STUDIES IN THE PHYSIOLOGY OF THE FUNGI[1]

I. Nitrogen Fixation

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Introduction and Critical Review of Literature

The problem of the fixation of free (atmospheric) or molecular nitrogen by the fungi has received attention at the hands of no small number of investigators, yet a careful study of the literature is sufficient to indicate that much further work —with the strictest regards for accurate methods—will be required before the problem is satisfactorily solved. For reasons developed later in this paper, we have felt the desirability of continuing, under different conditions, the investigations begun by one of us some years ago.

At the present time there can be no doubt entertained, of course, as to the capacity of the legume tubercle bacteria (*Bacillus radicicola* vars.) and certain soil forms (notably *Azotobacter spp.* and *Clostridium Pasteurianum*) to fix nitrogen. Here the amounts of nitrogen-increase in relatively small cultures under favorable conditions are so far above any regular experimental errors, and so consistently reported by careful workers, that the simple question of whether or not there is fixation is eliminated. On the other hand, there is much contradictory evidence as to the fact of nitrogen fixation by other bacteria and by the fungi, especially by the moulds and

[1] Note.—About half a dozen investigations are already in progress dealing with the physiology of the fungi, and it is proposed to give considerable attention to this phase of physiology during the next few years. The investigations would include certain aspects of nutrition and enzyme action, growth relations—especially the effects of environmental factors—and various phases of the general phenomenon of parasitism. On account of the continuity or relationship of many of the problems, it has seemed well to group these topics under the general title "Studies in the Physiology of the Fungi," of which the present article is No. 1. —B. M. Duggar.

other saprophytic species. Even those who report fixation for the last-mentioned fungi base their conclusions, in the majority of cases, upon amounts which are only questionably beyond the possibility of experimental error. The literature has been frequently reviewed, but for purposes of discussion in this and in forthcoming papers, it has been found well to give this detailed consideration. No account of N-fixation by bacteria is included.

The experiments of Jodin ('62) are now interesting merely in an historical way. He observed fungi, in an impure culture, to grow upon media, "très sensiblement exemptes de composés azotés organique ou mineraux." Employing the methods of gas analysis, he found that in a sealed vessel the amount of molecular nitrogen used was from 6 to 7 per cent of the oxygen consumed.

Hallier ('67) simply reported that he had often observed yeasts growing in a nitrogen-free medium, but he realized the necessity of quantitative data in order definitely to determine the fact of nitrogen fixation. From observations on the growth of mould fungi in nitrogen-containing and nitrogen-free media Nägeli ('80) concluded that mould fungi are unable to assimilate free nitrogen.

Frank ('92) states that he grew certain forms of *Penicillium* upon nutrient media lacking nitrogen and then tested the media for the presence of nitrogen, securing a positive indication. No quantitative work appears to have been done, nor are the quantities of medium employed in either case mentioned. Later he ('93) reported growing *Penicillium cladosporioides*[1] (=*Hormodendron cladosporioides*) during ten months in nitrogen-free media containing sugar. A culture on 65 cc. of solution is reported to have given a fixation of 3.5 mg. nitrogen. Insufficient details are furnished regarding methods employed and the use of controls.

Using *Aspergillus niger* and *Alternaria tenuis* as the basis of a test, supplementary to a more complete study of bacteria, Berthelot ('93) reported a fixation of 5.8 to 10.0 mg. in the

[1] In the discussion of literature the writers have written the names of the various organisms just as they are given in the original articles.

first-mentioned fungus, and 4.6 to 11.1 mg. in the last-named species. This appears to be the total amount fixed after growing for a period of months in 600 cc. of Cohn's solution with various sources of carbon. For 100 cc. the nitrogen quantities would therefore be .97–1.67 and .77–1.85 mg., respectively. Fixation by *Gymnoascus* is also mentioned. With respect to analytical methods the exact procedure is not given, and one might, perhaps, without being too critical, wish to have had assurances regarding the purity of cultures, the nature of the vessels ("ballons") used, and how the sample for analysis was taken, especially in view of the following remark made in regard to the amount of fixation in one of the series with bacteria: "Ils auraient été sans doute plus accusés si la dessiccation des matériaux n'avait pas fini par amener la mort des bactéries."

Puriewitsch ('95) used *Aspergillus niger* and *Penicillium glaucum* in a nutrient salt solution containing also 3 per cent tartaric acid, variable amounts of cane sugar, also small amounts of ammonium nitrate. He obtained a mean nitrogen fixation of 4.51 mg. for *Aspergillus,* and 3.26 mg. for *Penicillium.* It is not stated whether these amounts are calculated on the basis of 100 cc. of solution, or whether they were for 25–50 cc., the quantities which appear to have been used in the different cultures. At the same time he reported that the amount of fixation increased with the concentration of sugar, but did not increase in direct proportion to the increase in weight of the mycelium. It is not clear, though quite possible, that these results were obtained with pure cultures. Moreover, since in some of the cultures, at least, dry weight determinations of the fungous felt were made, the conclusion is unavoidable that herein might be a possibility of error. Likewise, the division of some of the experiments into "(a)" and "(b)" suggests that the whole of the solution was not employed in the analysis. The results were subsequently criticized by Czapek ('01) and Heinze ('06) on other grounds.

The fact that fungi may make an appreciable growth on media containing a very low minimum of nitrogen, without nitrogen gain, has been pointed out by some investigators,

and may adequately explain Fermi's ('96) statement to the effect that he was able to grow certain moulds and yeasts on nitrogen-free media without nitrogen fixation.

