from freshly attacked tissues suggested the possibility that the causal agent might be an organism of ultramicroscopic dimensions. Typically affected stems and leaves of the plant were thoroughly crushed in a mortar with a minimal quantity of distilled water. The grosser fragments of the decayed tissue were allowed to settle and the brown supernatant fluid then decanted off into an ordinary filter. This filtrate was then passed through a Masson porcelain candle and the bacterium-free filtrate thus obtained was transferred to a series of sterile test-tubes to facilitate subsequent filling of the syringe for different sets of inoculations. The sterility of the filtrate was tested in the usual way by transferring a number of loopfuls to sterile agar slants. As no growth came up on the control tubes the filtration was proved effective. With these filtrates, from which all visible microörganisms had been removed and which contained the postulated virus, hypodermic inoculations were made on the different parts of aucuba stems and leaves specified in detail under the descriptive account of the bacterial inoculations. The injections were made with the same precautions, under similar environmental conditions and on the different/

different categories of plants and cuttings previously noted for the bacterial experiments. These "virus" extract inoculations were in every case unsuccessful. Another possibility yet remained, that of the joint action of virus and bacterium. The idea that the disease might be the result of a symbiotic attack by two distinct causal entities each of which by itself would be incapable of producing infection prompted the use of mixed inocula prepared by combining filtrate with pure cultures of the bacteria in various artificial liquid nutrients. These inoculations performed, like their predecessors, with varied conditions, technique and experimental material were also abortive.

The Relation of Phomopsis Aucubae Trav. to Disease in Aucuba.

Phomopsis aucubae Trav. is a recognised saprophyte on Aucuba: its pustular fructifications on old dead twigs are familiar. The question naturally arises as to whether under certain conditions such as reduced vitality of the host this fungus may not be capable of assuming a parasitic rôle and invading fresh uninfected tissues adjoining the decaying shoots in which the fungus is situated. It has been mentioned already in the discussion of symptoms of aucuba disease how pure cultures of Phomopsis may be isolated from twigs, and also from some of the longer unbranched radical shoots, dead in the first instance from natural causes and only subsequently saprophytised by the fungus. The appearance of the decaying shoots in such cases is unmistakable, with the

occurrence/

occurrence of brown rot of the stem and the characteristic brown, thin, shrivelled nature of the adherent leaves; these features could scarcely be confused with the black glossy die-back caused by progressive necrosis down towards the base of the affected twigs in the local type of aucuba blight. In the older killed regions of plants suffering from aucuba disease Phomopsis also appears and ultimately forms pycnidia and, when the substrate is exhausted of its nutrient substance, perithecial stromata. With the object of putting the invasive powers of Phomopsis to the test inoculation experiments were made on healthy aucuba tissues. It was necessary to start, of course, by obtaining pure cultures of the fungus from isolations made from the older killed parts of blighted stems. This was easily effected by placing fragments of the infected tissues, excised from the inner portion of previously surface-sterilised twigs, on to ordinary nutrient agar. By hyphal tip transfers from the growth surrounding the inocula fresh poured plates were inoculated and typical Phomopsis mycelial forms were produced with the appearance of unenclosed conidial clusters of typical ellipsoidal biguttulate, alpha spores on the aërial mycelium. On a synthetic medium of sucrose 2 per cent., KNO_3 0.2 per cent., $MgSO_4$ $7H_2O$ and KH_2PO_4 0.25 per cent. each and washed agar 3 per cent. the mycelium was induced to form pycnidial aggregations from which moist yellow masses of alpha spores were later extruded. When viewed through the medium by transmitted light these spore fruits were seen to be associated with dark clumpings of the surrounding mycelium, while/

while the remainder of the surface of the medium was covered with a felt of white translucent mycelium. Healthy succulent shoots of aucuba, defoliated and placed in boiling tubes with a little basal distilled water, were autoclaved for 40 minutes at 15 lbs. pressure and on cooling inoculated with Phomopsis mycelium. After several days the surface of the inoculated twigs was covered with a large number of very small discrete pustules which later were erumpent to give long slender coiled spore tendrils of light lemon-yellow colour characterising the fructifications as typical pycnidia of Phomopsis (Plate VI, F & G). Similar spore horn extrusions were also observed on the stem and leaves of old blighted twigs, which had been held in the enclosed atmosphere of a moist chamber, indicating that the diseased leaves also are subject to later infection by Phomopsis. Having established the identity of the fungus isolated from old diseased twigs, and also having proved its identity with the fungus producing fructifications occurring naturally on the stem and leaves of blighted shoots in advanced stages of the disease, it still remained to be tried if this fungus when inoculated in pure culture into healthy aucuba shoots, was capable of leading a parasitic existence. The surface of the plant was sterilised with absolute alcohol at the points where inoculation was to be made and, after making the necessary incisions or abrasions, the wounded parts were overspread with sterile melted agar. Portions of the sporiferous aërial mycelium were then transferred to the prepared/

prepared sites of inoculation on stem, petiole and leaf lamina. The inocula were kept moist with sterile water and examined from time to time, but even after the elapse of three weeks there was no evidence that the fungus had attacked the healthy tissues adjoining those injured by the process of inoculation. The presence of superficial mycelium, on the other hand, indicated the continued viability of the fungus and its growth on the drops of exudate that had run down the stem from the gaping slit further emphasised the saprophytic nature of its relation to aucuba. Other series of experiments in which the stem apices were pierced with inoculating needles charged with fungal mycelium and the petiolar stumps of severed leaves smeared over with similar inoculum were no more productive of positive information with regard to the possible parasitism of Phomopsis although in many cases the observations were extended to several months during which period the inoculated twigs remained perfectly normal.

THE PATHOGENICITY OF BOTRYTIS CINEREA PERS.

During the preparation of tubes with sound healthy twigs for inoculation purposes it was observed that in a few cases, after the twigs had been under the moist enclosed conditions of the plugged tubes for several days a rapid brown rot took place. Subsequent investigation of this unusual type of symptom revealed a connection between its appearance and the presence of flowers in the fork of the affected stems.

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In a case such as that shown in Plate VI, A, we have the spread of necrosis from the peduncle into the limbs of the fork at whose junction the inflorescence is situated. The rate of advance of the infection was very rapid and the rotting of the attacked twigs sometimes completed in 4 to 5 days. Portions of diseased branches affected, in vitro, with this soft brown rot were transferred, after sterilisation of the surface from which the inoculum was to be taken, to the young expanding flowers of other enclosed twigs. Although no abrasion of the floral parts had taken place and the inoculum simply laid on, the infection was imparted and brown decay visible in the stem below by the third day (Plate VI, B). In contrast to the original infection from which the inoculum was obtained and in which the disease spread from the base of the peduncle upwards into the arms of the fork, the present infection affected only the stem below the peduncle spreading downwards into the older, more woody tissues. (Plate VI, B). From this it will be seen that the infection in both cases spread from the flower stalk but whereas in one case the disease advanced upwards into the young, succulent shoots of recent growth and showed no evidence of descending into the older parts below the junction, in the other instance for some unknown reason the infection progressed downwards into the more mature tissues of the stem leaving the apparently more susceptible succulent branches above the peduncle unaffected.

This virulent attack of healthy twigs with the production/

production of symptoms wholly unlike those hitherto observed in diseased conditions of aucuba seemed to indicate that here we were dealing with a causal agency widely different from that responsible for the production of the disease which is the primary object of investigation in the present work. Typical portions of the soft brown decayed tissues were planted on the surface of poured agar plates in order to effect the isolation of any causal organism that might be present. In all such isolations from different peduncular inoculations and from spontaneous infections at floral organs one species of fungus was consistently obtained but no microörganism was ever found in association with it. At first it appeared possible that we had here an example of parasitism by Phomopsis aucubae but a subsequent study of the cultural and morphological characteristics exhibited by the fungus showed that it bore no relation to the saprophytic Phomopsis, that occurs abundantly on diseased or decaying twigs in nature, nor indeed to any fungus previously recorded from the leaves and branches of aucuba (vide Oudemans[5]). For the reason that this species has not been mentioned in connection with aucuba as host and that, under the conditions of experiment noted above, the parasitism is of a particularly energetic type it was considered that a fuller description of the invading fungus and its effect upon the tissues of the host was desirable and even necessary if the pathogenicity of the fungus were to be given satisfactory proof.

Apart from the successful inoculations obtained by transferring/

transferring portions of the original lesions to the inflorescences of healthy twigs, infection was also produced by introducing pure cultures of the fungus into slits in the stem and scarifications on the leaves of healthy twigs which did not possess flowers. The inoculations were carried out in similar fashion to those with Phomopsis as described above. The surface of the organ to be inoculated was first of all sterilised, the incision of stem or abrasion of leaf surface, as the case might be, effected with sterile scalpel and the mycelium, usually with conidiophores attached, was transferred to drops of solidified sterile agar at the point of inoculation. In every case the disease was reproduced with striking rapidity. When inoculations were made at different points on the same stem the necrosis spread upwards and downwards from these points so that the amount of healthy stem rapidly diminished as the advancing margins of necrosis approached each other (Plate VI, C). It will be noticed from this example that infection produced primarily in the stem passes up the petiole into the leaf lamina (Plate VI, C and D). Conversely inoculation of the leaf leads to infection of the stem via the petiole. For instance the result of inoculating the mid-rib region of the lamina (as shown in Plate VI, D) is a spread of necrosis through mesophyll and vascular tissue alike down into the stem and upwards towards the leaf apex. On the same twig (Plate VI, D) inoculation of the apical part of the stem leads to the death of the growing point and the killing/

killing of the apical leaves by active invasion: a narrow band of healthy stem between the infected regions still remained when the photograph was taken, but less than 24 hours later it had been entirely penetrated by the brown rot. From these artificially inoculated infections the fungus was subsequently reisolated in pure culture and on comparison with that originally isolated from the diseased tissues it proved to be identical. Since the control twigs, treated in a similar way except for the omission of fungal inoculum, showed no symptoms of disease the postulates of Koch have been satisfied and the pathogenicity of the fungus consequently established.

