On two species of *Heterosporium* particularly *Heterosporium echinulatum.*

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(Schluß.)

Experiment VII.

The plant was one of those used in the previous experiment; but was turned round through an angle of 180° so that leaves not previously inoculated were presented to the experimenter.

On February 5th 1912 the plant was inoculated. A watery suspension was made from infected *Dianthus* plants collected from one of the nurseries at the beginning of the month. The conidia were brushed off from three diseased areas by means of a small paint brush into a watch glass containing a little tap-water. A drop of this liquid under the low power of the microscope showed it to contain numerous spores of *H. echinulatum.*

The lower leaves of the plant were painted over the whole of the upper surface with the water suspension of spores, and were marked with small copper-wire loops. Two days later, namely upon February 7th one inoculated leaf was cut off close to the stem, and taken up to the laboratory where it was laid over night in a petri-dish kept damp by means of a filter paper saturated with water. Free hand sections were made of the end portion of the leaf about 2 cm from the leaf-tip. The sections were killed in lacto-phenol, and stained in Bleu coton G 4 B. Two or three sections were obtained which showed spores lying upon the upper epidermis; the spores, however, showed no sign of germination. Sections were cut daily from this leaf to find out when the spores germinated, and when the penetration of the host took place. Although the leaf was servered from the parent plant, yet until the mycelium of *H. echinulatum* was found within its tissues, it would be useless to look for penetration in those leaves still on the parent plant. Thus this leaf afforded a time-limit for the infection to take place. The time required for the infection of the leaves not removed from the plant might be longer than that for the isolated leaf; but it could not be shorter.

On the 12th February 1912 sections were obtained, which, however, showed no spores, but mycelium which had penetrated the host tissues by passing direct through the cuticle and epidermis into the central tissue of the leaf. The mycelium or infecting hyphae had taken a very nearly straight course as far as the middle portion of the leaf, and was as yet unbranched. No infection by way of the stomata could be observed. The infection in this case took place in from six to seven days. Some of the leaves on the plant itself were now cut off, and examined in the same way. Penetrating hyphae were found, but spores were not seen, and the actual mode of penetration, except in one case was not observed. In this one case a fairly thick section was mounted obliquely, so that a sur-

face view of the epidermis was obtained as well as of the inner tissues of the leaf. This section showed an infection hypha which had penetrated the middle lamella between two adjacent epidermis cells (see fig. 47).

On the 4th March disease spots were seen due to the infections on the 5th February. The spots were greyish in colour with a dark spot in the centre, and extended to both sides of the leaf; their size was from 1—1.5 mm in diametre. In this case four weeks had elapsed between inoculation and the reappearance of the parasite.

Experiment VIII.

On March 8th another *Dianthus*-plant was infected in the same manner as before, the spores being taken from old disease spots in two cases, and from a pure culture in another. Three leaves were inoculated. The solution containing the spores also contained pieces of aerial hyphae, and aphid casts which helped to mark the places of inoculation. These areas were kept moist every day by the addition of a drop of water led by means of a paint brush on to the inoculation areas.

On the 16th March one of the inoculated leaves was cut off, the inoculated area was cut out, and free hand sections were cut. The sections were killed in lacto-phenol, and stained in Bleu-coton G 4 B. Many sections showed the presence of mycelium in the tissues from the epidermis to the middle portions of the leaf. In one or two cases the infecting hyphae had branched into two. In some instances the epidermis had been torn away in cutting, so that in these cases the actual manner of penetration could not be made out. In one or two sections, however, infecting hyphae were observed to pass direct through the epidermis (fig. 48). In one case an infection through a stoma was observed (see fig. 49).

The other two leaves were also removed, and their inoculated areas cut out and killed in weak Flemming solution.

Experiment IX.