Brefeld ('00) determined that cereals and grasses infected with species of *Ustilago* were unable to assimilate free nitrogen, but this type of negative evidence is valueless in the present discussion.

More extensive than any of the earlier work is that reported by Saida ('01) who investigated seven species, three of which (*Phoma Betae, Mucor stolonifer,* and *Aspergillus niger*) give nitrogen fixation both with and without the presence of combined nitrogen in the culture medium, one species (*Endococcus purpurascens*) requires the presence of combined nitrogen, and three species give negative results. In most cases the fungi were grown on 50 cc. of nutrient salt solutions containing dextrose or cane sugar, the source of nitrogen being a small quantity of $(NH_4)_2SO_4$ or of $(NH_4)_2CO_3$. In none of the four species except *Phoma Betae* is the amount of fixation more than about 2 mg. (.8871–2.0699 mg.). Fixation (varying from 1.1828 to 10.536 mg.) in *P. Betae* rises somewhat in relation to sugar content of the medium, although maximum fixation occurs in sugar beet decoction plus sugar. The exact method of handling the cultures is not described, but with the exception of possibilities mentioned later, the work seems to be above criticism.

Czapek ('01) states that *Aspergillus niger* does not fix free nitrogen. Later, as a result of numerous experiments on nitrogen-containing media Czapek ('02) again reports no fixation for this species. He declares that the work of Puriewitsch and Saida requires confirmation.

Studying the effects of a yeast and a mould on the nitrogen fixation of *Azotobacter,* Gerlach and Vogel ('03) conclude from analyses of the control cultures, in which each of these organisms was grown alone, that neither of the former fungi are capable of utilizing atmospheric nitrogen.

Koch ('03) was unable to demonstrate any nitrogen fixation for *Aspergillus niger* in a few preliminary experiments. He draws attention, however, to an experiment made by

Hiltner from which it would appear that *Lolium temulentum* (inhabited by an associated fungus) thrives equally well in quartz sand with or without nitrogen as fertilizer, while the fungus-free *Lolium italicum* develops much better when the quartz sand is fertilized with nitrogen than when the substratum is without such fertilization.

In the first experiments reported by Ternetz ('04) with the fungus isolated from the roots of certain *Ericaceae,* and later designated *Phoma radicis* vars., very slight nitrogen fixation was found. In 100–150 cc. of nutrient solutions containing dextrose .6–3.85 mg. represent the range of fixation.

Stimulated by the work of Saida and others, Heinze ('06) reports a detailed repetition of the work of Saida ('01) and Puriewitsch ('95), employing to a considerable extent the same organisms and the same solutions. The work seems to have been unusually extensive, but since it was in every case negative, no details are published. Heinze was apparently inclined in 1903 to consider the possibility that yeasts in certain stages may fix nitrogen, since he states (see review of Schulze's work by Heinze, Centralbl. f. Bakt. II. **10**: p. 675): "Schliesslich deuten mancherlei Beobachtungen der Ref. darauf hin, dass man auch event. bei den Hefen—und zwar in statu sporulandi—möglicherweise gerade bei den Vorgängen, bei denen die Spore nach Hansen wiederum zum Sporangium wird, mit gewissen mehr oder weniger stark ausgeprägten N-Assimilationsvorgängen zu rechnen hat." This earlier statement is apparently the basis of Lipman's ('11–'12, see p. 173) reference to Heinze's work.

Through pot experiments with seedlings of *Pinus montana* with and without mycorhiza, Möller ('06) concluded that the fungus associated with the roots of this species is unable to supply the host with nitrogen accruing as a result of fixation.

In continuation of her earlier work Ternetz ('07) has secured data of special interest, with reference to fixation, for *Phoma radicis* vars., likewise in a comparative way for a few other fungi and bacteria. The utmost care seems to have been observed with respect to the purity of materials, the use of necessary blanks in the analyses, and of controls in the

experiments. Cultivated during a period of 28 days on 50 cc. of nutrient solution the 5 races of *Phoma radicis* gave a nitrogen fixation ranging from 2.3 mg. in the lowest to 15.7 mg. in the highest. For *Aspergillus niger* and *Penicillium glaucum* the fixation was 1.9 and 2.8 mg., respectively. The usual high fixation was secured with *Azotobacter chroococcum* and *Clostridium pastorianum.* In spite of the fact that the methods employed are those generally recognized as unimpeachable, still attention should be drawn to the fact that in such cases as those of *Aspergillus* and *Penicillium,* where the fixation is only about 2 mg., the technique employed must be subjected to the closest scrutiny. It is noted that the felt was separated from the culture solution in the usual way, and further that the solution was then made up to the original volume (a procedure of vital importance when aliquot parts of the solution serve for analysis, and one seldom mentioned). Then from one-sixteenth to one-fourth, depending upon the amount of sugar present, of the total solution was taken for the analysis. In this way any small experimental error involved would have been multiplied 4–16 times.

Equally satisfactory in respect to method is the work of Froehlich ('08). Here again the methods are described in sufficient detail so that one is not left in doubt as to important parts of the technique. The organisms used lend a particular interest to the work, inasmuch as they were isolated from dead and decaying plant material and are fungi generally considered important in the decay of vegetation. Those selected consist of one species from each of four common genera. All were found to fix nitrogen to a slight degree, averaging as follows: *Alternaria* 3.34, *Macrosporium* 3.70, *Cladosporium* 2.26, and *Hormodendron* 1.93 mg. Many subsidiary experiments of interest are included.