After the life history of the parasite had been worked out in pure culture little difficulty was experienced in its identification as Botrytis cinerea Pers. The succesive cultural forms may be briefly referred to at this point. The primary mycelial growth from inocula on agar poured plates was extremely rapid and had almost covered the entire surface by the end of the second day (Plate VII, E). The flat, spreading growth form of the whitish-grey translucent mycelium is indicated in Plate VII, E, and under higher magnification (Plate VII, F) the mode of hyphal branching is shown. The next growth phase commences after the surface mycelium has covered the available nutrient surface; aërial hyphae then arise, (after 3 to 4 days) first of all at the limits of growth at the margin of the petri dish and the/

the junction of separately inoculated growths. From this aërial mycelium the typical Botrytis conidial fructifications are produced (Plate VII, G). The conidial heads are borne on stout brownish conidiophores differing in colour and texture from the surface mycelium. When the moist surfaces of agar slants are inoculated the mycelium forms a denser, white, compact felt from which there arises at intervals on the surface (after 4 to 5 days) discrete black sclerotia of characteristic botrytis form (Plate VII, H and I). On transference of these sclerotia to fresh agar slants they germinate to produce ordinary vegetative mycelium which later aggregates to form a fresh crop of sclerotia. Autoclaved twigs of aucuba when inoculated with the mycelium usually gave rise to both types of propagative body. The conidiophores grew out aërially from the drier top end of the inoculated twigs next the cotton wool stoppers: the sclerotia were formed externally on the epidermal surface at the lower moister end of the twig and dropped off as hard spherical brown masses on to the cotton wool on which the twig rested among the water at the base of the tube. In the leaves of twigs dead of botrytis rot sclerotia are formed as circular to irregular areas in the mesophyll. These sclerotial areas are more shiny and darker in colour than the diseased mesophyll around them and they arise as cushion-like structures with the raised outline towards the abaxial leaf surface. They constitute areas of varying size and shape in the mesophyll but do not appear to be/

be capable of bridging the larger veins but tend rather to become attenuated along one side of them. The nature of the infection was also studied histologically. For this purpose pieces of infected twigs, including the area of demarcation between diseased and healthy tissues, were fixed in Flemming's strong fluid. The appearance of the diseased twig in Plate VI, A, will serve to illustrate the type selected for detailed examination. The apices of the twin branches have been invaded and the infection has progressed a short way up the petioles of the apical leaves. On sectioning such a region longitudinally at a thickness of 4 μ and, after staining, observing under the lower power it was noted that the fungal hyphae had not penetrated up the tissues of the petiole far in advance of the externally determined limit of the rot. This is explained by the fact that the disease is of the parenchymo-vascular type and that as a result in the general invasion of the tissues the rate of advance will be immediately noticeable in the superficial layers since the progress of necrosis is marked by a succession of levels common to all tissues. The first material examined was stained with Flemming's triple stain, but this method failed to differentiate between host and fungus except for the hyphal tips of the latter at the foremost line of invasion (Plate VII, B). The denser granular cytoplasm of these leading threads retained the safranin to an extent that the older vacuolated mycelium and host tissues did not and this enabled the mode of progression of the invasion to be traced

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with precision. Promiscuous penetration of the tissues is thus effected, the parasite ramifying at one time in the intercellular spaces and then passing into the cell cavity (Plate VII, B). Staining the sections with Haidenhain's Iron Alum-haematoxylin and counterstaining with eosin in clove oil was also tried in an attempt to differentiate the mature hyphae but with even less success than with the previous stain. Material fixed in acetic-alcohol gave no better results by these methods. An excellent stain for the purpose, however, was found in a one per cent. solution of cotton blue with lacti-phenol as solvent. The sections were stained in this medium three days and differentiated for about half that period in pure lacti-phenol. The mycelium was then admirably distinguished from the host tissues (Plate VII, C and D). The fungal cytoplasm was stained a clear bright blue and the oil globules, which were stained black with the osmic acid of the fixative, increased the definition of the hyphal segments. What was tantamount to a polychrome effect was the staining of the host nuclei a pale greeny blue shade, thus adding another element of relief to the pathological picture. Since the tissue depicted in the photomicrograph (Plate VII, A) is from the apical part of what at the time of infection was a vigorously growing shoot it is almost entirely parenchymatous and the result of invasion is the production of a soft rot. In the procambial strands, however, the pathogen was observed to follow the line of the primordial vascular elements so that considerable lengths

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of the septate hyphae could be seen in longitudinal sections (Plate VII, C). In the medullary and cortical regions of the infected shoot the ramifications were more tortuous and the intracellular nature of the invasion was especially apparent in such tissues. This is clearly demonstrated in Plate VII, D where the nucleus of one cell is invested by the hyphae of the parasite.

Later, on examining some of the twigs that had died as a result of botrytis disease it was observed that in addition to the normal fructifications of this fungus there were present, more particularly on the leaves, the pycnidia and extruded spore tendrils of Phomopsis aucubae Trav. On the drier parts of the dead leaves the coiled spore horns were of a light orange yellow colour but where the surface conditions were moister the spore masses formed structureless pustular heaps of a pale buff colour. Under the microscope the contents of both varieties of extrusion were seen to be composed of typical biguttulate ovoid spores of Phomopsis. The occurrence of these spore formations on the aucuba twigs, post mortem, is farther evidence of the saprophytic powers of Phomopsis. It shows that that fungus is capable of utilising for its growth the remnant of nutriment that remains after the original pathogen has exhausted the tissues of its special requirements. The spores of Phomopsis are propably widely distributed on the surface of healthy aucuba plants but must await the activity of parasitic or other lethal agency before they can germinate with the chance of producing an internal mycelial system.

EXPERIMENTS WITH MIXED INOCULA.

In view of the fact that infection with Botrytis cinerea was the first means at our disposal of effecting the artificial production of disease in aucuba under controlled experimental conditions it was thought that advantage might be taken of such pathogenicity in determining if association of the bacillus of aucuba with the parasitic botrytis might not throw some light on a possible saprophytic rôle for the bacterium as all attempts to establish it as a primary invader had hitherto failed.

The inoculum was prepared by mixing the mycelium and conidia of botrytis with a watery suspension of the bacillus taken from an agar slant culture of recent growth. The same precautions as to surface sterilysis of the parts to be inoculated were observed as in previous experiments with botrytis and bacillus separately and the method of inoculating and the different regions inoculated were also similar to those before adopted. At the same time a comparable procedure was carried out on a number of other twigs employing botrytis alone as inoculating medium. Several shoots were similarly treated, but with the omission of the organisms, to act as checks. After several days those twigs inoculated with botrytis alone showed symptoms typical of the rapid brown rot and the final results were entirely similar to those obtained on previous occasions (cf. Plate VI, C and D). The control twigs remained perfectly healthy and normal throughout

the period of observation. The effect of the mixed inoculations, on the other hand, showed a remarkable contrast. The lesions produced were small and confined to a small area surrounding the seat of inoculation in the case of the stem and in the case of the inoculations on the leaf no signs of spreading disease were apparent at all and the actual injury appeared no greater than that caused mechanically in introducing the inoculum. It was further noticed that even where inoculated leaves were subsequently abscissed no botrytis infection occurred although fungal mycelium could be seen at the injured surface. In the stem the attack was so completely and effectively inhibited that the small lesions (Plate VII, E) after a time became dry and black by the collapse of the decayed tissues, and the epidermis thrown into longitudinal striae so that altogether the appearance was somewhat reminiscent of naturally diseased aucuba surfaces. The negative synergic effect was a little less marked when the mixed inoculum was placed on a flat wound surface as at a cut petiole (Plate VII, E, lower part of stem). Here also, however, the disease was ultimately suppressed and its rate of encroaching on the healthy tissues was at no point as rapid as the necrosis produced by the botrytis acting alone. No further study of the antibiosis exhibited by the botrytis-cum-bacillus inocula was deemed advisable at the present time as it did not appear likely to aid in explaining the mode of distribution of the bacillus in the more extensive lesions of general necrosis. The demonstration of the failure of botrytis to produce disease in the presence of this particular bacillus indicates that such

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a relationship may possibly exist in nature. The fact that such a proved and virulent parasite as botrytis has not been recorded from diseased or even dead twigs of aucuba may find an explanation when taken in conjunction with the widespread occurrence of the bacillus on aucuba lesions under natural conditions.

DISCUSSION.

An examination of the results detailed above will reveal no indubitable proof of the pathogenicity of any of the organisms isolated from lesions on, or observed in association with, diseased shrubs of aucuba. In the absence, therefore, of incontrovertible evidence the presentation of definite conclusions concerning the problem of the causality of the disease would be not only futile, but positively misleading. Nevertheless there are certain facts relating to these possible pathogens that admit of being construed in support of arguments which favour each in turn as being the aetiological agent. We shall discuss, then, individually the different views by which the casual claims of the several organisms might be regarded. Our attention will be directed first to the bacterium specifically characterised in an earlier part of this work.

The connection of this organism with the disease of aucuba rests solely on the fact of its repeated isolation, most often in pure culture, from the fresher parts of recently necrosed tissues./

tissues. There is also to be taken into consideration the resemblance to a bacterial blight which a general but not too intensive observation of the symptoms suggests viz: the obviously blighted and blackened appearance of affected bushes and the rapidity with which, in certain instances, these symptoms may appear. On the other side there is to be set a large number of points of a negative character which, however, are of not inconsiderable import and serve to dispose, almost entirely, of any indication of parasitism that the above data might convey. First we must record a complete failure to observe the organism in multifariously fixed and stained sections of both floral and vegetative tissues from typical and suspected (early) lesions. This involved the preparation of serial sections transverse and longitudinal from roots, stems, flowers (female) and leaves (midrib, marginal serratures and apices) in different stages of necrosis but in spite of the care observed in the different processes no traces were discoverable of bodies that might reasonably be described as bacteria. Likewise in carefully observed sections of untreated material from diseased stems mounted directly in water no motile bodies were to be seen wither in the tissues or in the surrounding mountant. When smears were made from the fluid expressed from pathological tissues and stained by Gram's and other methods it was also found that no organisms could be detected. In particular, an examination of the diseased tissues in section revealed no widespread appearance of bacterial cavities: intracellular material was generally absent and lysis of the middle lamellae/

lamellae did not seem to be a feature of the disease nor did penetration or swelling of the cellulose walls occur. Observation of diseased bushes before and during the growing season never revealed the presence of exudate upon any old or lately diseased parts nor was anything of such a nature given out when stems in process of necrosis were cut. It may also be mentioned that during the isolations, although encircling growths of the organism were obtained from the solid inocula of diseased tissue fragments implanted on solid agar surfaces, yet the number of colonies, dissociated from similar inocula, arising in the medium of plates poured from shake isolations on gelatin was always either exceedingly small or nil.. As the same condition was found even where the diseased tissue particles were finally more widely distributed on the plate by maceration in sterile distilled water previous to inoculation of the melted gelatin, it was concluded that the organism must be present in the tissues in relatively small numbers since it must be remembered that just a few viable individuals would be sufficient to produce the characteristic halo of liquefaction around the diseased fragments. There is, of course, the alternative view that the organisms may not be readily displaceable from the tissues; but in diseases of the blight type where bacteria are pathogenetically active it is usually necessary to proceed to dilutions of the second and third order before discrete colonies of the causal organism can be picked off so wide is its dispersion from the small amount of inoculum originally introduced. Finally there are the completely/

completely negative results of the inoculation experiments to be taken into account. The diversity of methods employed in introducing the inoculum and the exhaustive choice of sites for inoculation to which were superadded the experimental conditions that are considered to be supremely favourable to bacterial invasion, all failed to produce even the slightest manifestation of parasitism on the part of the organism. The weight of evidence appears, therefore, to be against the assigning of a causal significance to the organism. Its practically constant occurrence, however, and that in pure culture, in those regions of the plant which have become last affected by disease seem to indicate that it is a primary saprophyte of special habitat or at least that it is capable of such adaptive specialisation.

