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ARTIFICIAL ASSOCIATIONS OF NON-LEGUMES WITH DINITROGEN-FIXING BACTERIA AND THEIR POSSIBLE IMPACT ON FERTILIZER USE.

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1) SYMBIOSIS AND ASSOCIATION

Symbiosis has a clear meaning : the mutual support of two (or more) organisms, which belong to different species and are living at least temporarily in close contact. Association means a less well defined form of organization, sometimes with some characteristics of a true symbiosis. Therefore, several authors are using the term "hemisymbiosis", which should be avoided, because one either deals with a symbiosis or not.

In this context we are dealing with associations exhibiting dinitrogen-fixing capacities. It was proposed to call such an association "diazotrophic biocoenosis". Depending from the plant part engaged-root, leaf or stem- the general term biocoenosis will be replaced by "rhizocoenosis", "phyllocoenosis", and "caulocoenosis", respectively. We shall concentrate here on "diazotrophic rhizocoenosis", e.g. associations, in which the dinitrogen-fixing bacteria are found in and around the root system of the plant partner.

2) THE BACKGROUND : INCREASING DEMAND FOR FERTILIZERS, FINANCIAL RESTRICTIONS, AND THE POSSIBLE IMPACT OF GENETIC MANIPULATIONS

In the preceding paper Dr. Postgate has outlined the present situation. Just a few remarks shall be added, concerning mainly cereals, our main nutritive plants. The demand for cereals and with it the demand for fertilizers will increase at least till the year 2000. On the other side, the following remark, introducing the invitation for an international symposium on biological nitrogen fixation in tropical agriculture, which was held in 1981 at Cali/Colombia, seems to be characteristic : "Large scale production of nitrogenous fertilizers is no longer economically feasible, except in countries that have surplus natural gas. For the vast majority of countries nitrogen fertilizer is already, or soon will become, a costly import."

The discrepancy between demand on the one hand and financial and/or technical possibilities on the other, could be diminished, as Dr. Postgate has outlined, by the development of dinitrogen-fixing cereals created by genetic manipulations. If one follows our newspapers, confronting us with headlines like "Gene-technology on the field!", the problem seems to be solved . As a scientist with fifteen years experience in this sphere, one becomes much more sceptical. There are several reports on successfull gene transfer experiments in higher plants. Applying the criteria, with which conventional biology and molecular technologies have provided us, there still remain unresolved questions in all these cases (I include our own experiments). Until now it was not possible to establish beyond any doubt foreign gene material, for instance a bacterial gene, in higher plants in such a manner that it was carried from generation to generation by sexual propagation, e.g. by seeds.

An exception should be mentioned. In 1981 two teams succeeded in transferring the nif-operon, the bacterial gene set governing dinitrogen-fixation, into yeast. Yeasts are higher plants in so far as they show a cell nucleus. The geneticist knows that they are exceptional favorable objects in gene manipulations. Even in this case, however, the success was limited : the nif-operon was

introduced, but it did not function. There was no fixation of aerial nitrogen. The example demonstrates the difficulties with which one is encountered even if the nif-operon is transferred.

Compared with other higher plants, cereals offer additional difficulties in genetic manipulations. In the mostly propagated transfer system in higher plants isolated protoplasts are used as receptors. One tries to introduce the foreign gene material into them in a special form : integrated into a plasmid from crown gall bacteria, which can easily be taken up and evaluated by the plant cells. Unfortunately, until now it was not possible to regenerate protoplasts from cereals to new plants and, secondly, the just mentioned vector plasmid (Ti-plasmid) shows no interaction with cereal cells. Therefore, it will probably take much longer time for the first successfull gene transfer in cereals than in other higher plants. Whether another gene transfer system proposed by us, in which pollen is used to transfer the foreign gene material during the normal fertilization process, will overcome these obstacles remains to be investigated.

