Botany. — *The lichenisation of aerophilic algae.* By A. QUISPEL. (From the Botanical Institute, University of Leyden and the Laboratory of microbiology, Delft Technical Institute). (Communicated by Prof. L. G. M. BAAS BECKING.)

(Communicated at the meeting of February 28, 1942.)

Though the dual nature of lichens is generally recognized since the days of SCHWENDENER, there does not exist an equally great unanimity as to the way in which we have to look upon the mutual relations between the two components. The reason for this is, in the first place, the lack of physiological experiments. Physiology was a long time one of the most neglected chapters of lichenology and only in recent times a greater interest seems to have been taken in the physiological problems of lichens. For a better understanding of the symbiosis, however, it does not suffice to experiment with the lichen as a whole, but we have to work with the two isolated components: alga and fungus. Several authors have investigated the algae in pure culture, yet there are still many problems to solve. As to the fungal part, however, the experiments are very scanty. Though it appeared to be possible to cultivate this symbiont, as shown by the work of MOELLER (1887), WERNER (1927) and THOMAS (1939), the growth-velocity in vitro is so small that physiological work needs remained limited to preliminary or simple experiments. Moreover, as the fungi grow badly in liquids, quantitative work is impeded.

We believe to have found in the lichenized algal covers of Pleurococcus, Apatococcus and allied species, a better object of study in this respect. The observation that these algal covers are always mixed with symbiontic hyphae is due to SCHMID (1933); his endeavours remained limited to slide-cultures. During the running of our experiments the comprehensive work of THOMAS (1939) on the biology of lichen-components appeared, in which he describes an attempted isolation of these fungi as well. Though he does not say very much upon this subject, he mentions that growth was better than in the known lichen-fungi and that he had been able to isolate eight different species.

We isolated the hyphae from these algal-covers by the aid of hanging-drop cultures of a suspension in sterile tap-water, in which the hyphae developed, after which the drop was transferred to a tube with malt-agar, or by isolating a small group of algal cells with adhering hyphae with a small glas-capillary under the microscope. In this way several fungi were isolated, which resembled each other in many respects, but differed in details. The lack of typical fructifications did not enable us to make any definite classification. As an illustration the description of one of them is given below:

> Solid, compact, very hard thallus, with a dark greyish-black colour in the hyphae, which colour diffuses into the surrounding agar. Thallus elevated and lobated, on some media for the main part in the agar, with a height equal to its length. Thallus consisting of thin hyphae penetrating into the substrate, a central layer (consisting of interlaced hyphae with thick-walled, more or less rounded cells), an outer layer, in which the cells are long-drawn (10—15 μ long, 4,5—6 μ thick), with thick walls and delicate terminal branches rising into the air. Finally we want to mention the occurrence of large, globate cells and of oil-drops in old cells.

The other fungi differ from the described one in the intensity and nature of the pigment production, the cell-form in the central layer, the dimensions of the cells, the length of the aerial hyphae, etc. In one of the fungi these aerial hyphae consist of small ovoid cells, which loosen easily on suitable media, thus functioning as conidia.

Two points are of a special interest:

1. the characteristics described above are very similar to those described for certain true lichen fungi in literature. Here too we find mentioned the compact hard colonies, the elevated mode of growth, the darkening of the surrounding agar, the stratified thallus, the thick-walled cells, the occurrence of big globular cells (sometimes misinterpreted as algae), the oil production in the protoplasm-rich cells, the conidia production of the aerial hyphae, while many of the pictures given resemble my fungi in every respect. Moreover I isolated a fungus from a heavy lichenized, soredial cover of Cystococcus spec. as they are often to be found in the vicinity of towns. This fungus, which certainly is a lichen fungus (though soredial covers like these cannot be classified), could hardly be distinguished from some of the Pleurococcus and Apatococcus symbionts. The only lichen fungus which I isolated from a lichen (Xanthoria parietina (L) Th. Fr.) does not seem to be identical with one of them, although again there are many points of similarity. Though I cannot establish whether my fungi are identical with certain true lichen fungi, a close relationship with at least some of them seems to be beyond any doubt.