With regard to the possibility that fungal infection may be responsible for the production of disease symptoms, the case of botrytis parasitism may be briefly dismissed. This fungus was never on any occasion observed on bushes affected by disease nor was it ever isolated from the tissues of naturally occurring lesions. Although its pathogenicity for experimental plants was proved to be of a virulent type, as judged by the ease with which it was successfully inoculated and the rapidity with which the symptoms spread, yet it was noticed that it was only where the infective material was presented at unprotected surfaces, such as the exposed nectaries and stigmata of the female flowers or the incisions and abrasions employed in inoculation, that the fungus/

fungus was capable of invading the healthy tissues. The spread of superficial mycelium over the lower unaffected part of the twig shown in Plate VI, A, indicates that the hyphae are unable to penetrate directly the cuticularised surface of the intact stem. From these observations it may be safely concluded that botrytis is not the cause of the disease we are investigating and bearing in mind the symptoms produced by botrytis in artificially infected aucuba cuttings, it is also probable that this fungus does not saprophytise diseased aucuba plants in nature.

More serious consideration must be given, however, to the relations of Phomopsis in diseased conditions of aucuba. This fungus is a recognised and widely distributed saprophyte of aucuba, and the question naturally arises whether, under suitable conditions supervening in the host plant, it may not be able to adopt a more aggressive parasitic activity. This view does not receive support from the observations made during the course of the present investigation. From the freshly killed tissues where the cause of the disease, if fungal, might have been expected to be most active no fungi were ever isolated. In stained preparations of these regions it was likewise found impossible to trace the presence of fungal mycelium. Freehand sections of similar lesions observed directly, on mounting in water, were no more indicative of fungal invasion. A few inches farther down the dead stem, however, all these conditions were fulfilled. Phomopsis was then isolated, in many cases as pure cultures: the mycelium was observed in unstained mounts of transverse and longitudinal/

longitudinal sections of the stem at such points. Both parenchymatous and vascular tissues were interpenetrated by the hyphae. The results of trial inoculations with pure vigorously sporulating cultures of Phomopsis would seem to indicate that it is not able to overcome the natural resistance of healthy aucuba tissues. What did appear to be a case of weak fungal parasitism, however, was observed in one instance of which the following is a description. Situated on the still green and turgid lower part of a stem whose upper smaller branches had suffered die-back there was a circular patch of brown decay radiating from a lenticel. The portion of the woody stem including the lesion was removed and placed in lactic acid for several days to soften the tissues. Transverse sections were then cut through the focus of disease and double stained with safranin followed by picro-aniline blue according to Cartwright's (2) method. Fungal hyphae were then clearly observable under the microscope in the tissues underlying the lenticel and extending as far inwards as the cambium. This seems to indicate an ability on the part of fungi (probably Phomopsis) to encroach upon the enfeebled but not otherwise diseased tissues of aucuba. But the fact that the discoloration produced by this type of semi-parasitism was light brown, ab initio, tends to discredit farther the possibility that this type of attack is responsible for the blackened appearance of the host in the extended form of necrosis. The probability that some degree of impairment of the vitality of the host is necessary before even such local rots can arise is strengthened by the discovery/

discovery of fungal spores in the lenticels of healthy stems. Pieces of healthy shoots were cut and after thorough sterilisation of the surface followed by washing in sterile water transverse sections were taken from the region of the lenticels and transferred to the sterile agar surface of poured plates. After a few days, on examining the periphery of the sections under the low power, a varied fungal flora was observed emanating from the complementary tissues of the lenticels having arisen presumably by the germination of a spore collection at these points. Normally, therefore, the lenticels of healthy stems appear to harbour potential invaders but only when the sub-lenticellar tissues become weakened and susceptible can an extrance be effected. These are probably the channels by which phomopsis gains access to the decaying tissues of aucuba but the fact that it alone of all the fungi present superficially can appropriate entirely this medium for its growth or at least progresses so vigorously as to overcrowd its competitors indicates a relation of preëminent suitability of saprophyte to host.

The only other organism isolated from diseased parts of aucuba with anything approaching regularity was the yeast whose description has already been given. All the facts of isolation relating to this organism point to it as essentially an epiphyte. It was never observed in the tissues during examination of sections of diseased material and it is probable, therefore, that it has no reference to the cause of necrosis.

When we proceed from the microscopic to consider

the/

the invisible factors in pathology the problem becomes more vague and elusive. Having failed to establish the pathogenicity of any of the isolable or cultivable forms of life existing on or in the diseased plant we may reconsider the symptoms from the point of view of the ultramicrobic hypothesis. The postulate of virus as the causative agency in disease, in the absence of other demonstrable parasite, is, under certain circumstances, relatively easy to advance and even to indicate significant features supporting it in the symptoms but it is altogether more difficult to substantiate its correctness by experimental means or, conversely, to entirely disprove it. In the present case, therefore, we shall again be content to enumerate and compare the leading facts from this standpoint and leave the issue open till conclusive information shall be available.

There is always the possibility, however, that in the symptoms described we have included more than one disease of aucuba. For instance there are more points of analogy to virus infection discoverable in the external and histological appearances of the systemic or chronic type of disease before mentioned, than there are in the local necrosis of the terminal branches of other bushes which usually are able to survive this type of attack. Apart from the regions immediately diseased this latter type of plant presents an otherwise apparently healthy condition (cf. the left hand branches of the forks in the twigs marked A, Plate II). The pathological histology of the diebacks of such ultimate twigs shows that the acronecrosis (spreading from the stem apex downwards/

downwards in the stem and upwards into the apical leaves via petioles and mid-ribs) is not peculiar to any one kind of tissue but advances equally in all as the margin is regularly pushed forward into fresh tissue. There is abundance of inclusion in the dead cells but it is of a rather non-descript variety (Plate II, E) with no very characteristic form or structure. There is an absence of such material in the cells of the stem region in advance of the necrotic margin (Plate II, D). Leaves affected by tip necrosis (Plate III, A and B) show a similar lack of differentiation in the intracellular bodies of the generally necrosed tissues. The blackened aspect of blighted leaves and stems (in contradistinction to the light brown rot of the tissues by botrytis, for example, or by the fungal invasion of twigs isolated by abscission) is doubtless the result of the presence of this dense, opaque, homogeneous content of the necrosed cells (Plate III, C to F).

The distinctive features of systemic disease, however, are firstly the reduction in leaf size while the leaves are still green and turgid (Plate I and Plate II, B). But the dwarfed habit is more conspicuous where one member of an ornamental planting, for example, has become early affected and shows general symptoms. The whole form of such a plant is small and stunted; the growth, not only of the leaves, is retarded but the internodes are ill-developed and the stems thin and yellowish: necrosis is patchy and confined mainly to the lower parts of the shrub: the roots of such plants are also badly diseased. These

symptoms/

symptoms, of course, need not necessarily be interpreted as due to virus attack when we remember that the dwarfing due to a virose in plants is an expression of disordered translocation (caused by phloem necrosis) and is a condition likely to arise when any disease of a slow-acting character upsets the general course of nutritional movements. The morbid anatomy of the roots of these plants (Plate IV, A to D) displays an appearance different in some respects from necroses hitherto described, for a localisation of the necrosis is noticable in the phloem and outer cortical layers. In some of the diseased cells a more definite pattern is distinguishable in the presence of striate and reticulate inclusions; in others the cell material is either amorphous (as in stem die-back) or too opaque to admit of resolution (as in apical leaf lesions).

What may prove to be another form of lesion was discovered on some of the older leaves of a bush showing symptoms of the die-back category. These leaves had a general blanched appearance, with large light areas in addition to the usual mottling of the variegation, seen best in the mesophyll of the wings when viewed from the adaxial leaf surface. Closer observation revealed a brown but not very intense necrosis of the mid-rib spreading some distance into the mesophyll on either side (Plate VIII, A). These areas were studied in transverse sections of the mid-rib (Plate VIII, C). An opaque gum-like substance was present in the lumina of the upper epidermal cells but was absent from the lower epidermis. There was necrosis and a breaking/

breaking-down of the cell-walls around the vascular bundle of the mid-rib but the xylem and phloem themselves did not show evidence of extensive damage. The palisade tissue was more heavily diseased than the spongy parenchyma. Other signs of dissolution of the cells were evident in the aggregation of chloroplasts around the nuclei, the swelling of nuclear membranes and the ejection of chromatin granules. The same features were also found registered in longitudinal sections from the mid-rib region of similar leaves (Plate VIII, B). Whether this decaying condition of the leaf is symptomatic of disease or whether it represents a phase in natural processes of distintegration preceding leaf fall was a point not determined. In other cases, however, it was certain that such changes did not occur as a preliminary to abscission. Blackened, killed areas around the hydathodes and apices of similar leaves and also of leaves in other respects green and healthy were sectioned longitudinally in the hope that information might be gained regarding the way in which necrosis commenced since it had been observed previously that the necrosis at these points might later spread and involve larger areas of the lamina. The appearances, however, around the margins of the dark necrotic lesions gave no evidence of bacterial or fungal invasion and were otherwise no more illuminating on the cause of death than had been the similar examination of diseased material from other parts.

As reported previously the disease, if of virus origin, was not directly sap-transmissible as the negative results of/

of the inoculation experiments with bacterium-free filtrates of diseased tissue extract go to prove. When account is taken of the fact, however, that perhaps the majority of known viruses are not thus inoculable the above evidence on this point is seen to be only partial.