On the 25th March 1912 five leaves of a new *Dianthus* plant were inoculated with spores obtained from two PETRI-dish cultures on salep agar. It was ascertained that numerous spores were present. The inoculated areas were indicated with ink marks as in a previous experiment and after inoculation a bell-jar was placed over the plant. On the same day one old lower leaf, and one young leaf were removed from the plant, the cut ends gummed up with glycerine-jelly and taken up to the laboratory where they were placed in a PETRI-dish under a couple of glass slips such as those used for damp chambers[1]). These slips helped to keep the leaves flattened out. Through the circular opening in the glass slips the leaves were inoculated with the same material as above. Previous to inoculation the waxy coating of both leaves had been removed by gently rubbing the upper epidermis with a rag.

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1) See KLEBAHN (1), p. 489.

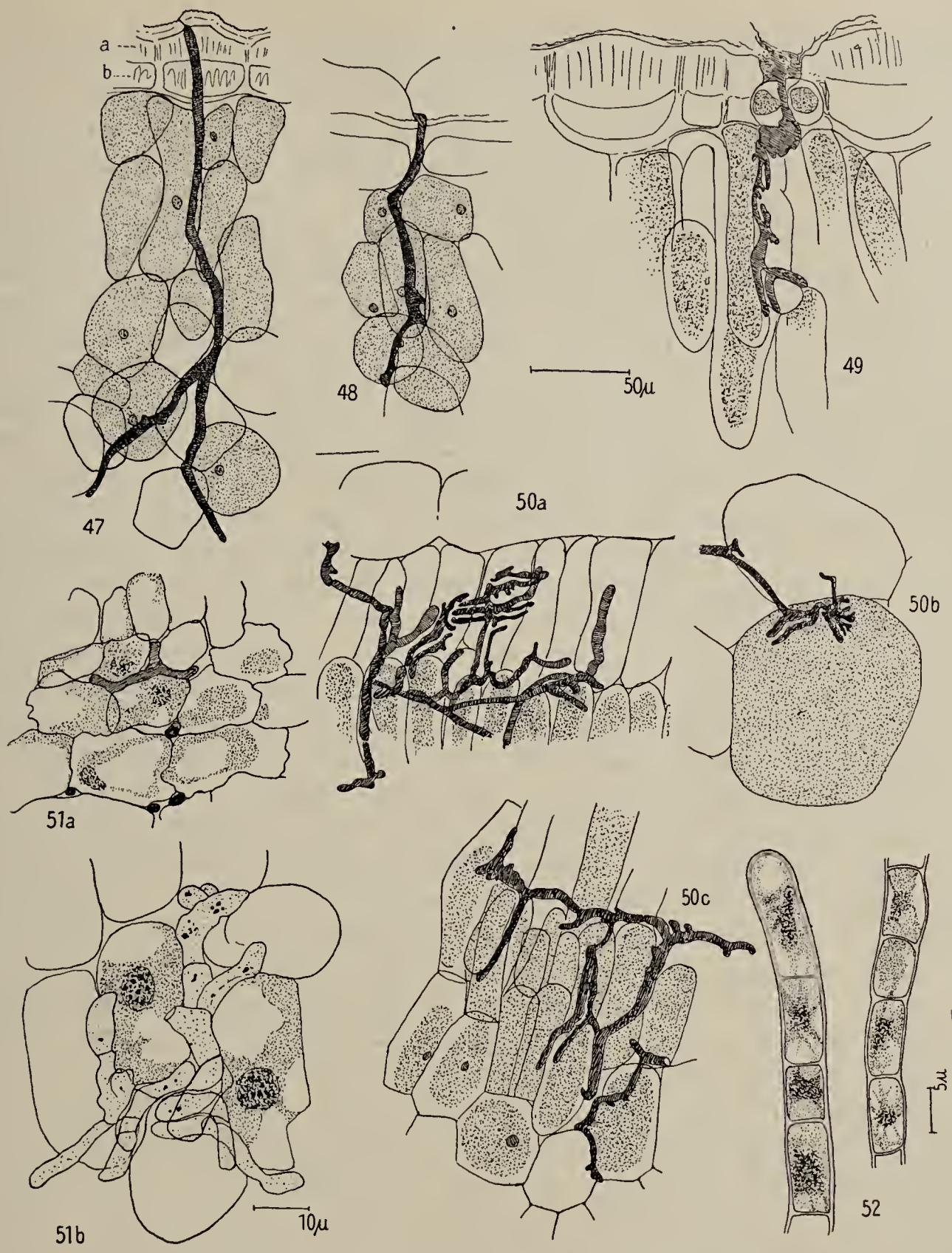
On the 1st April 1912 the two leaves kept in the petri-dish were examined; and on the younger of the two leaves which was thin, small brown spots were visible. On cutting sections and treating in lacto-phenol, and Bleu-coton, these spots proved to be well developed disease areas, conidia-bearing hyphae being found to have emerged from the stomata in some cases. Below each spot mycelium was found to be present in the leaf tissues. Penetration had evidently taken place some days previously. The mode of infection was not made out, but it was thought that in some cases this had probably taken place by way of the stomata. In the other leaf which was considerably thicker only two short infection hyphae were found. The manner of infection was not made out. As both leaves were treated under exactly the same conditions, the difference in the results must lie in the nature of the leaves themselves. The degree of resistance of the younger leaf had evidently sunk faster than that of the older, and thicker one, as in the former the fungus had developed at a rate, and in such a manner as to indicate that it was growing saprophytically rather than parasitically, whereas with the older leaf the behaviour of the fungus seemed to be similar to that under natural conditions.