The transfer of the bacterial nif-operon into higher plants, especially into cereals, is no short-term project. There seems to be, however, an alternative : artificial associations of non-legumes with dinitrogen-fixing bacteria. The procedure implies that the nif-operon remains where it functions best, within the bacteria, and that one tries to establish a diazotrophic biocoenosis between these bacteria and non-leguminous plants. The assumption was that mutual interactions between the bacteria and plant partner would take place, resulting finally in a higher yield of the non-legumes involved.

Evidence that this hope could be justified, was derived from two lines of investigation : field studies and test tube experiments.

3) ARTIFICIAL ASSOCIATIONS (DIAZOTROPHIC RHIZOCOENOSIS)

a. Field studies

In the last decade Dr. Döbereiner in Brazil detected natural associations between several tropical grasses, e.g. corn, severals species of millet, and sugar cane, and diazotrophic bacteria, e.g. Azospirillum brasilense and A. lipoferum. The bacteria concentrated in the rhizosphere and an apparently part of them was taken up into dead vessels of the root system. Considerable nitrogenase activity was detected using acetylene reduction test.

Just a word on nitrogenase. The bacterial enzyme of dinitrogen fixation was called nitrogenase. It reduces molecular N_2 to ammonia. Some other substrates are reduced as well, among them acetylene to ethylene. Acetylene reduction serves as basis for a simple assay on nitrogenase activity.

Based on the acetylene reduction assay it was calculated that up to 1 kg N/ha/day should be fixed by bacteria associated with the roots of some tropical forage grasses, and up to 2 kg N/ha/day by Azospirilla associated with the best maize lines. If one extrapolates over the whole vegetation period this would mean the fixation of approximately 100 kg N/ha. One would wonder whether under these conditions there could be any demand for industrial fertilizer ! One should take into consideration, however, that there are incertainties

in calculating the actual dinitrogen fixation from the values obtained by the acetylene reduction assay, and, furthermore, that the assay has to be run undermore or less unphysiological conditions. Therefore, one has to consider these data with some reservations. With high probability the actual nitrogenase activity was overestimated.

A better way to determine the actual nitrogen fixation rate would be the use of density labeled molecular nitrogen, N_2^{15}. Experiments of this kind were performed under field conditions just sporadically, so that the evidence gained by them was limited.

There remains, however, another more convincing way to evaluate the role of the bacteria : inoculation of the Gramineae with bacteria, that means the establishment of artificial associations.

To exclude any interference of other microorganisms with the association under study, usually experiments under sterile conditions will precede field inoculations. Under these conditions, Azospirilla and also Rhizobia were found to stimulate growth and development of several Gramineae. For instance, rhizobia enhanced up to 35% the dry weight of wheat which was kept under sterile conditions on highly supplemented vermiculite and soil media. Another example : Azospirilla were associated with corn (Zea mays) and foxtail millet (Setaria italica) under sterile conditions in Leonard-jars. In control assays, autoclaved bacteria were added. In all experimental assays nitrogenase activity was detected. The bacteria significantly increased plant dry weight and total N content of leaves. The values obtained reached from 50-100%, depending from the soil type used and from the amount of nitrogen fertilizers added. In poor soils containing low concentrations of available N, such as sand, there was a considerable higher increase than on loess, compared with controls on the same type of soil. On heavier soils containing more N, however, plant growth was much better. The percent increase was lower, but the absolute values of dry weight and N-content were higher than on poor soils.

The next stage were experiments under non-sterile conditions. Wheat plants associated with Azospirillum lipoferum in Kick-Braukmann-jars on medium soils ("Einheitserde") showed a 5-8 % increase of grain yield compared with controls treated with killed bacteria.