Several further observations of a more general nature made during the course of the investigation now fall to be recorded. In the first place it was noticed that the injuries sustained commonly called forth a phellogenic reaction on the part of the host. Such reaction cannot, however, be regarded as specific - as indicative of bacterial, fungal or virus infection - since the cork barrier is the regular method by which the plant seeks to limit and exclude any type of lesion whether the result of mechanical injury or infective activity. It was observed to occur in aucuba where a cork layer arrested the downward advance of necrosis in the die-back of twigs; and a similar layer has been noted in the case of the"physiological" exclusion of non-infected twigs by the process of shading-out and "natural die-back". The activity of a cork cambium was also in evidence where the cutting off of superficial lesions had occurred as in the warty excrescences on otherwise sound stems and in the smaller lateral lesions of diseased roots which might be regarded as analogous to the stem type just mentioned. Although cork cells situated at the level of abscission of the peduncles are formed as an entirely natural consequence of the failure of the fruits to develop yet they are rather significant from the point of view of possible disease transmission through the nectaries and stigmas as these are the only/

only really unprotected surfaces on the plant. This lack of protection is, however, not so great as appears at first sight. When the first stages of decay, following the failure to set berries, are initiated in the inflorescence the cork cells are already beginning to form an impervious barrier at the base of the flower stalk. Then, though the conditions are favourable in one sense for the inroads of saprophytic or weakly parasitic fungi or bacteria, as in the case of physiologically severed twigs, the path to the interior of the plant is blocked by the abscission layer which on the fall of the peduncle seals over the scar and is even less likely to permit the successful germination of fungal spores. In the diseased condition of aucuba we are studying in the present work the above reference to the abscission mechanism of the peduncle is hardly necessary for typical twig blight was observed on bushes on which no flowers appeared.

Another rather remarkable feature of shrubs displaying systemic symptoms of disease was that the apical leaves of unnecrosed twigs could remain green and turgid for a long time after the lower part of the main branch on which they were supported had been girdled and killed. Whether this phenomenon indicated that water was passing upwards through the dead portion of the stem is uncertain, but it was observed that some healthy twigs supported in flasks containing unsterilised tap water showed wilting of stem and leaves on their cut ends becoming fungally infected and the channels of absorption thereby blocked with/

with mycelium.

Since exhaustive examination of the diseased tissues yielded no suggestion of parasitism intelligible to the writer it was conceived possible that the injuries might be due to the activities of animal bodies. A selection of slides of various organs in necrosis and especially of the diseased roots were, therefore, submitted to a protozoologist for examination but he reported that he could trace the presence of no objects interpretable as of animal origin.

One aspect of the problem remains to be dealt with in conclusion. The possibility of a non-parasitic cause of the disease must not be neglected. In the first place it could be the result of physiological injury - a deficiency of certain constituents in the soil solution or a superabundance of others. The diversity of habitat (soil types) of diseased bushes appeared to rule out this possibility. A study of the hydrogen ion concentration of the soil in which typically diseased specimens were growing likewise disposed of this explanation. The pH of a number of samples of such soils from depths of 2, 4, 6 and 8 inches below the surface gave values ranging from 5.4 to 6.0 but the average value (which was also the mode) was 5.8. These observations, then, do not support an explanation based on abnormality of the edaphic factors of the environment.

When we turn to consider the possible effect of climatic variation a solution appears more imminent for it will be remembered that the severity of the symptoms and the extent of the injuries/

injuries sustained by the plants were by no means constant features from year to year. Linked to such possible variants as temperature, moisture relations etc. an explanation of synchronous variation in the manifestation of disease is hypothetically justifiable. This explanation is particularly applicable to the twig blight type of disease since such symptoms were more or less general over all shrubs in any one year, and the attenuation or virulence of disease was likewise uniformly observed on all aucuba plants as these factors varied from year to year. The hypothesis of winter injury would also accord well with the observation that the growing points are the most susceptible parts, as appears probable from the necroses spreading from these foci. However feasible such explanation of low temperature effects may appear it completely fails to explain the systemic condition of disease where only a few plants are affected and their immediate neighbours remain healthy. That is, where the symptoms are general and the shrub systemically affected, climatic rigor can be, at most, only one of the factors contributory to disease although it may be the initial or predisposing cause of the development of farther irregularities within the plant. In any case, whatever the correct explanation of the causal agent, the fact of this variation has been shown to exist and, whether the fluctuations in the external environment are directly responsible or whether they merely facilitate the action of other pathogenic factors, it must be taken into account if the explanation is to be complete.

SUMMARY

1. A description of the symptoms of a disease in Aucuba japonica Thunb. is given. In most cases the injury takes the form of a blight of the terminal twigs, in others a more systemic affection was observed.

2. The histology of typical stem, leaf and root lesions was studied but revealed no certain evidence of parasitic invasion.

3. Isolation from the more recently necrosed parts yielded a constant and, in most cases, pure growth of a motile bacterium.

4. Inoculation experiments with this organism gave negative results.

5. The organism is given specific characterisation and details of its life history are described.

6. The action of the extracted products of bacterial growth was tested on sections of sterile aucuba tissue without positive result.

7. Bacterium-free filtrates of the expressed sap from diseased plants were inoculated into healthy tissues but no lesions were produced.

8. Phomopsis aucubae Trav., occurring generally on the dead parts of aucuba bushes, was unsuccessfully inoculated on vigorous healthy shoots.

9/

9. The parasitism of Botrytis cinerea Pers. was established for experimental cuttings but this fungus was never observed in naturally occurring forms of aucuba disease.

10. As botrytis has not previously been mentioned on aucuba this new type of infection is described symptomatically and histologically.

11. The results of inoculation with mixed inocula of aucuba bacillus and botrytis pure cultures indicate than an antibiotic relation exists between these organisms. The inhibition of botrytis infection by the bacillus may afford an explanation of the non-appearance of botrytis rot of aucuba in nature.

12. The relative claims of the organisms, isolated from decayed tissues, to be regarded as significant in the production of necrosis are enumerated but no attempt has been made to formulate definite conclusions regarding the possible pathologic rôle of any of them.

13. It is suggested that differences in the degree of incidence of necrotic injury from year to year may be related, directly or indirectly, to the variation of climatic forces.

I am indebted to Dr. S. G. Jones, Lecturer in Mycology in the Department, for suggesting the problem and supervising its investigation. Through the courtesy of Professor C. H. Browning facilities for/

for part of the bacteriological work were made available at the Pathology Department of the University and Western Infirmary. The investigation was made possible by the receipt of a Grant from the Department of Scientific and Industrial Research.

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EXPLANATION OF PLATES.

PLATE I.

Photograph of systemically affected aucuba plant showing dwarfing of apical leaf groups not yet necrosed. Comparison may be made with the normal foliage of the healthy shrub at the left of the picture.

PLATE II.

Aucuba japonica Thunb.

A. Branched twigs showing typical die-back in one limb of each fork. The limit of stem necrosis is indicated by an arrow in each case. In the twig on the left the disease has been arrested at the junction of bifurcation; as is usual in this form of disease; in that on the right the advancing margin of necrosis has not yet terminated its course. (x $\frac{3}{4}$).

B. Blighted twigs from systemically diseased bushes. Note the dwarfed appearance of the leaves, the killed apices and patchy necrosis of the stems. (x $\frac{2}{3}$).

C. Illustration of phases in the natural death and subsequent decay by saprophytic fungal attack, without precedence of blight symptoms, of shaded-out abscissed twigs. The twig on the left (1), severed at the site of natural abscission, showed signs of physiological isolation, yellowing of stem and mesophyll, etc., but no trace of disease or fungal infection was present: that on the right (2) shows a late stage of disintegration by Phomopsis, the fructifications of which are beginning to appear at the dead apex; the adherent leaf was light brown and papery, and the stem was similarly discoloured. (x $\frac{3}{4}$).

D. Longitudinal section, at the necrotic junction, of a stem in process of local die-back. Note collapse of pith tissues into lamellae and the presence of intracellular masses in cortex and outer pith zones slightly in advance of medullary lamellation. (x 32).

E. Inner cortical cells, from a section similar to that described in D, more highly magnified to show the amorphous (undifferentiated) nature of the cell inclusions. (x 1,000).

PLATE III.

Aucuba japonica Thunb.

A. Young apical leaves from vigorous unbranched radical shoots of current season's growth exhibiting tip necrosis. (x $\frac{7}{8}$).

B. The middle leaf of the trio shown in A magnified $4\frac{3}{4}$ times. The general death of vascular and mesophyll tissues may be noted, also the occurrence of necrotic spots in advance of the more regular line of demarcation between diseased and healthy parts of the leaf.

C. Transverse section of the leaf lamina passing through an isolated necrotic spot similar in position to those observed in B. Note the general opacity of the lesion due to the filling of the lamina of necrosed cells by a dense black gummy material. The abaxial epidermis over the lesion is unaffected but its adaxial counterpart is diseased beyond the region of mesophyll involved. (x 75).

D. A higher power representation of the edge of a leaf lesion similar to that described in C. The irresolvable density of the typically diseased cells shades off through a few intervening cells at the margin into the normal cells of the healthy mesophyll. (x 850).

E. Longitudinal section of the diseased mid-rib of a leaf in tip necrosis at the most advanced point affected. There is a tendency for the necrosis in this case to spread first along the vascular elements. (x 75).

F. Part of a section, similar to that described in E, magnified to show the intracellular contents of the tracheids and adjoining cells at the cross-over from the normal to the pathological cell structure. Note the general resemblance to the conditions observed in D with opaque lumina succeeded by a more diffuse discoloration of the protoplasm in cells undergoing morbid change. (x 850).

PLATE IV.

Aucuba japonica Thunb.

A to D. Pathological Histology of the Root in Systemic Disease.

A. Longitudinal section of a diseased root taken at the junction of necrosed and healthy parts as determined by external appearance. Pathological content is observable in the exodermal and hypodermal layers and in the outer peripheral cells of the stele: the intermediate cortical cells are normal. (x 197).

B. Superficial layers from a section similar to that described in A. The cell bodies display a granular to reticulate pattern. (x 400).

C. Longitudinal section from a root (description as in A), showing the internal limitation of necrotic symptoms to the phloem. (x 197).

D. Portion of the stele, from a section similar to that described in C, showing the striate to opaque nature of the material occluded by the sieve-tubes in necrosis. (x 400).

PLATE V.

Bacillus of aucuba.

A. Poured plate isolation of the organism by halos of pure growth from stem sections of diseased aucuba twigs implanted on agar. (x $\frac{3}{4}$).

B. Surface and buried colonies of the organism on beef infusion agar. Photographed by oblique transmitted light from a three days' old culture to show homogeneous structure and entire margin of surface colony. (x $1\frac{3}{4}$).

C. Surface and buried colonies of the organism on gelatin. The surface colonies show large areas of surrounding liquefaction: the buried colonies are small and their position marked by the occurrence of small pits of liquefaction at the surface of the medium. Age of culture and photography as in B. (x $1\frac{1}{2}$).