Zikes ('09) conducted extensive experiments to determine the free nitrogen relations of a yeast-like organism isolated from the leaves of laurel and called by him *Torula Wiesneri,* which he cultivated on flasks containing 300 cc. of culture fluid. He employed the Dumas method of analysis, filtered off the fluid from the yeast cells, and made separate determina-

tions. He reports a fixation of 5.1–6.5 mg. per liter, .51–.61 per 100 cc. of culture solution, which, however, he regards as satisfactory positive evidence.

No investigator has obtained figures comparable to those of Latham ('09). This work was well conceived and arranged with a view to determining the effect of zinc sulphate on the nitrogen fixation of *Sterigmatocystis nigra* (*Aspergillus niger*) abundantly supplied with combined nitrogen.[1] The results published exhibit a variation ranging from a nitrogen loss of 42.5 mg. to a fixation of 205.1 mg. per culture, on 50 cc. of medium. In view of all the earlier and later studies made on fixation by this fungus, granting at the same time, of course, possible differences in strains, it can only be surmised, perhaps, that miscalculations are accountable for these unusual results. It would appear that in making the analyses she employed aliquot parts of the culture solution, and likewise divided the felt. Such a procedure, however, only suggests possibilities and cannot explain the results. In the case of maximum fixation, 677.3 mg. of nitrogen are reported fixed in felt and solution per gram of dry felt produced. This is an amount incomparably greater than anything elsewhere obtained.

Duggar and Knudson ('11) reported only by abstract upon extensive series of experiments in which *Aspergillus niger*, *Trichoderma lignorum* (erroneously given as *T. lignicola*), and several species of *Basidiomycetes* were employed. Various nutrient media were used, including synthetic nutrient solutions, leaf decoctions, and decayed leaves ground to a fine powder. None of the cultures showed a difference in the N-content over the controls sufficient to indicate fixation, whether with or without combined nitrogen. It may be stated that this work was not published in detail by reason of the uniformly negative results. It was intended to pursue the work further using ground leaf mould and similar materials as nutrient media, but difficulties in obtaining uniform samples

[1] The nutrient salt solution employed was the well-known Richards' solution consisting of NH_4NO_3 1 gm., KH_2PO_4 0.5 gm., $MgSO_4$ 0.25 gm., $FeCl_3$ trace, and sugar 5 gm., except that the amount of the nitrate in the different series varied from 115.4 to 160.3 mg. per culture, or 50 cc. of solution.

for analysis on such media were almost insuperable. The desirability of growing the *Basidiomycetes* on solid media was obvious, but under such circumstances it would have been necessary for greatest accuracy either to analyze the entire contents of such bulky cultures or to take account of change in weight of materials due to loss of CO_2 and H_2O produced in respiration.

Löhnis ('10) seems to report having found N-fixation in *Torula,* but his work was neither extensive nor reported in such way as to give the details of the methods employed.

Pennington ('11) worked with two species of *Penicillium,* two species of *Fusarium, Aspergillus niger,* and *Alternaria sp.* A variety of experiments was arranged in liter flasks containing 100 cc. of solution. Employing accepted methods he obtained no nitrogen fixation in a first series of experiments, although there was some growth on media practically without nitrogen, that is, with scarcely perceptible amounts, due, he believes, to impurities in the dextrose employed. In another extensive series no differences between the flasks containing the moulds and the controls were sufficient to indicate fixation. In one case *Penicillium* gave an apparent fixation of .88 mg. In considerable part his work was planned to confirm or disprove that of Latham ('09). The results are therefore peculiarly interesting, especially as he has apparently observed great care to eliminate all possibilities of error, and in advance thoroughly tested his ability to determine the nitrogen content of the cultures accurately.

Medisch ('10) observed some growth of the fungus *Hypocrea rufa* in solutions to which no nitrogen was added. He reports a gain of 1.05–2.45 mg. nitrogen in 50 cc. distilled water. This preliminary experiment was followed by others in which, under purified air, the organism was grown on various culture solutions. These included solutions containing no nitrogen, nitrogen as "potassium humate," and as NH_4NO_3. The results indicate that, whereas in the first case, with nitrogen present only as an impurity, the fixation was 1.74–3.23 mg. in 100 cc., in the humate solution the gain was 3.15–4.61 mg., and in the solution containing NH_4NO_3 the gain was

2.45–3.06 mg. He considers these quantities as possibly within the limit of experimental error. Unfortunately, various details regarding the handling of cultures and the methods of analysis are omitted.

Lipman ('11–'12) made an extended study of the relation of certain yeasts and fungi to nitrogen fixation, employing 7 species of yeast, 5 pseudo yeasts, and *Mycoderma,* also *Aspergillus niger, Penicillium glaucum,* and *Botrytis cinerea.* In the first extensive series, omitting the moulds, there was a slight gain of nitrogen in practically all cases, but only in a single case, a pseudo yeast, was this more than 1.0 mg. In a second series 9 out of 15 forms gave a gain of 1.05–2.28 mg., while 6 forms yielded less than 1 mg. increase of nitrogen. In the final series, which includes the fungi, the yeasts exhibited gains of .07–3.78 mg., while the fungi ranged from .05 to 2.38 mg.