As far as field studies are concerned, the results were contradictory. Unfortunately, unsuccessful experiments are published very scarcely, so that one has to rely on personal communications. There are, however, some reports on successful inoculation experiments from Belgium, Brasil, Canada and Israel. The experiments in Israel are exemplary for this type of experimentation. During 1979-1980 eighteen field trials were carried out in three different regions, under different environmental and soil conditions. Soils were inoculated by directly spraying a bacterial inoculum. The bacteria used were several strains of Azospirillum brasilense, the plant partners corn (Zea mays), several millets (Panicum miliaceum, Sorghum bicolor, and Setaria italica), and wheat (Triticum turgidum and T. aestivum). Control plots remained untreated. Highest grain yield increases up to 35% over non-inoculated controls were obtained at intermediate levels of nitrogen fertilizers initially added.

Undoubtely in several field experiments there were positive effects of an inoculation with Azospirillum. There is no proof, however, that all these effects are due to the dinitrogen-fixing activity of the bacteria added. Another possibility could be the excretion of plant growth stimulants by the bacteria. Appropriate controls would allow to exclude this possibility. Unfortunately, in the field experiments the controls were kept untreated. Therefore it remains an open question whether the effectiveness of the bacteria was due to dinitrogen fixation, to the production of plant growth stimulants, or-most likely- to both of them. Just as in the jar-experiments, further work should include controls treated with killed bacteria, or even better with nif-mutants of the respective bacteria. Such nif-mutants are unable to fix dinitrogen, but normal in respect to the production of hormon- like factors.

To summarize this point : Compared with the work on, for instance, the legume- rhizobium symbiosis, field data concerning the diazotrophic rhizocoenosis cereals - bacteria are rather scarce. Therefore it seems difficult to give generalizing statements. Nevertheless, the tendency is unmistakable that diazotrophic bacteria enhance plant growth and development. It should be stressed, however, that the best results were obtained following addition of medium concentrations of N-fertilizers. Industrial fertilizers are by no means superfluous !

b. Test tube experiments

Once supposed the beneficial role of diazotrophic bacteria in the development of non-legumes, the mechanism which could be operative in such associations remained completely unclear. Some insight could be gained by in vitro studies.

Let us start with rhizobia, the root nodule bacteria of the legumes. As late as 1976 a leading textbook stated : "It is only following the establishment of the nodule with the commensurate differentiation of the bacteria into a bacteroid and the production of leghaemoglobin that nitrogen fixation is possible."

One year before the appearance of the book, however, this "dogma" was broken down. Since 1975 one knows that <u>non-leguminous</u> plant tissues are able to induce nitrogenase activity in rhizobia without any nodule formation or leghaemoglobin synthesis. Later on we could demonstrate that nitrogenaseinduction by non-leguminous tissues was possible through a membrane which was impermeable for bacteria, a result interpretable with the existence of inducing factors excreted by the plant cells. Further transfilter experiments using density labeled aerial N (N_2^{15}) demonstrated that the nitrogen fixed by the bacteria passed the membrane in the form of ammonia and was channelled into the normal nitrogen metabolism of higher plants. These results demonstrated that cells of non-leguminous plants were profiting from the nitrogen fixed by the rhizobia.

For a possible practical use, first of all one had to replace the tissue cultures by plants. In first experiments we used a model plant, the petunia, later on young plants of nutritive value, tomatoes and wheat. With all these species, essentially the same results were obtained : In the agar media used, the rhizobia concentrated glove-like around the roots and fixed in a polar end manner on the surface of root cells, especially root hairs. Light and electron-microscopical studies revealed that part of the bacteria invaded living root cells. In all these artificial associations nitrogenase activity developed. Following removal of the plants from the association, nitro-

genase activity rapidly declined, indicating the necessity of inducing plants. Also in these experiments nitrogenase induction was possible through membranes which were impermeable for the bacteria. Control assays with bacteria alone showed no or negligible nitrogenase activity.

Induction was prevented by the addition of rifampicin, an inhibitor of bacterial RNA-polymerase, that means of primary genetic activity. Therefore, induction involves the activation of the above mentioned nif-operon, the gene set governing nitrogenase production.