D. Surface and buried colonies of the organism on gelatin after prolonged sub-culturing on agar. Note the raised growth of the surface colonies and the absence of liquefaction. Photographed by reflected light from a three days' old culture. (x 1).

E. Film from a two days' old agar streak culture showing flagella. Stained by Kirkpatrick's method. (x 2,400).

F. Film from culture similar to that used for E and similarly stained. The rods possess from one to four polar flagella (x 1,600).

PLATE VI.

The Parasitism of Botrytis cinerea Pers. on Aucuba japonica Thunb.

A. Experimental cutting of aucuba from boiling tube. Botrytis infection has taken place through the exposed stigmata and nectaries and the rot has spread from the peduncle into both limbs of the fork as far as the levels indicated by the arrows. The growing points of the twig have been killed and the disease is spreading to the petioles of the apical leaves. (x 2).

B. Infection has occurred as in A, but the necrosis has invaded the older, more woody, part of the stem below the peduncle to the point designated by the arrow: the younger, more succulent branches, springing from the junction of inflorescence and main stem, have not been affected. (x $\frac{2}{5}$).

C. Result of artificial inoculations on an aucuba twig with Botrytis mycelium isolated from lesions similar to those depicted in A and B. The necrosis has travelled up and down the stem from the seats of inoculation and up the petiole to the base of the leaf lamina. The part of the stem comprised between the arrows shows the rapidly diminishing green healthy portion that remained at the time the photograph was taken. (x $\frac{2}{3}$).

D. Botrytis invasion of aucuba twig. Artificially induced lesions photographed four days after inoculation with sporulating mycelium of botrytis. The sites of inoculation were (1) the mid-rib at the mid-laminal region, (2) the open surface of the petiole of an apical leaf deprived of its lamina and (3) the stem just above the lowest node shown. The limits of the general tissue rot which resulted are defined by the arrows. (x 1).

E. Specimen twig of aucuba, inoculated with mixture of botrytis and the bacillus of aucuba, illustrating the antibiotic relationship of these organisms. The invasive powers of botrytis are modified and finally suppressed (lower inoculation at cut petiole) or almost entirely inhibited from the beginning (small sunken lesion marking site of upper inoculated stem incision). (x 1).

F and G. Autoclaved aucuba stem (from healthy succulent unaffected branch), inoculated with pure culture of Phomopsis aucubae Trav. isolated from older parts of the typically blighted stems occurring naturally. The coiling spore tendrils of the fungus are shown issuing from the pycnidial ostioles. (x 9).

PLATE VII.

A to D Intracellular habit of the invading mycelium (of botrytis) in the killed stem tissues of aucuba.

A. Low power of longitudinal section of aucuba stem showing general distribution of the parasitic botrytis mycelium in diseased growing point and apical petioles (x 36).

B. The densely granular advance hyphae of the infection penetrating the host cells (from a region of an apical petiole slightly beyond that shown in A). Stained Flemming's Triple Stain. (x 830).

C. Segmented mycelium running longitudinally in the embryonic conducting tissues near apex of diseased stem (taken from a section similar to that described in A). (x 780).

D. Hyphae ramifying in the cells of the killed cortical region of a stem (from a section similar to that described in A). The more lightly stained circular bodies are the host nuclei. (x 780).

A, C and D. Sections from material fixed in Flemming's Strong Fluid and stained with cotton blue in lacti-phenol.

E to I. Stages in the Life History and Growth Forms of Botrytis Cinerea Pers.

E. Reisolation of the fungus in pure culture on agar. Inocula derived from artificially inoculated aucuba stem lesion (three days' old culture). (x $\frac{3}{4}$).

F. Habit of the surface mycelium showing mode of branching of the hyphae (Marginal growth from culture described in E). (x $4\frac{1}{2}$).

G. Conidial heads of the aërial mycelium. Note typical clusters of Botrytis conidia. (x 11).

H. Sclerotial groups standing out from dense white parent mycelium. (x $\frac{3}{4}$).

I. Group of sclerotia (from these described in H) magnified to show surface morphology and droplets of exuded moisture (from ten days' old sub-culture on agar). (x 4).

E to I, all photographed by reflected light.

PLATE VIII.

Aucuba japonica Thunb.

A. Group of leaves showing discoloration (necrosis) along their mid-ribs and expanding slightly into the adjacent mesophyll. Dark necrosed spots may also be detected at the extreme apices and hydathodic tips of some of the leaves. (x 1).

B. Longitudinal section of a necrosed mid-rib from a leaf similar to those shown in A. Necrosis is most noticeable in the palisade tissue, and black occlusions can be observed in the cells of the upper epidermis. (x 45).

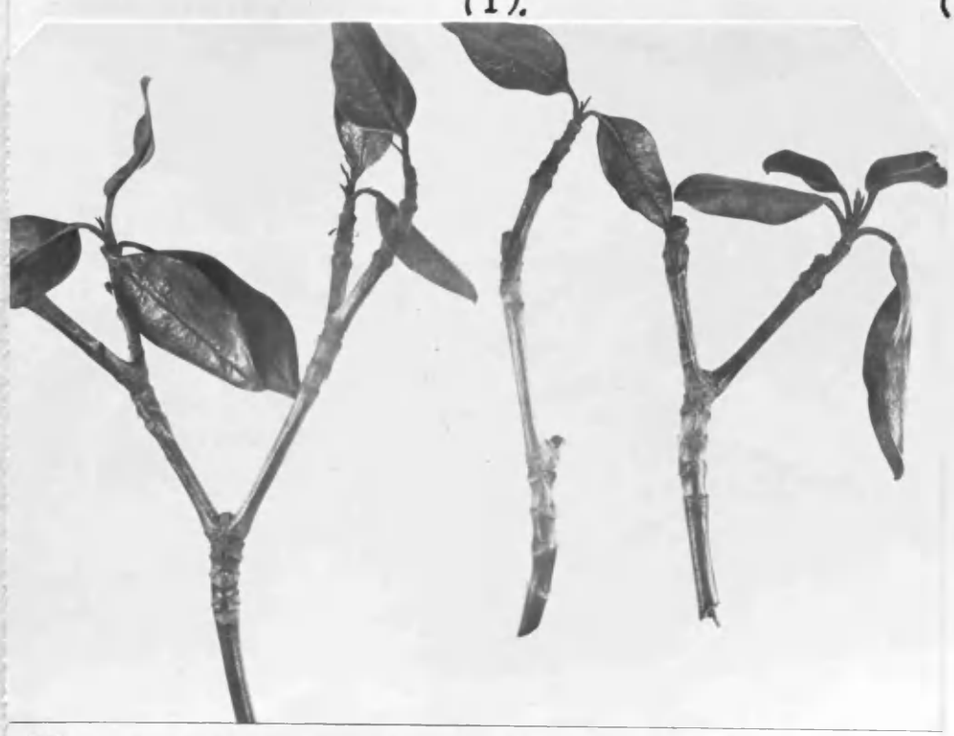
C. Transverse section of a necrotic mid-rib from a leaf comparable to those in A. The tissues around the vascular bundle appear to be disintegrating, otherwise the pathological features are as described for B. (x 45).

PLATE I.

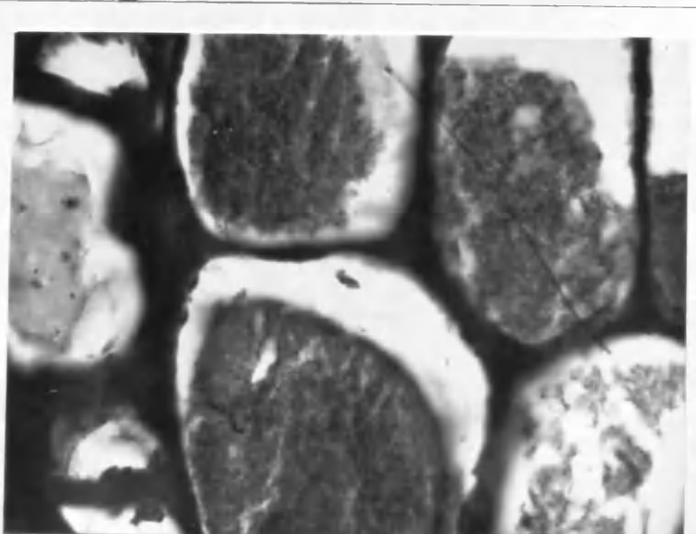
PLATE II.

A

(1). (2).

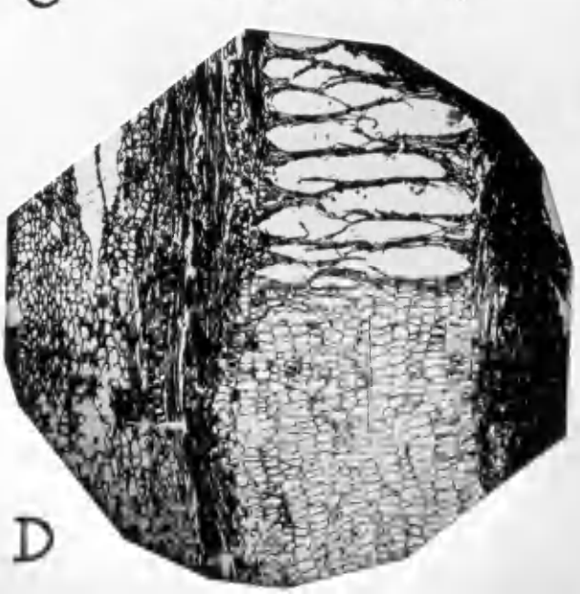


B



E

C (1). (2).

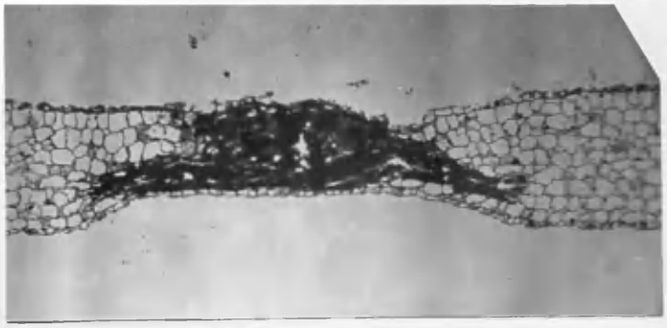


D

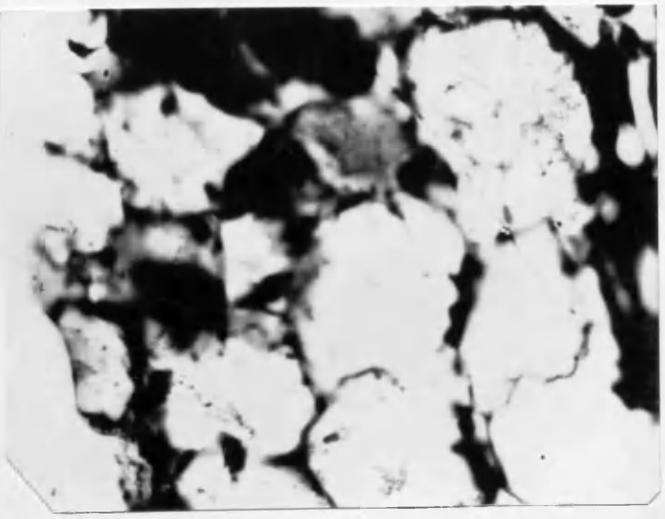
PLATE III.