Preliminary to her studies in nitrogen fixation Stahel ('11) made an extensive trial of fungi on media low in nitrogen. She isolated 54 species, largely from decaying vegetation, and then grew these on various media, including silica jelly without combined nitrogen. Five species were found to grow well on the last-named medium, while 22 made some growth. In the studies regarding N-fixation it is determined that the 5 species which grew on nitrogen-free media are capable of fixation, likewise 4 of the organisms from the group growing indifferently also possess this capacity. The method of handling the flasks is not given in detail, but it appears that the mycelium was filtered off from the solution and separate analyses made. The fungi were grown on 200 cc. of nutrient solution, and, in general, the amount of fixation was related to the initial nitrogen content. While the method suggests some possibilities for error, yet in some cases the amount of fixation is certainly well above the usual experimental errors. It is to be noted, however, that the following amounts represent questionable fixation: *Aspergillus* .41 mg., *Penicillium* .50 mg., *Botrytis* .46 mg., *Melanoma* .46 mg., *Epicoccum* .41 mg., *Bispora* .61–1.44 mg.; while the following give higher re-

sults: *Alternaria* 1.02–5.55 mg., *Hormodendron* .36–5.0 mg., *Macrosporium* .23–5.91 mg.

Experiments conducted by Kossowicz ('12) which seemed to suggest N-fixation in the case of certain species of *Saccharomyces, Monilia candida,* and *Oidium lactis* were subsequently repeated by him ('14) under more nearly standard control conditions. The results were interpreted as entirely negative. Besides the organisms previously employed, he used also *Aspergillus niger, A. glaucus, Penicillium glaucum, P. brevicaule,* and a species of each of the following, *Botrytis, Mucor,* and *Isaria.*

Will ('12), reporting work of Scheckenbach, declared the capacity of certain species of *Torula* to grow upon nutrient media lacking nitrogen, likewise to fix atmospheric nitrogen when little or no combined nitrogen was supplied. There is, however, with the experiments reported, little evidence that sufficient precautions were taken in the arrangement of suitable controls.

The capacity of *Blastoderma salminicolor, Torula sp.,* and "pastorianus" yeast to fix nitrogen has been mentioned by Lindner ('12),[1] but to what extent this work was quantitatively executed cannot be determined from the data at hand.

Goddard's ('13) investigations parallel those of Froehlich, Stahel, and, to a certain extent, those of Ternetz. He isolated 15 species of fungi from the soil, and tested each of these with respect to nitrogen fixation, grown on 50 cc. of a culture solution comparable to the nutrient media employed by other investigators. Every possible precaution seems to have been taken to insure accuracy. The fungi were grown 48–70 days. With no organism in any series were there indications of consistent gains over the initial nitrogen content. Species of *Aspergillus* and *Penicillium* were included in these studies.

In connection with his investigations of mycorhiza problems, Peklo ('13) isolated 3 species of fungi, 2 being species of *Penicillium,* and one an indetermined form. Each of these was grown on Winogradski's solution plus dextrose for 1–2

[1] The reference given appears to be an abstract of a more extensive report which is at present unavailable.

months. For each species he claims positive results, the fixation ranging from 0.8575 mg. in the lowest to 1.8615 mg. in the highest, per 100 cc. of solution. The inference seems to be that in each case a single inoculated culture or a single control was usually employed. It is of interest to note that aside from the few analyses made, the fungous felt and the solution were separately analyzed.

Traaen ('14) made no quantitative studies to determine N-fixation, but he observed the growth of 4 fungi on media practically nitrogen-free, and as a result of the very weak growth he came to the conclusion that under the conditions they could not possibly utilize atmospheric nitrogen.

Using strains of *Aspergillus niger* and *Penicillium glaucum,* Chambers ('16) was unable to demonstrate any N-fixation. He employed Folin's micro-Kjeldahl method, growing the organisms in long Jena test-tubes and making the determinations without transfer of any portion of the culture.

Methods

The organisms used in this work were *Aspergillus niger,* a strain long employed in various physiological experiments in this laboratory; a species of *Penicillium,* isolated from leaves and corresponding closely to Thom's idea of *P. expansum; P. digitatum,* isolated from a decaying orange; *Macrosporium commune,*[1] isolated from dried grass culms; *Phoma Betae,* a culture obtained through the kindness of Mr. E. C. Rittue, Los Angeles, California; and for comparison three forms of *Azotobacter,* as follows, all three being furnished by Dr. J. G. Lipman, *A. vinelandii, A. chroococcum* (from Kansas soil), and *A. chroococcum* (from Colorado soil).

Except as to the source of nitrogen and carbon, there has been no great dissimilarity in the mineral nutrient solutions employed by European investigators. The Cohn solution or a modification of it has been the basis of much of the foreign work. We wished to have some of our experiments follow fairly closely the work of Saida, therefore we have used in

[1] The morphological and cultural characters of this organism will be described in a subsequent paper.

some of the experiments (series 1A–4A, table 1) his solution as regards concentration of mineral nutrients. It is as follows:

KH_2PO_4	.4	gram
$MgSO_4$	.4	gram
$CaCl_2$	.2	gram
H_2O	100	cc.

This is designated solution A.

To this has been added $(NH_4)_2SO_4$ or asparagin as a source of nitrogen, and dextrose or saccharose as a source of carbon. In most of the work, however (series 5B–15B, 17B–22B, table 1), it has seemed well to use a modification of the formula known as Richards' solution, used especially by Miss Latham in securing the extraordinary results to be referred to later. The modification consists merely in varying the sources and amounts of nitrogen and carbon furnished, these last being the same as employed with "solution A" above.

The Richards' solution consisted of:

KH_2PO_4	.5	gram
$MgSO_4$	.25	gram
$FeCl_3$		trace
H_2O	100	cc.

This is designated solution B.

Stock solutions of each constituent were made up of appropriate strength, usually such that an equal quantity of each was required for any culture.