On the 2nd April all the other inoculated leaves were removed and the inoculated areas cut out and four of these areas after being cut each into two pieces and killed in chromo-acetic acid solution, were imbedded in paraffin of 52° C melting point in the usual way. The fifth piece was at once cut and examined after treatment in lacto-phenol, and Bleu-coton. No germinating spores or infecting mycelium could be found in any of the sections.

From the above recorded infection experiments the following conclusions may be drawn: The mycelium of *H. echinulatum* in the tissues of *Dianthus* is intercellular. Infection takes place from 6 to 7 days after inoculation, either by hyphae which pierce the epidermis probably by way of the middle lamella between two epidermis cells, or through the stomata. Conidia are again produced by the parasite after a period of from 3—4 weeks growth within the tissues of the host-plant.

IV. Examination of diseased tissues of *Dianthus*.

For this purpose original material from the nurseries both fresh and in alcohol, and artifically infected plants were used. The material to be imbedded in paraffin was killed in Juel's fluid, boiling alcohol, acetic alcohol, dilute Flemming solution, and in chrom-acetic acid solution. The last proved the most satisfactory. For free-hand sections, the material was killed in alcohol or in hot lacto-phenol. The alcohol material stained best in the combination Fuchsin-lichtgrün, or gentian violet-orange G. That treated in lacto-phenol was invariably stained in Bleu-coton G 4 B, which was sometimes followed by orange G, but excellent results were obtained with the Bleu-coton alone and the most satisfactory observations were made from this material. This is a plasma stain, and hence is taken up by the cytoplasm of the cells of both the host and the parasite. The hyphae of the parasite are more deeply stained than are the host cells, hence it was possible to wash out the Bleu-coton from the host cells, and leave the hyphae stained; the



Figs. 47—52: *Heterosporium echinulatum.*

Fig. 47: Showing a young infection hypha which has pierced the upper epidermis between two epidermis cells of *Dianthus caryophyllus.* The figure shows the thick outer walls (*a*) of the epidermis cells overlaid by the cuticle and its waxy coating and the striated side walls (*b*). (Free hand section treated in hot lacto-phenol and bleu coton G4B.) — Fig. 48: Similar to fig. 47. — Fig. 49: Showing an infecting hypha penetrating the host tissues by way of a stoma. — Figs. 50*a*, 50*b*, 50*c*; Showing the mycelium of *H. echinulatum* ramifying in the palisade tissue (*a* and *c*), and in the spongy parenchyma (*b*). (Free hand sections treated in hot lacto-phenol and bleu coton G4B.) — Fig. 51*a*. The edge of a disease area, showing the intracellular mycelium among the spongy parenchyma. (Paraffin section stained in DELAFIELD's haematoxylin.) — Figs. 47—51*a*: × 215. — Fig. 51*b*: Taken a little way behind the edge of a disease area. (From a paraffin section stained in DELAFIELD's haematoxylin (× 600).) — Fig. 52: Hyphae of *H. echinulatum* from a hanging drop-culture, stained in HAIDENHAIN's iron alum haematoxylin, showing uninucleate cells (× 1000).