A

B



C

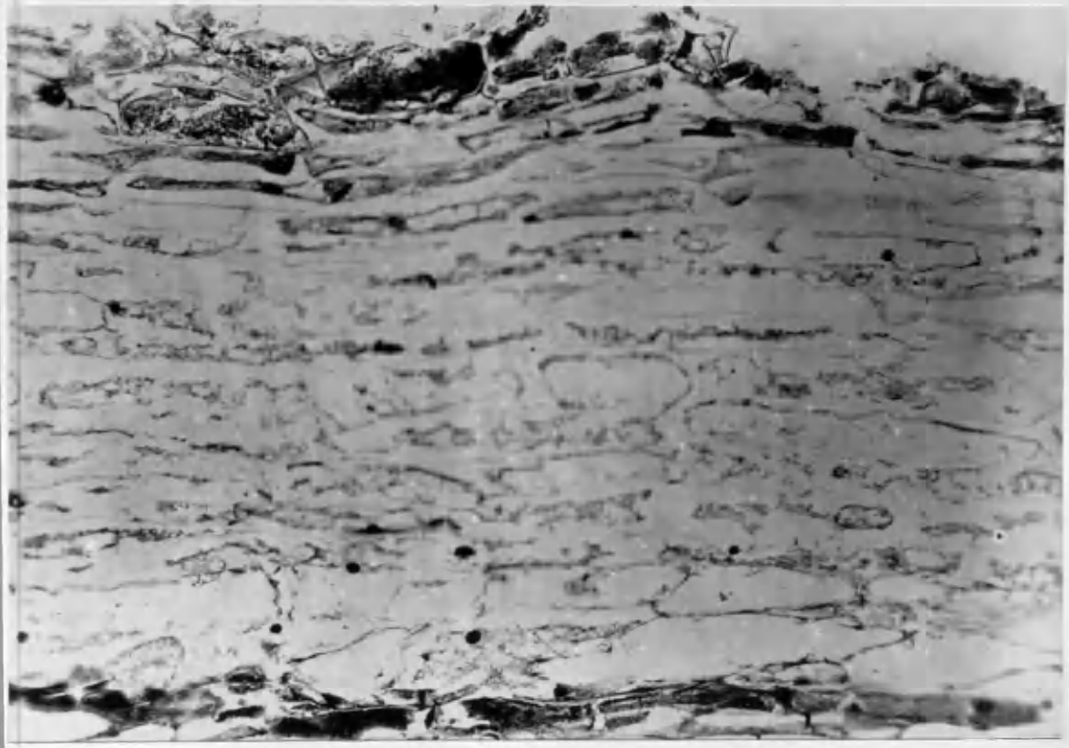


D

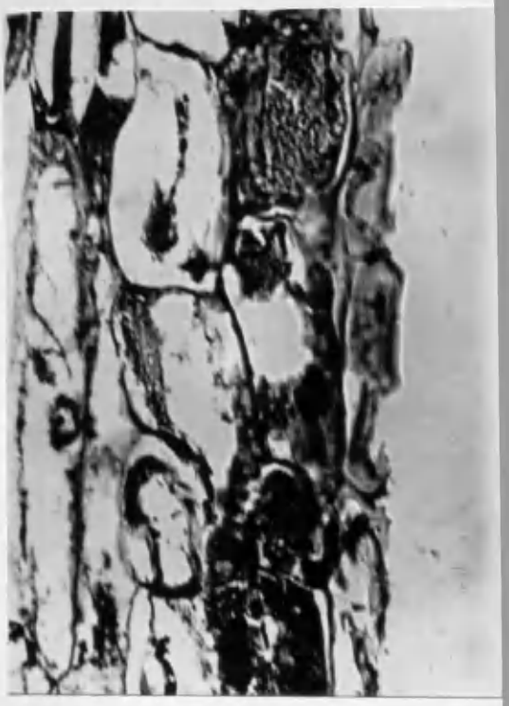
E

F

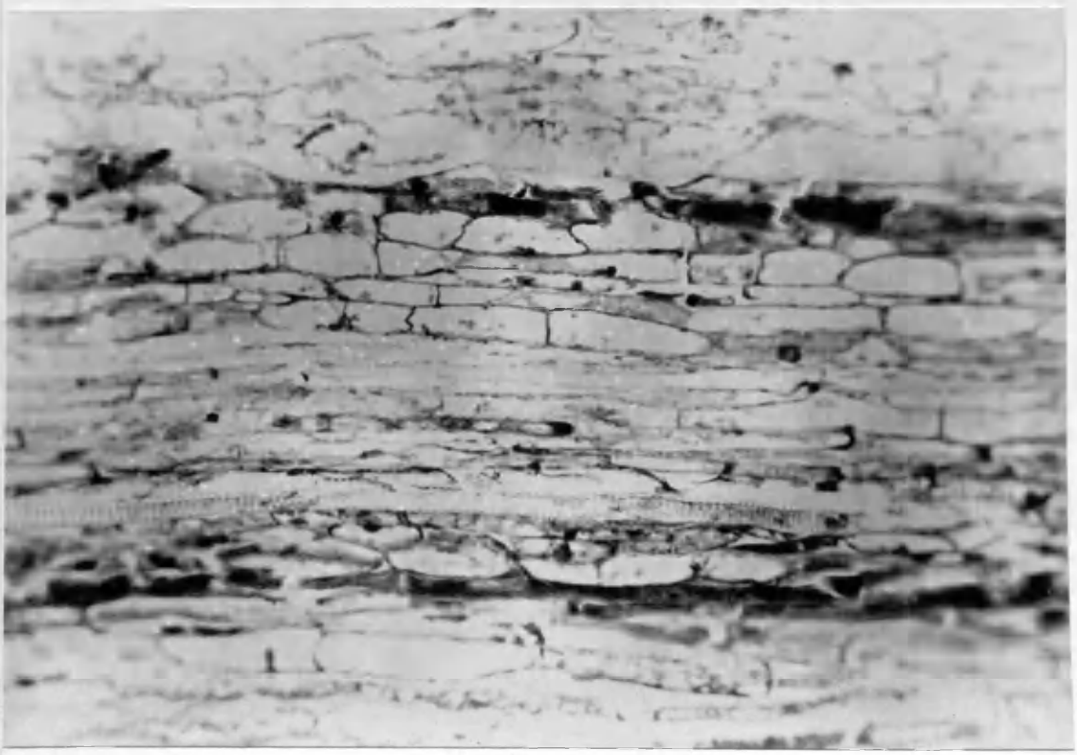
PLATE IV.



A



B



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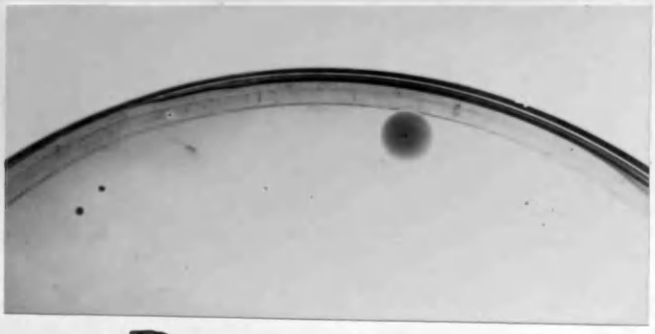

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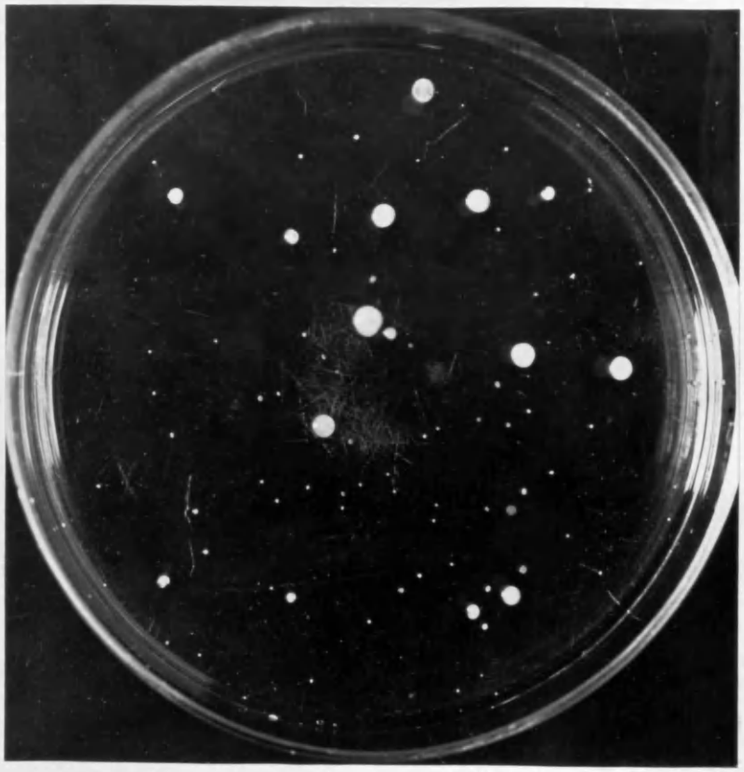

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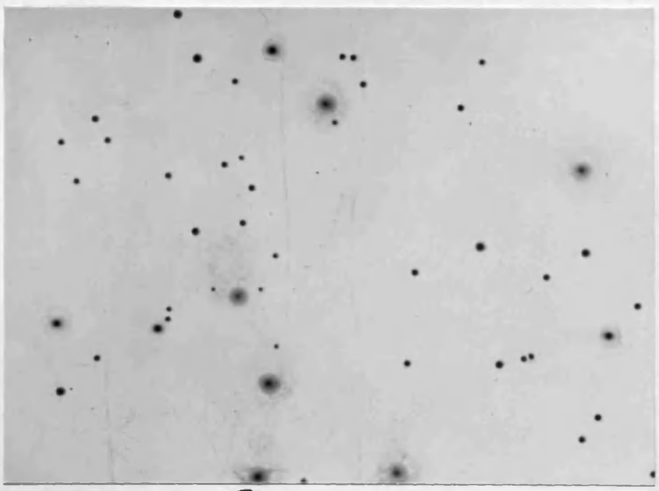