In further experiments the rhizobia were replaced by Azospirilla, the bacteria used in jar and field experiments. The plant partners tested (wheat and millet) induced nitrogenase activity in Azospirilla as well. Nitrate inhibits nitrogenase activity in Azospirillum. Studies of the nitrate level in our associations revealed, that the induction of nitrogenase activity was not due to the removal of repressing nitrate by the growing plants.

Recently, we succeeded in inducing nitrogenase activity by wheat conditioned media. Wheat plants were grown in dialysis bags filled with glass beads and liquid culture medium. Wheat showed an excellent growth on this special hydroculture, much better than on agar. The wheat culture system was surrounded by agar, which was inoculated with the bacteria. In such transfilter associations the wheat plants induced nitrogenase activity in the bacteria. Once more, this transfilter induction tells in favour of inducing substances excreted by the roots. It was possible to demonstrate the existence of these inducing factors : the root conditioned medium was decanted from the glass beads and used for induction assays. It induced nitrogenase activity in Azospirilla whereas control media did not. From the practical as well as from the theoretical point of view it would be most interesting to investigate these inducing factors.

At least in the test tube wheat and other non-leguminous plant species are able to induce nitrogenase activity in diazotrophic bacteria. It would be of high practical importance to find out whether such an induction could take place under field conditions as well. The point is that nitrogenase induction in the test tube was possible in the presence of nitrate. Supposing field conditions this would mean that nitrogenase induction and exploitation of aerial nitrogen would be possible even in the presence of N-fertilizers added. The fact, that under field conditions the best results were obtained using medium concentrations of fertilizers would well fit to this hypothesis. Further studies in optimizing field associations have to take into consideration nitrogenase induction by the plant partner. Anyway, applying the right concentration of N-fertilizers to the right cereal-bacterium association, there will be no exclusion of biological dinitrogen fixation by industrial N-fertilizers, especially nitrates.

3) POSSIBLE IMPACT ON FERTILIZER USE

The results obtained so far justify the hope that artificial associations of cereals with dinitrogen-fixing bacteria will function under field conditions satisfactorily. Convincing evidence for its global importance, however, is still missing. Let us overlook this gap and speculate what the impact on fertilizer use would be, if the hope just expressed could be realized.

First of all, a low input strategy has to take into consideration not only fertilizers, but also quite other factors (Dr.G.L. Nickel, lecture at the University of Hohenheim, May 1982)

1. Irrigation
2. Fertilizers
3. Pesticides
4. Soil amendement
5. Tillage

None of these factors can be considered separately. Let us assume that N-fertilizers could be replaced at least partially by the activity of diazotrophic bacteria. One of the consequences would be an increasing demand for pesticides, which were tolerated by both partners of the associations, cereals as well as bacteria. This would imply a thorough screening of the existing pesticides or the development of new ones. No doubt that the chemical industry will follow all developments on this sector with Argus' eyes !

Now to the use of fertilizers. It is selfunderstanding that a reduced use of N-fertilizers does not mean that no fertilizers at all are needed. As far as the N-fertilizers are concerned, rough estimates of their possible reduction in artificial association range from one to two third of the amounts normally added. One should not overlook, however, that such estimations are based on first positive results, so that they are from the romantic initial phase of the work and, therefore, could reflect overestimations. By energetical reasons, it seems highly improbable that loose associations will be ever as effective as the symbiosis of, for instance, legumes with their rhizobia. In the root nodules there is an intimate contact between the bacteria and the plant tissues, which are providing the rhizobia with the energy needed for dinitrogen fixation. There is nothing comparable in the associations. The role of the bacteria, which are invading the roots of cereals, remains to be elucidated, especially under quantitative aspects of their dinitrogen fixation.