2. Yet the growth velocity, even of the fungus from the soredial Cystococcus cover, is much better. After inoculating a malt-agar plate with a suspension of hyphae, we obtain after two weeks already colonies with a diameter of 4—8 mm. Moreover they grow excellently in liquid culture solutions. In suitable liquid media they form (in ERLENMEYER flasks of 300 cc, provided with 100 cc liquid) mycelia with a dry weight of 1—1,5 gr. after a two month incubation. Most probably our fungi are to be regarded as relatives (or perhaps even strains) of true lichen fungi, which are less adapted to the symbiosis and in consequence growing better in pure culture. An investigation after the physiological properties seemed to be of a great importance for the problem of the lichen symbiosis, though of course we have to be very cautious to apply the results obtained to true lichens, as here conditions will be more complicated than in our more primitive (or reduced?) alga-fungus symbiosis.

Though the investigation after these properties still is in full progress we want to mention here the most important facts, which have come to light.

Whilst the fungi are developing well in media like maltextract, the development on synthetic media is very scanty. The application of some accessory substances in the form of yeast extract seemed to be necessary. Usually this beneficial effect appeared to be caused by aneurin.

TABLE I.

Dry weight of the mycelia of the fungi Pl I, isolated from a Pleurococcus cover and C, isolated from a soredial Cystococcus cover, cultivated on CZAPEK–DOX solution (25 cc in ERLENMEYER flasks of 50 cc with different concentrations of aneurin (MERCK).

Aneurin concentration	0	5	10	15	γ p. 25 cc
Dry weight Pl I	20	310	145	100	mg
Dry weight C	38	90	105	100	mg

The growth requirements of some other fungi appear to be more complicated, as here the beneficial effect of aneurin does not seem as pronounced as that of yeast extract.

It seemed indicated to investigate whether the algal partner could provide this vitamin in nature. A strain of Apatococcus minor Edl. from the collection of the laboratory for microbiology at Delft and three cultures of lichen gonidia (from Xanthoria parietina (L) TH. FR. Physcia pulverulenta (HOFFM.) NYL. and Parmelia acetabulum (NECK) DUB., which were isolated according to the method of JAAG (1929) and cultivated on BEIJERINCK agar with 2 % glucose, appeared to develop well on aneurin-free media, so that the supposition seemed justified that these algae were able to synthesize this vitamin. The convincing proof was obtained by the following experiment:

ERLENMEYER flasks of 50 cc provided with 25 cc CZAPEK–DOX solution without aneurin were inoculated with the algae mentioned above and cultivated in the light. After a month small green globules were clearly visible all over the bottom of the flasks. Then they were inoculated with the same amount of a suspension of the fungus Pl. I, together with two sterile CZAPEK–DOX media, one with and one without aneurin as a control.

TABLE II.

Control	Apat. minor	Xanthor. par.	Parm. acet.	10 γ % aneurin
25	85	90	330	440 mg dry weight

These figures leave no doubt that the relatively small amount of algae present in the solution already had a marked influence. So it is evident, that the algal symbiont provides its fungus partner with the required aneurin. This is the first observation which makes it probable that in the lichen symbiosis, like in so many cases of symbiosis, the exchange of accessory growth substances plays an important role.

As a source of carbon the fungi can make use of different sugars, starch and poly-alcohols. Among the last mentioned the fitness of erythritol, one of the most prominent reserve-substances of the proto-pleurococcoid algae and of certain lichen-gonidia is of a special interest. As a matter of course there are specific differences between the fungi in this respect.

Organic and inorganic compounds may be used as nitrogen sources. On nitrogen-free media the fungi show no development, even after the addition of traces of sodium molybdate. In media with small quantities of yeast extract as only source of nitrogen the development, however, was so abundant that it warranted the determination of the nitrogen content as compared with uninoculated control media by an ordinary KJELDAHL method.

TABLE III.