For *Glomerella Gossypii* a modification of the Uschinsky solution, as indicated in table 1, was employed, since this had been found satisfactory for this organism through other workers in the laboratory. For the various strains of *Azotobacter* a soil-compost extract containing mannite was employed. Three hundred gm. of potting soil and 100 gm. of well-fermented compost were each extracted for 2 hours with 1 liter of water, then filtered, and the filtrates combined. To the mixed extract was added for each 100 cc. the following constituents: K_2HPO_4 .05 gm., $CaCO_3$ 1 gm., and mannite 5.0 gm.

Kjeldahl flasks of 500 cc. capacity were used as culture vessels in all cases, and into each were placed 50 cc. of the solution required.[1] The idea of using the Kjeldahl flasks for the cul-

[1] In the bacterial cultures 100 cc. of solution were employed.

tures enabled us to make the nitrogen determinations of both inoculated and uninoculated flasks from the entire contents of the flasks, therefore to dispense entirely with any transfers of culture solution or fungous felt, and to avoid the possibility of errors thus ensuing.

All glassware was cleaned by standard methods; nitrogen-free double distilled water was used; and Merck's reagents. Every experiment was set up in triplicate, also with three controls; that is, for every series in which a different fungus, a different amount or source of nitrogen or of carbon was used, there were 6 cultures, 3 of which were inoculated and incubated, while the remaining 3 were inoculated, autoclaved to kill the spores (since they served as controls), and were then incubated with the others.

The inoculations were made from cultures on potato agar, fresh cultures only being employed as a source of spores or mycelium. The inoculation procedure was as follows for those forms producing spores: Numerous spores were transferred to a flask containing 100 cc. sterile H_2O. This was agitated until there was an evident spore suspension, and this then pipetted out with a sterile pipette into a second sterile flask. From this last flask 1-cc. portions were transferred with sterile pipettes to each flask in the series. The controls were then autoclaved for 15 minutes at 15 pounds pressure. That the method was entirely satisfactory is shown by the fact that there was only a single case of contamination in all the series employed and no case of growth in any of the controls. Similarly, in the inoculation of the series with *Azotobacter,* loops of the organism were diffused in sterile water, then $\frac{1}{5}$-cc. portions were placed in each flask by means of a sterile graduated pipette. All transfers were made with the greatest precaution in a steamed transfer room. In the case of *Phoma Betae,* where no spores were produced, small masses of hyphae of approximately equal size were inserted into each flask.

Repeated tests have shown that in the incubator rooms for the length of time which these experiments were permitted to run there is no detectable amount of combined nitrogen absorbed by flasks of the culture solution or by flasks of dis-

tilled water. Both from this fact and further from the nature of the controls it was unnecessary to place the cultures in a chamber arranged to protect against combined nitrogen.

The data presented in this paper on the determination of nitrogen were obtained either with the Kjeldahl-Gunning method,—using mercury in addition to potassium sulphate,—or, where nitrates were involved, with the Förster modification of the method mentioned. In some preliminary work an extended attempt was made to utilize the Folin micro-Kjeldahl apparatus, but that proved inapplicable to the present work for the following reason: The amount of culture solution which it is possible to use with this method is small, and doubtless would be too small to yield convincing results in view of the present confusion regarding the question of nitrogen fixation in the fungi. In the light of the results obtained by Puriewitsch, Saida, and others, all of whom used from 50 to 100 cc. of culture solution, it seemed essential to employ the "macro" method and to deal with cultures as large as practicable.

In the pioneer work of Jodin ('62) gas analysis methods were employed for the determination of nitrogen fixation, therefore through the indirect method of nitrogen loss in the culture chamber. Since that time all the work which may claim a right to be considered quantitative has been made with the Kjeldahl method, or with some modification of it, usually the Gunning. That this method is sufficiently accurate to detect any amount of fixation worthy of the name is evident, since an experienced analyst can usually secure results which often check to within .2 mg. However, if one does not observe all possible precautions, errors may creep in which will yield widely varying results. Chief among these possibilities in the problem of nitrogen fixation are the following:

1. Impure chemicals.
2. Accuracy of standard acid and alkali.
3. Indicator.
4. Completeness of digestion and distillation.
5. Loss of nitrogen in the transfer of the culture material, or felt, from one flask to another.

6. Multiplication of the experimental error through taking an aliquot part of the fungous mat or culture solution and upon the determination from this basing a calculation for the whole.

7. Inadequate controls.

Analyzed chemicals may be obtained always, but these should be checked by running blank experiments. Standard acids and alkalis should be checked up by at least two methods. Nevertheless, slight discrepancy in the standard affects the actual rather than the relative analytical results, provided the same solutions are used for the nitrogen determinations whether they grow the fungus or are used as controls. Certain indicators have, in the presence of ammonia, what might be called a "running" end-point; that is, the color change occurs through a fairly wide range of H-ion concentration. After trying several indicators for this work alizarin red (Alizarin sulfonsäure Natrium, Merck) and cochineal were found to give the best satisfaction. The former in .1 per cent aqueous solution was used.

The error due to incomplete digestion or distillation, while easily guarded against, may sometimes occur, if care is not observed. It was the practice here to continue the digestion 15 minutes after the mixture had become colorless. A full hour was given to distillation, since this interval proved entirely sufficient as shown by tests from time to time.