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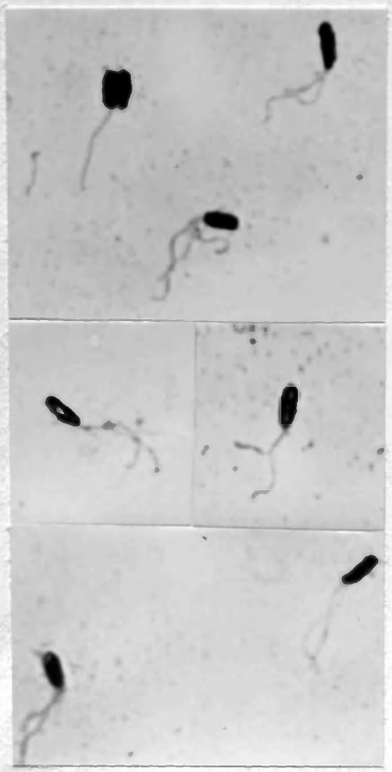

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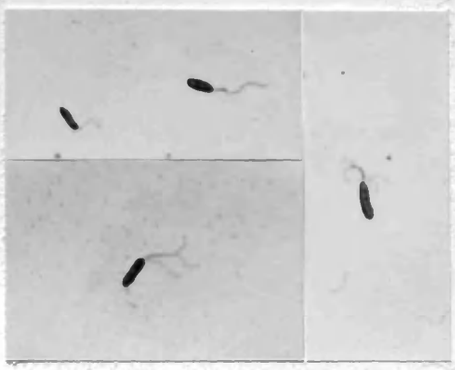

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PLATE VI.

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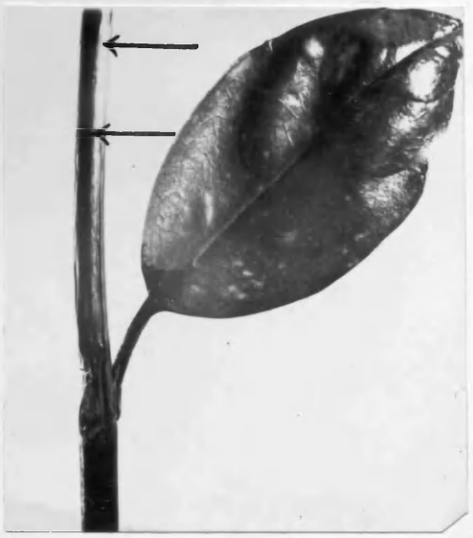

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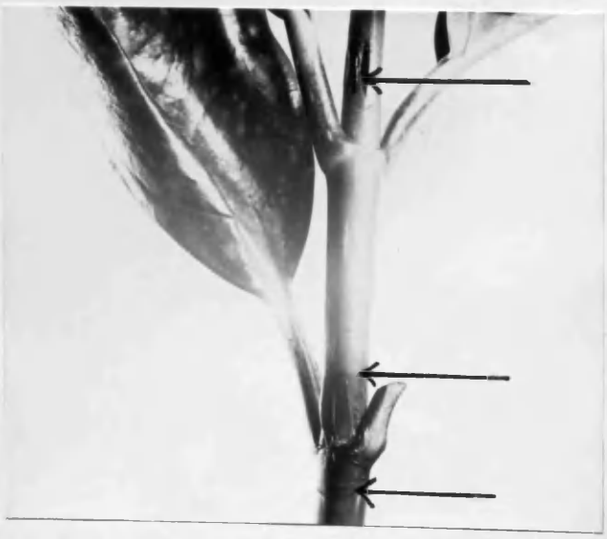

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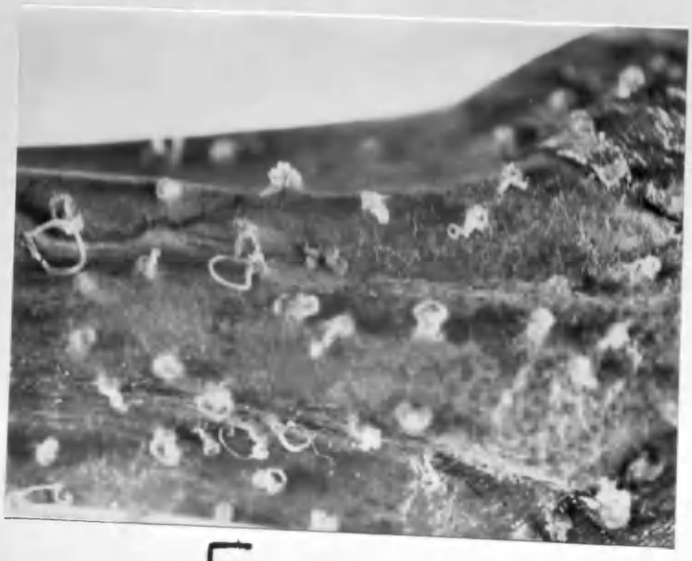

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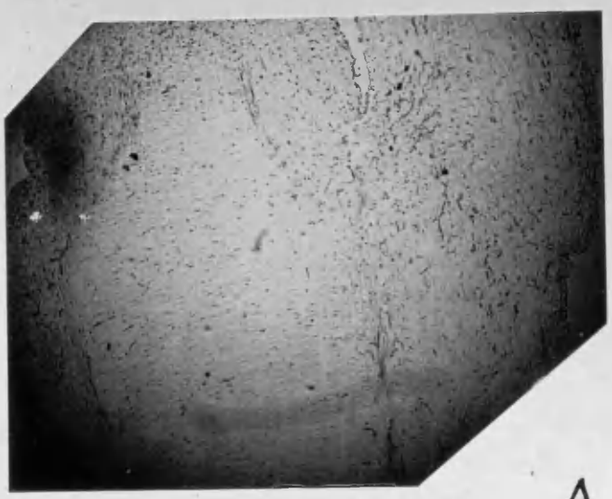
PLATE VII.



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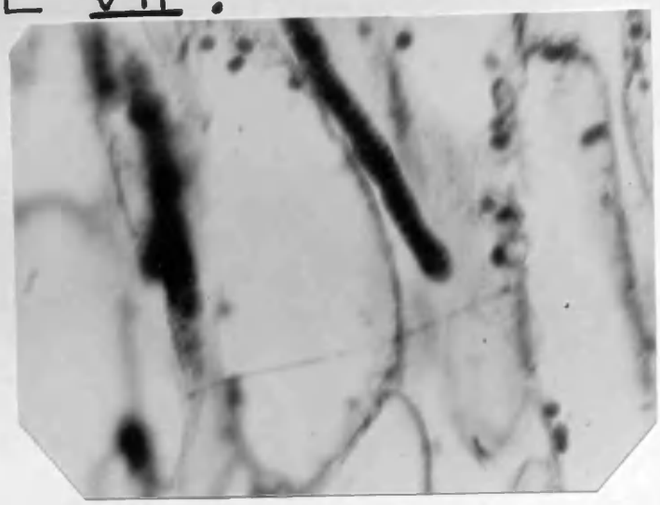

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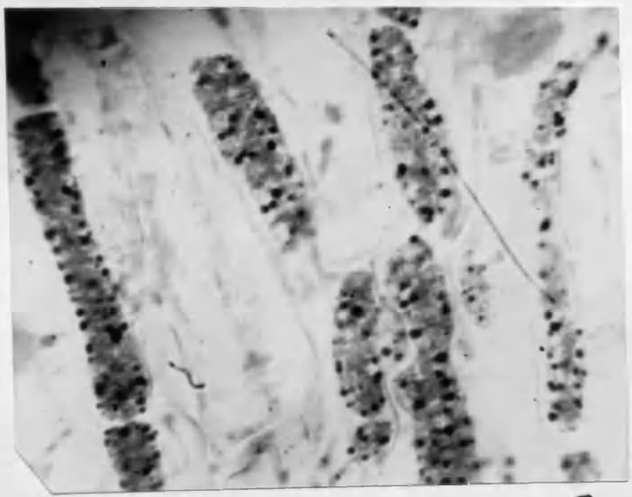

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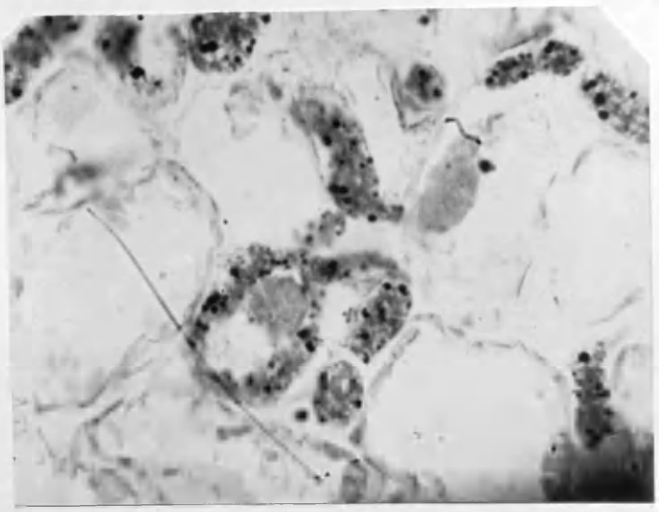

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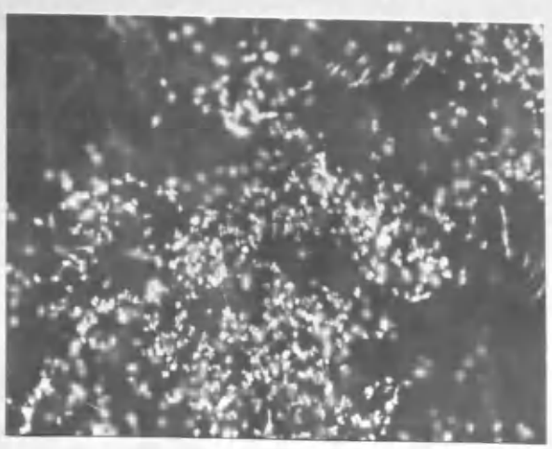

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PLATE VIII.