Dry weight and nitrogen content of the fungi Pl 1, Pl 2 and C after cultivation in ERLENMEYER flasks of 300 cc provided with 100 cc nitrogen-free CZAPEK–DOX solution with 1 cc yeast extract after two months incubation.

Fungus	Pl I	Pl 1	Pl 2	C
Dry weight	860	1240	860	510 mg
Nitrogen content (mycelium + medium)	7.39	12.25	12.42	11.95 „
Nitrogen content uninoculated medium	8.05	12.59	12.83	11.99 „

So any assimilation of atmospheric nitrogen is altogether out of the question. On the contrary, we observe in all cases a disappearance of nitrogen as compared to the nitrogen in the uninoculated control, which may be easily explained by the evaporation of some volatile nitrogen compounds during the two months of cultivation. The fungi appear to develop well in solutions relatively poor in nitrogen.

An extensive search was made after the metabolic products of these fungi in relation to an eventual production of lichenic acids. These remarkable, taxonomically important products, which are produced by most lichens in often very considerable amounts were formerly regarded as specific products of the symbiosis. The most convincing arguments for this assumption were that they had been never found in any other organism and especially the old experiment of TOBLER (1909), in which this investigator showed that the lichenic acid parietin (physcion) was only produced by the fungus in pure culture after synthesis with his algal partner.

More recently, however, RAISTRICK c.s. isolated from some very common moulds like Aspergillus and Penicillium a great number of remarkable metabolic products, which in many cases bore a striking resemblance to certain lichenic acids, while finally he could identify one of the metabolic products of Aspergillus glaucus Link. with the lichenic acid parietin. Whilst these observations made it very probable that many of the lichenic acids were the result of the metabolism of the fungus alone, the decisive proof was only very recently given by THOMAS (1939), who was able to demonstrate the existence of parietin in pure cultures of the fungus components of Caloplaca and Xanthoria species and stictaurin in the fungus partner of Candelariella vitellina (EHRH.) MULL. ARG. Whether all lichenic acids are the product of the fungus alone still remains doubtful, in consequence of the chemical diversity of these compounds (see compilation of ASAHINA 1939).

It seemed interesting to investigate whether we could find among the metabolic products of our fungi, which are to be regarded as relatives of the lichen fungi and from which we can cultivate in a relatively short time such great quantities, lichenic acids or allied substances. To this purpose they were cultivated on the most divergent ways: in media with varying carbon compounds, with varying nitrogen sources, by varying the percentage of the salts (especially N and P), by cultivation in oxygen rich air, cultivation at varying temperatures, in the light, in solutions, agar or on plaster of Paris, soaked in culture solution, etc. After two months cultivation they were examined as follows: the culture solution was tested with $FeCl_3$ upon phenolic compounds, so was the alcoholic or acetonic extract of the fungus mycelium, moreover this extract was evaporated on a watch-glass to detect crystalline substances and to the same purpose a small piece of mycelium was sublimated in a KLEIN–WERNER micro-vacuum sublimation apparatus.

In none of these ways we could detect in any culture the presence of a lichenic acid or similar substances. This was the most astonishing for the fungus C, which had been isolated from the heavy lichenized, soredial algal cover. By extracting this cover with acetone, evaporating the extract on a watch-glass and after washing away fats and chlorophyll with benzene I obtained a crystalline product, which could be purified by crystallizing from alcohol or ether. This substance shows the following characteristic colour-reactions: the so called homo-fluorescein reaction, characteristic for orcin derivatives, to wit the red colour with NaOH and chloroform and the green fluorescence after pouring this solution into water, furthermore a red colouration with $FeCl_3$, a yellow colour with KOH, a brownish-red colour with concentrated H_2SO_4, a yellowish-brown colour with paraphenylenediamin, a yellow colour with benzidin and with anilin. The substance becomes brown at 240° C. and carbonizes at 260° C. It crystallizes as short, white needles. Most probable it is identical with salazinic acid or an allied lichenic acid. Yet we were not able to detect this substance, which is easily recognizable by its marked colour-reactions, in any of the cultures of the fungus symbiont isolated from this cover.