Note! All the drawings except figs. 17—24 were drawn with the help of the large ABBÉ drawing apparatus. Ocul. ZEISS 2, 3, 4. Obj. ZEISS A, C, E, $\frac{1}{12}''$ immersion.

host cells could then be stained with orange G if desired. This was seldom done, however, as an excellent differentiation was given by the deep blue of the mycelial hyphae. and the pale blue of the host cells. With gentian violet and orange G, the hyphae retained the violet while the host cells took up the orange stain. With fuchsin and „lichtgrün" the hyphae were stained red, and the cells of the leaf green. In all cases the nuclei of the host cells retained the first stain of the combination; but it was found impossible to stain the nuclei of the hyphae owing apparently to the fact that firstly the membrane of the hyphae also retained the stain to the same degree as the nuclei, and that secondly the stain washed out from the membrane apparently at the same rate as from the nuclei. In a transverse section through a spot, the hyphae at the edge which were yet young and densely filled with cytoplasm stained deeply, while those in the centre appeared fainter in colour, owing to the small amount of cytoplasm present in the older cells of the hyphae. The microtome sections were cut from 5—10 μ thick and stained in Delafields haematoxylin, Heidenhain, and the combinations, gentian-violet and orange G, and Fuchsin and „lichtgrün". The iron-containing stains proved unsatisfactory in most cases, a great deal of black precipitate being thrown down in the hyphae. In no cases were haustoria made out to be penetrating the host cells; but very often at the edge fo a disease area the hyphae were seen to branch in a curious manner round about the host cells (fig. 50*a*, *b*, *c*). Many parallel hyphae with curious thickenings and swellings were found at these points arising from one main hypha. These parallel-running branches seemed to wrap round the cells and reminded one somewhat of the internal anatomy of a lichen thallus. The resemblance between a lichen and the young edge of the disease spots, and the young infection areas was to be further observed in the relation between the hyphal cells and host cells. In good preparations the host cells in and about an infection area i. e. an area with young infection hyphae, or the edge of a disease spot of any age, were not shrunk or abnormal in any way by the presence of these hyphae, and a symbiotic relationship seemed to exist between host and fungus at these points (fig. 51*a* and *b*). It was only in the centre of fairly old disease areas that the host cells were destroyed. The destruction of the host tissues at these points was nearly complete, the host cells being replaced by a much branched mycelium. The harder parts of the vascular bundles of the host and the epidermis were the only tissues to be recognised in these areas. In places beneath the epidermis of both sides the parasitic hyphae had formed clumps of a pseudoparenchymatous nature; it was from these that the numerous conidiophores, and aerial hyphae were given off through the widened and split stomata to the exterior. The mycelium was throughout intercellular, and multiseptate. The nuclei were made out in one case which was a hanging drop-culture killed in chrom-acetic acid, and stained in Heidenhain (fig. 52) the membranes of some of the young hyphae not being stained so that under the oil immersion the nuclei of the individual cells could be made out. The cells of the hyphae appeared to be uninuclear.

In the above quoted paper by Reed and Cooley on *Heterosporium variabile* the parasitic mycelium is given as intracellular although their microphotograph is by no means convincing.

V. The occurrence of the disease and the winter cultivation of carnations.

The disease makes its appearance in the late autumn during and after periods of damp. If the weather remains dry with little rain, the disease may not appear much before winter; on the other hand if the weather has been at all wet and the atmosphere consequently kept damp for some time, the carnations will be noticeably infected in the early autumn. In fact seasonal and climatic conditions seem to play a great part in the degree and extent of the outbreak. During any period of damp at any time of the year some spots of *H. echinulatum* may be found upon the lower leaves of the carnations, but the disease is always very much more pronounced during the winter months, and is scarcely to be noticed during the summer. During wet summers the disease spots are more frequently met with than during dry ones, and in dry winters the carnations are less affected than in wet winters.