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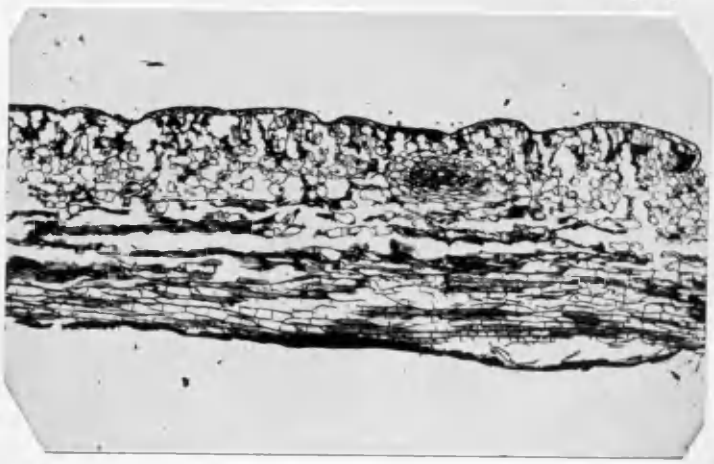

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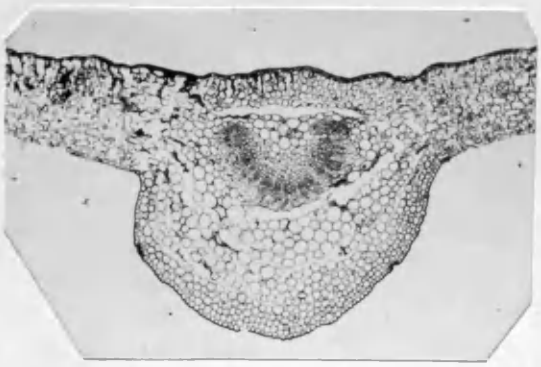

C

A STUDY OF THE FOLIAR ENDODERMIS IN THE PLANTAGINACEAE.

(Addendum: January 1934.)

Introduction.

In view of the generality of the claim of the foliar endodermis as a family character of the Plantaginaceae already advanced by the writer[3] it was considered that the investigation of this anatomical feature might be profitably extended by the examination of the remaining genus (Bougueria) of this family as well as by the inclusion of a number of additional, and climatically varied, species of the genus Plantago itself.

Material and Methods.

As all the immediately available sources of fresh material of plantaginaceous species were exhausted previously in the work alluded to above[3] recourse was had in the present instance to the dried leaves of herbarium specimens suitably softened by the chemical pretreatment recommended by McLean[2] as an efficacious preliminary to the sectioning of such desiccated material. This method was regarded as much the more satisfactory after the soda-glycerine soaking mentioned by Howarth[1] as well suited to the preparation for sectioning of dried herbarium leaves of Festuca ovina L. had been tried with very indifferent results.

The McLean technique consists essentially in the treatment of the material by the following well defined stages: the removal of air from the shrivelled tissues by absolute alcohol

for a period determined by the thickness of material involved: transference to distilled water through graded alcohols, the softening process being hastened by maintaining the water at a temperature up to about 70 degrees Centigrade after the final change: steeping in an 8 per cent. aqueous solution of Potassium Hydroxide for about a week, preferably under reduced pressure after half the period of soaking has elapsed, the final volume of fluid being roughly one third the original: neutralisation with a 15 per cent. aqueous solution of Acetic acid changed several times: washing with distilled water till on standing it remains neutral to litmus: after transference to 60 per cent. spirit the material is readily hand-sectioned when supported in pith.

Considering that many of the leaves investigated by the aid of such treatment were upwards of a hundred years old the almost complete removal of discolouration from the tissues and the resumption by even the thinner cellulose walls of their normal contours bespeak the excellence of this method for the purpose designed.

The further treatment of the sections so obtained was carried through on lines entirely similar to the procedure fully described in the above reference(3): that is the sections, after subjection to the appropriate action of eau de javelle, were stained firstly by a heated solution of Sudan III in spirit and glycerine to bring up the suberin lamellae of any secondary elements that the endodermis might possess and secondly by the

Gentian Violet of the glycerine jelly mountant to determine the presence or absence of primary endodermal characters as distinguished by the Caspary band. Where these endodermal structures did occur the staining was equally effective with, and the appearances of Caspary strip etc. wholly typical of those obtained in the leaves of the undried specimens of the species already examined.

The material dealt with in the present work comprised, in the genus Plantago, the following eight species:

P. anilata Drumm.
P. arenaria Waldst. et Kit.
P. eriopoda Torr.
P. gnaphaloides Nutt.
P. lagopus Linn.
P. minuta Pall.
P. varia R. Br.
P. virginica Linn.

and in the monospecific genus Bougueria the type species B. nubicola Decne.*

The anatomy of the leaves of these species was studied after preparation of the leaves according to the procedure outlined above, by the same methods as formerly, except that no sections were taken from the apical region; that is the mid-laminal and basal zones alone were examined.

Results.

The results of observation will now be given in detail for each of the species designated.

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* The material of the Plantago spp. was derived from the Herbarium of the Botany Department, Glasgow University, through the kindness of Professor Walton; that of Bougueria was obtained at the Herbarium of the Royal Botanic Garden, Edinburgh by the courtesy of the Regius Keeper, Sir William Wright Smith.

B. nubicola. The leaves of this species are tri-nerved and distinctly dorsiventral in structure with two to three layers of adaxial palisade on top of the laxer mesenchyme. In transverse section the lesser as well as the principal veins at the mid laminal region showed a complete primary endodermis a condition which was repeated exactly at the level of the leaf base.

P. anilata. A complete primary endodermis was here again characteristic of all the vascular tracts from the mid-rib and larger lateral veins down to the relatively small veinlets sectioned transversely in both the mid and basal parts of the leaf.

P. arenaria. In the mid-lamina a complete primary endodermis was present round all the bundles from the mid-rib to the smallest visible in transverse section. At the leaf base a complete endodermis likewise surrounded all the veins but traces of suberin were discernible in some of the endodermal cells of the mid-rib and large laterals, especially on the radial and inner tangential walls of such cells, a condition which might be described as the initial stages of an incipient secondary endodermis.

P. eriopoda. There was an entire primary endodermis investing all the lesser veins as well as the mid-rib and main laterals and it was absent only from the finest vascular strands. Contrary to the usual mode of increase in endodermal differentiation in Plantago spp. the stage of development encountered in sections of the leaf base showed this species to be anomalous, for, while the smaller bundles were still surrounded by an uninterrupted primary

endodermis, that of the mid-rib and larger laterals showed an unexpected degree of incompleteness. It is also worthy of note that at these lower levels on the leaf the measure of completeness of the endodermis was up to a point in inverse proportion to the relative size and anatomical importance of the various bundles as presented in transverse view. Since this condition prevailed in all the specimen leaves of this species so examined it would appear that such aberrance is specifically characteristic.

P. gnaphaloides. The leaf of this species is tri-nerved and at the mid-laminal level possesses a complete endodermis associated with mid-rib, large laterals and veins of the next order, and even down to the smallest strands: the mid-rib endodermis and that of the main laterals was partially in the secondary phase. The secondary condition is more pronounced in the leaf base region where it is attained completely by the endodermes of mid rib and large laterals while the smaller veins contain several such secondary elements in their endodermal sheaths. It was noted that, even where the endodermis might be completely secondary, the individual suberin lamellae were not uniformly developed over the whole inner surface of the cells but were thinner at, and sometimes apparently absent from, the outer tangential walls.

P. lagopus. Round all the bundles at the mid laminal region of the leaf there was an endodermis complete and primary except at the mid-rib where a few secondary cells were observed: in one

instance, for example, three out of the twenty four cells comprising the mid-rib endodermis were in the secondary condition. At the leaf base the secondary stage of endodermal development was now well marked round the mid-rib and main laterals but was still only partially complete: a primary endodermis invested all lesser veins at this level.

P. minuta. In transverse section no endodermal characters could be detected in connection with the veins at the mid laminal level of the leaf. Similarly the vascular system of the leaf base region was characterised by the entire absence of a recognisable endodermis although it must be recorded that in certain examples there appeared a slight amount of lignin staining towards the inner side of the radial walls of a few cells of the mid-rib bundle sheath which might possibly represent the vestiges of Caspary strip formation. The presence of a very much reduced primary endodermis was, however, never definitely established in any part of the leaf of this species although typical portions of specimen leaves were cleared in eau de javelle, mounted whole after staining with ammoniacal basic fuchsine, and carefully examined for traces of endodermal development.

P. varia. The endodermis is absent from all veins at the mid-laminal level of the leaf. Even at the base of the leaf no endodermis whatever was formed round the lesser bundles while the mid-rib and largest lateral veins possessed only a vestigial primary endodermis with but a few cells in each of those bundle

sheaths displaying signs of characteristically identifiable Caspary strips. Apart from the absence of the significant Caspary strips the cells of these bundle sheaths seemed otherwise to conform, histologically and in their arrangement and typical regularity, with the constitution of a normal endodermis.

P. virginica. A complete endodermis is present round the vascular bundles, including even the smallest veins, of the leaf of this species at its mid laminal zone: it is mainly secondary round the mid-rib and larger laterals. Sections of the leaf base showed a higher degree of differentiation in the endodermis of the bundles for it was completely secondary round mid-rib and main laterals and also round those smaller bundles situated between the mid-rib and the other main veins but round the smaller veins near to the leaf margins the secondary endodermis was incomplete by the presence of varying numbers of purely primary cells or the endodermis of such bundles might be totally composed of primary elements.

Conclusion.

This farther investigation of the occurrence and internal distribution of the foliar endodermis in the Plantaginaceae increases substantially the evidence in favour of regarding this feature as characteristic of the family. Especially also is its presence in Bougueria significant of the mutual bearing of floristic considerations and vegetative histology on the question of natural relationship.

Summary.

(1) The species considered above with respect to the nature of the foliar endodermis possessed by each may be grouped conveniently under three heads: firstly the small section, comprising P. minuta and P. varia, where the endodermis was extremely reduced in character or even doubtfully present: secondly those species, including B. nubicola, P. anilata, and P. eripoda, in which the endodermis though present never proceeded past the primary phase of development: and finally the group of species, P. arenaria, P. gnaphaloides, P. lagopus and P. virginica, which showed varying degrees of the development of a secondary stage in their endodermal system.

(2) The inclusion of Bougueria, the third and last genus of the Plantaginaceae, and the investigation of an additional eight species of Plantago in the present work affords strong presumptive justification of the view that the foliar endodermis determined for so representative a range of species must be of universal occurrence throughout the remaining, uninvestigated portion of the family.

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January, 1934.

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2. McLean, R.C. The Utilisation of Herbarium Material. New Phyt., vol. XV, Nos. 5 and 6, 1916.

3. Trapp, G. A Study of the Foliar Endodermis in the Plantaginaceae. Trans. Roy. Soc. Edin. Vol. LVII, Pt. II, (No. 18), 1933.