Moreover it appeared that some proto-pleurococcoid algal covers too (namely those consisting of Apatococcus minor EDL.), though lichenized in a relatively small degree, were in the possession of a remarkable metabolic product, which may be isolated as follows:

the algal cover, scraped from an old stone wall is extracted in an extraction funnel with acetone; the acetone is destilled off and the remaining product vigorously shaken with benzene and filtered through a glass filter. In the filter remains the rough product, which may be purified easily by crystallization from alcohol or from ether. It is a beautiful white substance, consisting of very long, threadlike crystals, melting at 139° C., soluble in most organic solvents, best in chloroform and pyridin, less in hot alcohol, acetone or ether, slightly in these solvents when cold. No colour-reactions could be detected. It is without any doubt identical with the "acide phycique", discovered by LAMY in 1857, which substance was chemically investigated in the laboratory of Prof. Dr. G. VAN ITERSON by J. G. VAN DE SANDE (1927 unpublished). We have tried to

establish the chemical constitution of this compound, making use of VAN DE SANDE's data for comparison.

The empirical formula of the substance followed from elementary analysis performed by Mr. P. J. HUBERS, laboratory for Organic Chemistry, University of Amsterdam, which showed the following values in two determinations:

C 69.22 %	H 11.33 %	N 3.64 %
C 69.78 %	H 11.37 %	N 3.68 %

From this we calculate the tentative formula as $C_{23}H_{45}O_4N$. This formula should yield C 69.13 % H 11.35 % N 3.51 %. Two molecular-weight determinations (melting-point depression in Camphor according to RAST) yielded 432 and 396 (calculated for $C_{23}H_{45}O_4N$: 399.59). VAN DE SANDE calculated from his determinations elementary formulae with yet more C and H atoms.

The substance which we shall provisionally name "apatococcin" shows, in alcoholic solution, a neutral reaction and cannot be shaken out of its chloroform-solution either by bases or by acids. Boiling with a dilute alcoholic solution of NaOH, or the action of cold concentrated NaOH, saponifies the substance. A foamy solution ensues, which, after acidification, yields a crystalline precipitate, consisting of very long, threadlike needles, m.p. 160° C. The elementary analysis of this product by Mr. P. J. HUBERS showed:

	C 68.28 %	H 11.26 %	N 3.94 %.
Calculated for $C_{21}H_{41}NO_4$:	C 67.89 %	H 11.13 %	N 3.77 %
$C_{22}H_{43}NO_4$:	C 68.53 %	H 11.24 %	N 3.63 %

Probably, therefore, in the original apatococcin an acid group was esterified, either with CH_3 or C_2H_5. A methoxyl and ethoxyl determination in this product by Mr. HUBERS yielded 9.50 % OC_2H_5 or 6.55 % OCH_3 (calculated for one group OC_2H_5 11.28 % and for one group OCH_3 7.77 %). Therefore, the presence of one group $COOCH_3$- (or $COOC_2H_5$) in apatococcin appears probable.

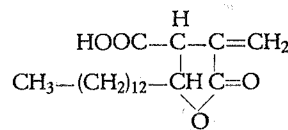
The substance seems saturated, in solutions neither permanganate nor bromine are decolorized (already stated by VAN DE SANDE). As to the position of the nitrogen it may be stated that the substance has neither alcaline nor alcaloid character as it does not form salts with dilute or strong acids and does not give any alcaloid-reactions in solutions. The nitrogen cannot be removed by saponification and action of HNO_2 does not seem to change the substance. It is, therefore, neither a simple acid-amid nor a primary or secundary amin (which is in good accordance with the data of VAN DE SANDE).

By action of phenylhydrazin the substance remains unchanged so that most probably it does contain neither an aldehyde nor a ketone group.

According to VAN DE SANDE the substance can be acetylated by boiling with acetic anhydride and a trace of sodium acetate for some days. In repeating this experiment, however, most of my substance deteriorated.