In the critical review of literature it has been emphasized that many of those investigators reporting nitrogen fixation for the fungi have limited their nitrogen determinations to aliquot portions of the culture solution. The total nitrogen was then calculated. Summarizing some of the points to which attention should be drawn, it is found that Puriewitsch ('95), Saida ('01), Ternetz ('07), and Froehlich ('08) all filtered the solution from the fungous mat, determined the nitrogen from a portion of the solution, and calculated for the whole solution. The mat nitrogen was determined separately. Stahel ('11), Peklo ('13), and others, after separating solution from fungous felt, evaporated the culture medium to small bulk (following the addition of acid) and determined the

total nitrogen. To this was added the amount of nitrogen found in the felt. Lipman ('11–'12) did not separate the mat from the medium, but transferred the whole to a digestion flask, and later to a distilling flask, determining the total nitrogen in one lot. All the cases cited above involved one or more transfers of material, since the fungi were usually grown in Erlenmeyer flasks or similar receptacles, and the contents filtered or transferred before digestion, thence usually a second transfer to a distilling flask. It was with the end in view of eliminating the possibility of error in this direction that the method already described was employed, i. e., of growing, digesting, and distilling in the same flask and without transfer.

Where the digestion of nitrates was involved in the culture solution, the previous investigators have used, almost without exception, the Gunning-Jodlbauer method—phenol or salicylic acid and zinc dust being employed for the reduction of nitrates. In our work the Förster modification was employed, since certain workers have found difficulty in obtaining all the nitrate by the former method. Indeed, it was this difficulty which first led Förster to use sodium thiosulphate as a reducing agent. If all nitrates are not reduced a serious error is, of course, involved, one which, moreover, makes for a difference between controls and inoculated flasks. The results may be presumably correct for the converted or assimilated nitrogen of the mycelium (or products excreted therefrom), low figures resulting for the nitrate of the culture media. If an error were present, then, it would be related somewhat closely to total growth or to sugar consumption, factors determining nitrate consumption. It is equally true that the capacity to fix nitrogen by a fungus, if possible, might also be related to the capacity for growth under the particular conditions.

In the use of the Förster method at first certain difficulties were experienced. In preliminary work the recovery of nitrogen from a water solution of KNO_3 was easily accomplished within experimental error. When, however, a nitrate was added to a soil, compost, or plant tissue decoction the results were invariably low. It was found necessary to add

more sodium thiosulphate (3 gm. instead of 2) and to allow 10–15 minutes after its apparent decomposition had taken place before digestion was continued. This illustrates the possibility of error in a method that is not thoroughly tested in connection with the peculiar conditions at hand.

No difficulty was experienced in obtaining results with the Gunning-Kjeldahl modification that checked within experimental error. Some trouble, through frothing, will be experienced in the actual digestion, however, where the culture media are high in sugars. This may be overcome by boiling (after adding 15 cc. of concentrated sulphuric acid) slowly for an hour or more, then adding more acid together with 15 gm. of potassium sulphate and 1 gm. of mercury. In our work it became necessary at times to add a third lot of 15 cc. acid—the same amount being always added to both fungus-containing flasks and controls.

Distillation was carried out through block tin tubes which had been in use sufficiently long to obviate the possibility of error through absorption of ammonia—a point observed with new tin by several investigators. The standard acid and alkali were restandardized at short intervals. The same lot of chemicals was always used throughout a single series to insure parallel treatment with both fungus-containing flasks and controls.

Experimental Results and Discussion

The results of our experiments are presented in some detail in table I. It is necessary to note that while the quantities given in column V were obtained by careful weighings, they represent only approximately the quantities present in the solution as determined by analysis (see columns VI and VII). In any series the controls are as nearly perfect as we were able to arrange, that is, the solutions in all the flasks in any one series were taken from a single vessel of the culture medium, the complete mixing of the different constituents in the culture solution being given special attention. In column I the letters A and B given in connection with the series numbers refer to the two nutrient salt solutions employed as de-

scribed on page 424. Where no letters are given there are sufficient indications in column v to identify the culture medium employed. Data for all of the flasks analyzed are included in the table in order that the extent of the experimental error may appear just as well as the average of the determinations made.

TABLE I

NITROGEN FIXATION IN CERTAIN FUNGI AND BACTERIA

I	II	III	IV	V	VI		VII		VIII
Ser. no.	Organism	T. °C.	Per. of gr. days	Sources of N and C supplied, per cent	Total N in flasks containing fungi, mg.		Total N in control flasks, mg.		Diff. = N-fixation mg.
					Comp. data	Average	Comp. data	Average	
1A	*Aspergillus niger*.....	30	30	.7 asparagin 3.6 dextrose	62.510 62.545 63.140	62.732	62.510 62.335 62.300	62.382	.350
2A	*A. niger*.....	30	30	.7 asparagin 1.8 dextrose	61.215 61.985 62.545	61.915	61.915 59.710 61.915	61.180	.735
3A	*A. niger*....	30	30	.014 asparagin 10.8 dextrose	2.135 1.925 1.820	1.960	2.310 1.995 1.750	2.018	—.058
4A	*A. niger*.....	30	30	.7 asparagin 18.2 dextrose	60.305 59.955 59.990	60.083	60.375 60.585	60.488	—.405
5B	*A. niger*.....	30	30	.014 $(NH_4)_2SO_4$ 3.6 dextrose	1.435 1.575 1.400	1.470	1.470 1.505 1.435	1.470	———
6B	*A. niger*.....	30	30	.7 $(NH_4)_2SO_4$ 18.2 dextrose	70.385 70.420 70.455	70.417	70.490 70.490 70.560	70.547	—.130
7B	*A. niger*.....	30	30	.014 $(NH_4)_2SO_4$ 18.2 dextrose	1.715 1.715	1.715	1.680 1.750 1.715	1.715	———
8B	*Macrosporium commune*.....	25	30	.014 $(NH_4)_2SO_4$ 18.2 dextrose	2.030 2.153	2.091	1.925 2.065 1.995	1.995	.097
9B	*M. commune*	30	7	.7 $(NH_4)_2SO_4$ 18.2 dextrose	70.385 70.420 70.315	70.373	70.420 70.560 70.665	70.548	—.175
10B	*M. commune*	30	7	.014 $(NH_4)_2SO_4$ 18.2 dextrose	1.755 1.890 1.785	1.810	2.065 2.205 1.925	2.065	—.255
11B	*M. commune*	25	30	.0035 $(NH_4)_2SO_4$ 18.2 dextrose	.613 .753 .770	.712	.858 .683 .700	.747	—.035