Even if the lower estimation seems more realistic, even if "only" one third of the N-fertilizers hitherto added could be saved, fundamental consequences could be awaited. In highly industrialized countries with their abundant use of N-fertilizers the danger of pollutions will be reduced, an effect which will be welcomed from the ecological point of view. On the other hand, in developing countries with practical no use of industrial N-fertilizers it could be worth while to calculate whether they are still a "costly import". It makes a difference whether one has to add 100 or only 65% or even less of N-fertilizers . Depending from the actual amount of fertilizers needed in associations it might be well that the use of N-fertilizers will increase world-wide seen ! The chance seems to be given, that in associations of cereals with diazotrophic bacteria biological and industrial dinitrogen fixation will complement each other, a further example that ecology and economy are by no means incompatible.

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TA/82/15 Artificial associations of non-legumes with dinitrogen-fixing bacteria and their possible impact on fertilizer use, by D. HESS, Hohenheim University, Germany

DISCUSSION : (Rapporteur : L.J. CARPENTIER, IFA)

Q - Mr. E.G. PULLEN, The Phosphate Co-operative Company of Australia Limited, Australia

We are interested in the results of the field studies (Section 3a) where "soils were inoculated by directly spraying a bacterial inoculum", particularly for forage grasses, rice and wheat.

1. Would gypsum granules or fertilizer granules make a suitable medium for dispersal of the bacterial inoculum?

2. What are the conditions for maintaining the activity of the bacterial inoculum?

3. For a given initial fertilizer nitrogen level, what is the expected yield increase due to the bacterial inoculum?

A - Q-1. and Q-2

In the case of Azospirilla no solid or granular inoculum was used hitherto. Bacterial suspensions are sprayed directly on to the field. The bacteria are usually taken during the active multiplying stage. It is obvious that, if later laboratories and fields are far apart, another method of application will have to be used and the question of granular inoculum will then come up again.

A - Q-3. Concerning the amount of fertilizers to be saved, it depends on the rate of application. Spectacular results can be obtained when wheat plants are cultivated in a N free medium. Without N the plants are white-yellowish. The addition of Azospirilla makes them appear normal again in a few days. Apparently 100% of N fertilizers could be saved. However, if differences between plants grown on N free medium with and without Azospirilla are conspicuous, the result in terms of yield is very limited, and in a N free medium the yields remain very low. In case of high N rates, 140 kg/ha or more, N activity is stopped and no fertilizer can be saved. Calculations based on field experiments show that, when applying moderate rates, 60-80 kg/ha N, the addition of Azospirillum gives the same results as in the case of 80-100 kg/ha N. It means a saving of 1/3. However these are individual data and much more should be done before one can really rely on them.

Q - Mr. T.P. HIGNETT, IFDC, USA

1. Is there any work being done on the influence of placement of chemical nitrogen fertilizer on associative N fixation?

2. or on the use of fertilizer as carrier for bacteria?

3. What is the influence of P, K, S and other non-N fertilizer on associative fixation?

A - 1. In field studies, as far as nitrate or ammonia is involved, the bacterial activity is reduced, so that the benefit from the use of bacteria is also reduced if too much fertilizer is applied. The same can be found in test tubes. When too much nitrate is added, then the nitrogenase activity,

hence the N fixation, is stopped.

2. Not yet. It would be a most interesting combination.

3. It was not extensively investigated because usually, at least in test tube experiments, optimum rates of these different substances are given and only the amount of N is varied.

Q - Mr. K.H. WALTER, Adelaide & Wallaroo Fertilizers Ltd, Australia

In view of the fact that sulphate is easily leached from the soils and that legumes require as much sulphur as phosphorus, it appears to me that the manufacture of sulphur free fertilizers is unwise.

1. If your research is successful, does it mean that it could lead to a decline in demand for sulphur free fertilizers?

2. What micronutrients did you add to your agar medium?

A - 1. This question should be answered by specialists of legumes or sulphur.

2. There is a long list of these micronutrients, all of which are added in the cultural medium we use.