From the elementary formula it is probable that apatococcin possesses a long paraffin chain, which also would account for the foamy character of the sodium salt.

Though the investigation will be continued we expect that the substance may show a relationship to certain lichenic acids such as protolichesterinic acid (ASAHINA 1939), which acid, however, does not contain any nitrogen.



protolichesterinic acid.

In an apatococcus cover the substance is easily recognizable by the very characteristic curved threadlike crystals obtained on sublimation (fig. 1).

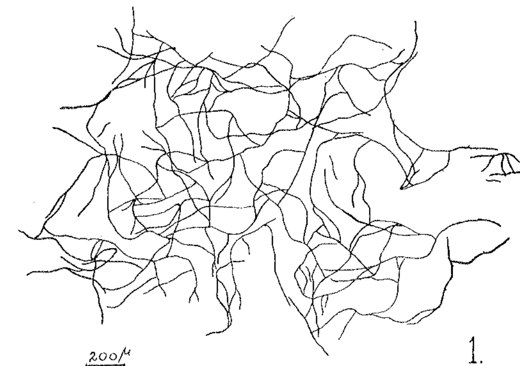


Fig. 1. Camera-lucida drawing of a sublimate from an apatococcus cover.

As, moreover, this substance could not be detected in any of the fungi, a number of pure cultures of the alga Apatococcus minor Edl. were sublimated in a KLEIN–WERNER apparatus. Initially the results were negative, but finally the crystals were clearly visible in the sublimate from some old cultures, which had partially died off from the extreme heat during the summer months (fig. 2). In a micromelting-point apparatus the melting-

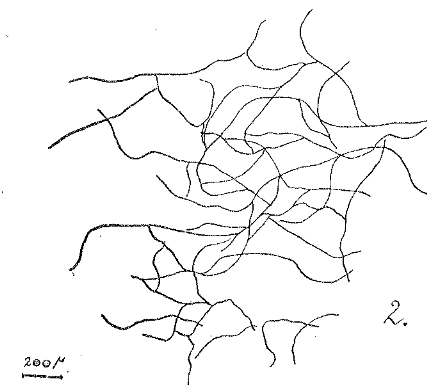


Fig. 2. Camera-lucida drawing of a sublimate from a dead pure culture of Apatococcus minor Edl. (cultivated on BEIJERINCK agar with 2 % glucose). Both sublimates were carefully washed with water.

point of some of these crystals could be determined as = 129° C., which agrees reasonably well with the melting-point of "apatococcin" (139° C.), when we take into account the impurity of a sublimate like this. The double refraction of the crystals is too weak to be useful as a characteristic.

So it is very probable that the "apatococcin" is a metabolic product, which is made by the alga Apatococcus minor Edl. without the help of its fungal symbionts, a result which was to be expected, when we take into account the domination of the alga Apatococcus over the fungus in the covers and the specificity of apatococcin to Apatococcus covers, though these covers are in the possession of different, non specific fungi. Though it does not appear to be the alga which functions as a gonidium in most lichens, the results mentioned point strongly in the direction that, in considering the lichenic acid

problem, we have to pay more attention to the algal part of the lichen than we were apt to do.

It was already a well-known fact that many lichenic acids consist of a lichenic acid s.s. (e.g. lecanoric acid) esterified with erythritol, which was already known as the metabolic product of the alga. Here we have an indication that some other substances as well may have an algal origin.

In some preliminary experiments we added apatococcin to cultures of some of our fungi on media poor in nutritive substances. No reaction was observed, the fungus being apparently unable to use this substance in its metabolism.

Conclusion.

The fungal symbionts in lichenized algal covers can be cultivated with more succes than true lichen fungi. Their great similarity to the latter makes it probable that they are related to certain true lichen fungi and that this alga-fungus symbiosis is comparable to the lichen symbiosis. In consequence they form an excellent object for the study of this symbiosis. The fungi are unable to fix atmospheric nitrogen. They cannot develop without aneurin, which they obtain, in nature, from their algal partner. In none of the cultures on various media, the presence of lichenic acids or similar products could be detected. On the contrary, it appeared that the alga Apatococcus is the producer of a remarkable metabolic product, called apatococcin, with the tentative formula $C_{23}H_{45}O_4N$. Some chemical properties of this substance are described. A relationship with certain lichenic acids is suggested. The investigation is continued.