TABLE I (Continued)

NITROGEN FIXATION IN CERTAIN FUNGI AND BACTERIA

I	II	III	IV	V	VI		VII		VIII
Ser. no.	Organism	T. °C.	Per. of gr. days	Sources of N and C supplied, per cent	Total N in flasks containing fungi, mg.		Total N in control flasks, mg.		Diff. = N-fixation mg.
					Comp. data	Average	Comp. data	Average	
12B	*Penicillium digitatum.*	30	42	.7 $(NH_4)_2SO_4$ 18.2 dextrose	70.455 70.630 70.455	70.513	70.630 70.560 70.665	70.618	—.105
13B	*P. digitatum*	30	42	.014 $(NH_4)_2SO_4$ 18.2 dextrose	2.555 2.590 2.660	2.602	2.660 2.765 2.765	2.730	—.128
14B	*Penicillium expansum*	25	35	.7 $(NH_4)_2SO_4$ 18.2 dextrose	71.890 72.065	71.978	71.540 71.575 71.785	71.633	.345
15B	*P. expansum*	25	35	.014 $(NH_4)_2SO_4$ 18.2 dextrose	2.415 2.345 2.450	2.403	2.415 2.450	2.433	—.030
16	*Glomerella Gossypii*..	25	30	Uschinsky sol. cornmeal decoct.	7.665 7.455 7.770	7.630	7.630 7.875 7.770	7.758	—.128
17B	*Phoma Betae*	25*	25	mangel decoct. 10.0 sucrose	26.635 27.370 26.985	26.997	23.765 23.695	23.730	3.267
18B	*P. Betae*....	25*	25	sugar beet decoct. 10.0 sucrose	16.975 16.870 19.110	17.652	14.665 14.595	14.630	3.022
19B	*P. Betae*....	25*	25	.7 $(NH_4)_2SO_4$ 18.2 dextrose	70.840 70.735 70.525	70.700	68.845 69.020 68.915	68.927	1.773
20B	*P. Betae*.....	25*	25	.014 $(NH_4)_2SO_4$ 18.2 dextrose	2.275 2.030	2.153	2.030 2.415 2.100	2.182	—.029
21B	*P. Betae*....	25*	89	mangel decoct. 10.0 sucrose	53.340 52.570 53.060	52.990	45.220 45.255	45.238	7.752
22B	*P. Betae*....	25*	89	sugar beet decoct. 10.0 sucrose	31.010 31.360	31.185	25.585 25.655	25.620	5.565
23†	*Azotobacter vinelandii*	25	28	soil-compost sol. 5.0 mannite	46.515 46.480 46.445	46.480	5.810 6.405	6.108	40.372
24†	*A. chroococcum*..... (Colorado)	25	28	soil-compost sol. 5.0 mannite	24.570 22.085 22.365	23.007	5.810 6.405	6.108	16.899
25†	*A. chroococcum*..... (Kansas)	25	28	soil-compost sol. 5.0 mannite	22.586 21.735 26.705	23.675	5.810 6.405	6.108	17.567

* 22-25°C.

† In these cases only were 100 cc. of culture solution employed; in all other cases 50 cc.

From our results it is clear that under the conditions of the experiments no fixation can be claimed for *Aspergillus niger, Macrosporium commune, Penicillium digitatum, P. expansum,* and *Glomerella Gossypii.* For the most part, with these fungi, the differences between the various members of any series, including the controls, represent variations which might be expected, and the fact that the averages of the controls are slightly above or below those of the flasks containing the fungi is of little significance.

With *Phoma Betae* the case is different. Here the assimilation of free nitrogen seems definite. The quantities obtained vary from practically 0.0 to 7.75 mg. per 50 cc. of culture medium. All cultures on sugar beet and mangel decoction exhibit a nitrogen increase which points definitely to free nitrogen assimilation. It should be noted that these cultures represent series maintained for a shorter and a longer period of time; those maintained for the longer interval yielding higher fixation quantities than those cultured for the shorter interval. In one series, 19B, where the source of nitrogen is .7 gram $(NH_4)_2SO_4$, the nitrogen difference is perhaps sufficient to indicate nitrogen fixation. At any rate, if we regard fixation as occurring in this solution, it is fair to explain the absence of fixation in series 20B, in which only .014 $(NH_4)_2SO_4$ was employed, as due to the small amount of growth occurring in the last-mentioned series. As would be expected, fixation is somewhat related to the length of the period of growth and to the extent of growth. The results with *Phoma Betae* were so unexpected, in view of the long series of negative values obtained with other fungi, that a further check upon the work was introduced in the following way: A known amount of KNO_3 was added to a series of flasks containing 50 cc. of the sugar beet medium, and analyses were then made to ascertain with what accuracy this nitrogen could be determined. No difficulty was experienced in recovering this nitrate nitrogen, as shown by the data in table II.

Furthermore, it seemed well, as a result of the experiments with *Phoma Betae,* to employ by comparison certain organisms known to have nitrogen-fixing power. Accordingly, the

selected strains of *Azotobacter* were tested, and all yielded positive results of satisfactory magnitude, as shown in table 1.