The spots at first appear no bigger than pin-points, and are grey in colour. The tissue both of the spot and of the surrounding portion is not sunk or shrivelled in any way. They are only to be made out upon one side of the leaf; in the case of the infection experiments they only appeared upon the upper surface. After a few days, however, the spots have increased in area, and when thay have attained a diametre of 1—2 mm, they are plainly visible on both sides of the leaf, indicating that the parasitic hyphae have spread from one surface of the leaf to the other. In the middle of each spot is to be seen a scanty aerial mycelium, composed of long wavy hyphae, which are for the most part spirally coiled. This aerial mycelium appears upon both sides of the leaf. Conidiophores begin to appear when the disease spot has attained a diametre of between 2 and 3 mm, and are dark olive green in colour, and dispersed among the central patch of aerial hyphae. The spot increases in size until it extends from one margin to the other. The conidiophores are arranged in fairly regular concentric circles around the first central patch. Very often cases were seen in which two fairly complete circles of conidiophores were produced upon the disease patches, but no spots were seen with conidiophores quite so regularly arranged as would appear to be the case from Rostrup's figure.

Cuttings made in late summer for next year's plants were also sometimes found to be infected, although at the time of cutting, the plants appeared quite free from the disease. During the winter months some of these young cuttings are found to be diseased. The spotted leaves of these are removed whenever they are found.

In any given bed of carnation plants which are always grown nearly touching each other, whether of old plants or of cuttings, a few are always present which are infected with *H. echinulatum*. The parasite may be in the stage of infection, or between that and the production of spores, or may have actually produced spore-bearing disease patches. The material for infection always seems to be present and as many of the lower leaves of the carnations plants can be easily wetted, the transmission of the disease from one leaf to another and from one plant to another by means of wind and rain can be thus accounted for. It was found in the infection experiments that it was possible to lodge a drop

of water containing spores upon the upper surface of the very youngest of leaves, so that in the case of young cuttings rain may account for the mode of inoculation. During the winter months both the young plants for next year and those of the same year's growth are kept under glass During wet and damp weather these plants will be found upon examination to be covered with a number of moulds besides *H. echinulatum* chief of which is *Botrytis* sp.

The longevity of the spores produced under natural conditions was not exactly determined, but they can apparently germinate after some weeks to judge from the fact that material with large spots brought from the nurseries into the laboratory and kept for a fortnight, gave a suspension of spores in water, all of which germinated; some of these spores must have been at least a month or five weeks old. In infection experiment VI, the spores used for inoculation were obtained from a clean-culture 6 weeks old and proved capable of infection. Infection from the ground is perhaps also possible: spores fallen on to the ground from diseased plants, afterwards removed, may be splashed by rain upon the leaves of freshly planted carnations. Infected leaves collected in the autumn and winter months were kept in the open during the winter; and some of these placed in guaze bags were hung from a horizontal pole, so that they swung a little way off the ground. Others were placed in pots with a wire netting above them to prevent them from being blown away. These wintered leaves were examined in the middle of April, but no trace of spores of *H. echinulatum* was found. Perithecia-like bodies were present which upon examination proved to be filled with an oil-like substance, and might have been perithecia or pycnidia in a young stage. Upon soaking in water over night, the old disease spots could be recognized as black decayed patches. Some of these were cut out and placed in petri-dishes on plum-juice agar; no *H. echinulatum* ever made its appearance in these cultures; on the other hand saprophytic moulds were plentiful.

The material bearing the Perithecia-like bodies was not further observed and the question as to whether they might possibly be the ascus stage of *H. echinulatum* was not entered into.

VI. Conclusion.

1. A new species of *Heterosporium* has been found upon the lower leaves of *Beta vulgaris* and has on this account been given the name of *H. Betae.*

The infection experiments showed that the forms described as *Heterosporium Betae* and *Hormodendron*-sp. are saprophytes or perhaps very weak parasites, wounded and dying tissue only being invaded by the mycelium of the *Heterosporium.* The parasitic nature of *Heterosporium echinulatum* was again conformed.