I want to thank Prof. Dr. G. VAN ITERSON for his valuable help and for allowing me to make use of the unpublished work of VAN DE SANDE and Prof. Dr. F. KÖGL who kindly gave some critical remarks as to my work on the chemical constitution of apatococcin.

LITERATURE.

ASAHINA, Y., Fortschr. Chem. Org. Naturstoffe **2**, 27 (1939).
JAAG, O., Thèse Geneve. (1929).
MÖLLER, A., Thesis Münster i. W. (1887).
RAISTRICK, H., Ann. Rev. Biochem. **10**, 571 (1941).
SCHMID, G., Flora **128**, 211 (1933).
THOMAS, E. A., Beitr. Krypt. Flora. Schweiz. **9**, H. 1 (1939).
TOBLER, F., Ber. D. Bot. Ges. **27**, 421 (1909).
WERNER, R. G., Thèse Paris Mulhouse. (1927).

Botany. — *On the influence of Colchicin upon the anthers of Carthamus tinctorius L.*

By Miss J. M. KRIJTHE (from the Laboratory of Genetics, Agricultural Institute, Wageningen). (Communicated by Prof. L. G. M. BAAS BECKING.)

(Communicated at the meeting of February 28, 1942.)

Although the literature on the influence of colchicin on living matter is voluminous, only a few papers deal with the effects of this substance upon flowers and inflorescences. Some of these papers only mention morphological characteristics such as pollen- or stomatal size, from which measurements often deductions are drawn as to tetra- or polyploidy of the material, often without cytological control.

Adequate cytological research has been published by LEVAN (1939), WALKER (1938), DERMEN (1938) and SATÔ (1939) — all on monocotyledons. The above authors followed — with minor variations — the following procedure; the entire inflorescence was treated for 5—6 days with a colchicin-solution of 0.1—1 %. Attention was almost exclusively directed towards changes in nuclear division, to wit: the absence of the spindle and chromosome-pairing (the chromosomes, however, dividing), with the subsequent absence of cell-division, by which absence abnormal large cells appear. These cells either show a large, tetraploid nucleus or several small nuclei.

This may be demonstrated not only with pollen grains, but also with unicellular staminal hairs. SATÔ mentions the appearance of irregular and incomplete cell-walls, without detailed description of their nature.

Material.

The present paper deals chiefly with phenomena observed in the inflorescences of the safflower (Carthamus tinctorius L.).

The safflower, a composite belonging to the Cynareae, appeared to be a favourable object because of its short vegetation-period (3—4 months), its profuse flowering (30 inflorescences per plant) and the relatively small number of chromosomes (haploid 12).

Method.

It was originally attempted to obtain tetraploid plants by the treatment of seeds and young seedlings with colchicin. As this proved to be unsuccessful (only two pairs of leaves developing subsequent to the treatment showing effects), young inflorescences (3—5 mm cross-section) were used.

The involucre was pushed aside by means of pincers, after which the cavity above the individual flowerets was filled with a colchicin-agar (0.4—0.8 % colchicin), or an aqueous solution of colchicin (10 drops aqueous 0.2 % solution) was applied for three consecutive days. Controls received 1 % agar, or water.

The involucre closed after treatment. The controls showed normal growth. The effects described seem, therefore, due to the colchicin applied.

The treated inflorescences were enclosed for three days in parchment bags, to prevent dessication. The fixation of the flowerets took place either in NAVASHIN's or CARNOY's fluid, between 7.30 and 11.30 a.m. The sections were cut to a thickness of 10 μ and stained with HEIDENHAIN-haematoxylin or with gentian-violet.

Results.

1. *Morphological changes.*

Already after one week a broadening of the entire inflorescence could be observed.