It will be observed in table 1 that slight discrepancies seem to occur between different series in respect to the amounts of nitrogen recovered—where the different series contained presumably the same amounts of initial nitrogen. This, however, is only an apparent discrepancy, since, as previously

TABLE II

RECOVERY OF NITROGEN AS KNO_3* ADDED TO SUGAR BEET CULTURE MEDIUM

Trials	Controls (no N added) mg.	13.8 mg. N added as KNO_3 mg.	N recovered mg.	Difference mg.
1	14.532	28.227	13.695	—.105
2	14.49	28.048	13.550	—.250

* 13.8 mg. nitrogen as KNO_3 added to 50 cc. sugar beet decoction + 10 per cent cane sugar.

mentioned, this work extended over a considerable period of time, and although the same lot of culture solutions was used for the control flasks and for the flasks in which the organisms were grown in any one series, it was nevertheless necessary to make up new solutions from time to time for the different series. The different series are therefore only approximately comparable.

Now since N-fixation occurs in organisms otherwise so physiologically different as *Azotobacter, Clostridium,* and *Bacillus radicicola,* why may it not occur in all fungi and bacteria, it has been asked time and again. Final answer can be given only in accordance with the results of properly planned and carefully executed experiments. Moreover, it has been shown abundantly that fixation is relatively uncommon among bacteria, the capacity being possessed largely by those groups mentioned above. As has been indicated, among others, Saida, Ternetz, and Stahel have reported fixation for *Aspergillus niger.* In the cases referred to the results are scarcely greater than might occur as experimental errors. This could not apply, however, to the results with *Phoma radicis* and apparently not to those with *Phoma Betae.* Confirmatory evidence

from our own results has certainly designated the Phoma group of organisms as worthy of further careful study.

With respect to the accumulated data for *Penicillium, Macrosporium, Alternaria,* and other saprophytic moulds occurring in the soil or upon decaying vegetation, it can only be said that the data fall into the same category as that for *Aspergillus niger.* We do not take issue with those reporting fixation, but we feel that in view of strong negative evidence regarding many of these fungi, further assurance must be given that the results may not be explained on the ground of experimental errors. We are quite well aware that the admission of the data for *Phoma Betae* has virtually thrown open the whole question for any and all fungi, yet we can find no grounds upon which adequately to criticize either our own results or those of Ternetz with another species of this genus.

Accepting the evidence for certain species of *Phoma,* in what direction shall we seek for organisms similarly endowed? Naturally related genera among the *Sphaeropsidales* would first be suggested, on purely morphological grounds. Again, for a long time physiologists have seen possibilities in organisms which have undergone such adjustment as characterizes the mycorhizal fungi generally. Up to the present time there has existed considerable uncertainty concerning the isolation and determination of the species which produce mycorhiza. Ternetz alone has demonstrated a *Phoma* as a root organism of this type. Peklo's studies lead him to believe that *Penicillium* and an undetermined fungus are involved. In this case, as already noted, a very weak nitrogen fixation was reported. It is not intended, however, in this connection to discuss the various indications respecting mycorhizal fungi. Attention may be drawn to the fact that the predominant presence of *Basidiomycetes* in forests and meadows early suggested the association of these forms with the roots of higher plants. In recent times species of *Tricholoma, Lactarius, Cortinarius,* and *Boletus* have been strongly suspected of being important in the development of mycorhiza.

The fungi are the primary agencies whereby vegetation is usually disintegrated or brought through the first stages of

decay. If it should be positively demonstrated, therefore, that the fungi concerned in this disintegration are at the same time capable of fixing an amount of nitrogen sufficient to prove of practical value, then it would be clear that agricultural practice might be modified in many ways to make greater use of this possibility of nitrogen enrichment accompanying the decay of herbage. As a matter of fact, however, the amount of fixation, as we have seen, reported for *Alternaria, Macrosporium, Cladosporium, Aspergillus, Penicillium,* etc., even by those recent investigators who claim fixation, is very slight—indeed, for such organisms it is usually considerably below 5 mg. per 50 cc. of solution. Assuming that there might be as much fungous felt in 1 cubic foot of ordinary soil as in 100 cc. of a culture[1] and that in both cases the amounts of fixation might be equal, we would have as a maximum 10 mg. nitrogen fixed per cubic foot or 420,000 mg. per acre, 1 foot deep, that is, 420 grams per acre, or about one pound. When it is recalled that in many cultures of *Azotobacter* the fixation has been as high as 50–200 mg. per 100 cc., and when it is further remembered that in the soil the conditions favor quantity of bacterial rather than fungous growth, we may perhaps gain some conception of the impracticability of claiming an economic relation in respect to nitrogen for such fungi.

Summary

1. A review is given of all available literature relating to nitrogen fixation by the fungi.

2. Culture and analytical methods are discussed, and suggestions are made with a view to the elimination of certain possible errors involved in this type of work.

3. Nitrogen fixation could not be demonstrated for *Aspergillus niger, Macrosporium commune, Penicillium digitatum, P. expansum,* and *Glomerella Gossypii.*

4. In cultures of *Phoma Betae* on mangel and on sugar

[1] This seems highly improbable, in the light of recent discussion of this point; compare the following: Conn, H. J. Relative importance of fungi and bacteria in soil. Science N. S. **44**: 857–858. 1916.

beet decoction with sugar a nitrogen gain of 3.022–7.752 mg. was established, which seems definitely to indicate fixation.

5. Comparative studies of strains of *Azotobacter* exhibit the usual relatively large fixation of nitrogen in the culture media.

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