2. The mycelium of *H. echinulatum* in the tissues of *Dianthus* is intercellular and without penetrating haustoria. Infection takes place in from 6—7 days after inoculation, either by hyphae piercing the epidermis, probably the middle lamella between two epidermis cells, or by way of the stomata. Conidia are again produced by the parasite after a period of from 3—4 weeks growth within the *Dianthus* tissues. The hyphae

of *H. echinulatum* are multiseptate and uni-nuclear. The carnation disease is spread by the conidia of *H. echinulatum* transported by means of wind and rain from one leaf to another, or to another plant, or fallen spores upon the ground may be transported in like manner to new plants. Perithecia-like bodies were found upon overwintered material of *Dianthus*. Owing to their being still in the early stages of development it was impossible to decide whether they were perithecia or pycnidia. The question whether these bodies had any connection, whatever with *H. echinu'atum* was not entered into.

3. Growths differing both in colour and in form were produced by one and the same fungus mycelium upon different nutrient media. These differences were noticeable in petri-dish and hanging-drop cultures, and existed between the aerial mycelia, the surface mycelia, and the sunken mycelia.

4. The conidia of *H. Betae* and *H. echinulatum* are produced acropetalously by a budding process, firstly from the heads of the conidiophores, and secondly from the first formed conidia. In both the bent and knoted appearance of the conidiophores is due to the prolongations which began from the first head, each head being capable of producing one prolongation placed at an angle to the previous one. Chains of spores containing three spores in a chain are common in *H. Betae*, and usually three such chains are produced upon one head. In the young conidiophores of *H. echinulatum* a few chains containing two spores are sometimes present, but these are seldom seen in the mature conidiophore.

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Referate.

Guilliermond, Les Levûres. (Paris, O. Doin, 1912.)

Cet ouvrage résume l'état actuel de nos connaissances sur les levûres, au points de vue morphologique, cytologique, biologique, systématique, phylogénique.

Il est divisé en deux parties, la première générale, la seconde spéciale. Dans la première partie l'auteur étudie particulièrement le développement, la sexualité des levûres et leur cytologie, questions que ses travaux out particulièrement contribué à éclairer.

La physiologie des levûres a été traitée avec la collaboration de M. A. Policard; elle comporte l'étude de la composition chimique des levûres, de leurs diastases et toxines, de leur alimentation, de leur respiration, et de la fermentation alcoolique, dont les diverses théories sout clairement exposées. Un chapitre est consacré à l'étude des relations des levûres avec le milieu extérieur.

L'action des agents physiques extérieurs, le parasitisme et les propriétés pathogènes sont particulièrement bien exposés. Un autre chapitre traite de l'origine des levûres et de leurs affinités. Cette question, qui doit sa solution aux recherches de l'auteur, est exposée d'une façon claire et complète. Deux chapitres sont consacrés à la description des méthodes employées pour la culture, l'isolement, la détermination des levûres, un autre à l'étude de la variation chez ces champignous, un autre encore à celle de leur classification.

Dans la 2e partie l'auteur étudie en particulier les diverses Levûres, puis les levûres douteuses (*Torula*, *Mycoderma*, etc.) et enfin les Levûres pathogènes et quelques champignons voisins des levûres, tels que les *Endomyces* et les *Pseudomonilia*.

Un copieux index bibliographique et de bonnes tables terminent cet ouvrage, qui est appelé à rendre les plus grands services, en mettant à la portée de tous des données éparses et souvent peu accessibles aux botanistes. R. Maire (Alger).

Marchand, H., Sur la conjugaison des ascospores chez quelques levûres. (Compt. Rend. Soc. Biol., Paris 1912, **71**, 410—412.)

L'auteur a retrouvé chez *Saccharomyces ellipsoideus*, *S. validus*, *S. intermedius*, *S. turbidans*, la copulation des ascospores décrite antérieurement