Solar protein

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Our major food plants may be divided into two types—those which need nitrogen fertilizer (cereal grains, such as corn, wheat, and rice) and those which do not (alfalfa, soybeans, and other legumes). The latter group produces protein by manufacturing its own nitrogen in a process that uses energy from the sun—hence, the term "solar protein."

Technology may someday provide an inexpensive source of nitrogen fertilizer. In the meantime, as fertilizer prices rise and supplies become limited, we may have to turn to the sun for more of our protein production.

Certain bacteria and blue-green algae are the only organisms known to convert atmospheric nitrogen gas into a form suitable for plant growth—a process, called biological nitrogen fixation, requiring energy from the sun. Legumes, such as alfalfa, clover, and soybeans, although without the necessary hereditary traits to supply their own nitrogen fertilizer, can become self-sufficient for nitrogen by attracting and "domesticating" symbiotic soil microbes that have the nitrogen-fixation (Nif) genes. Such genes code for the crucial enzyme nitrogenase that catalyzes the synthesis of ammonium ion (NH_4^+) from atmospheric nitrogen gas (N_2). Nif genes are also carried by a wide assortment of free-living bacteria in soil near plant roots—bacteria that may indirectly provide some nitrogen for the plant.

Root nodules on legumes contain the symbiotic bacteria which supply nitrogen to the host plant and receive carbon and energy from the host. In essence, through infection by a beneficial bacterium, the host plant acquires a new set of genetic traits—the Nif genes.

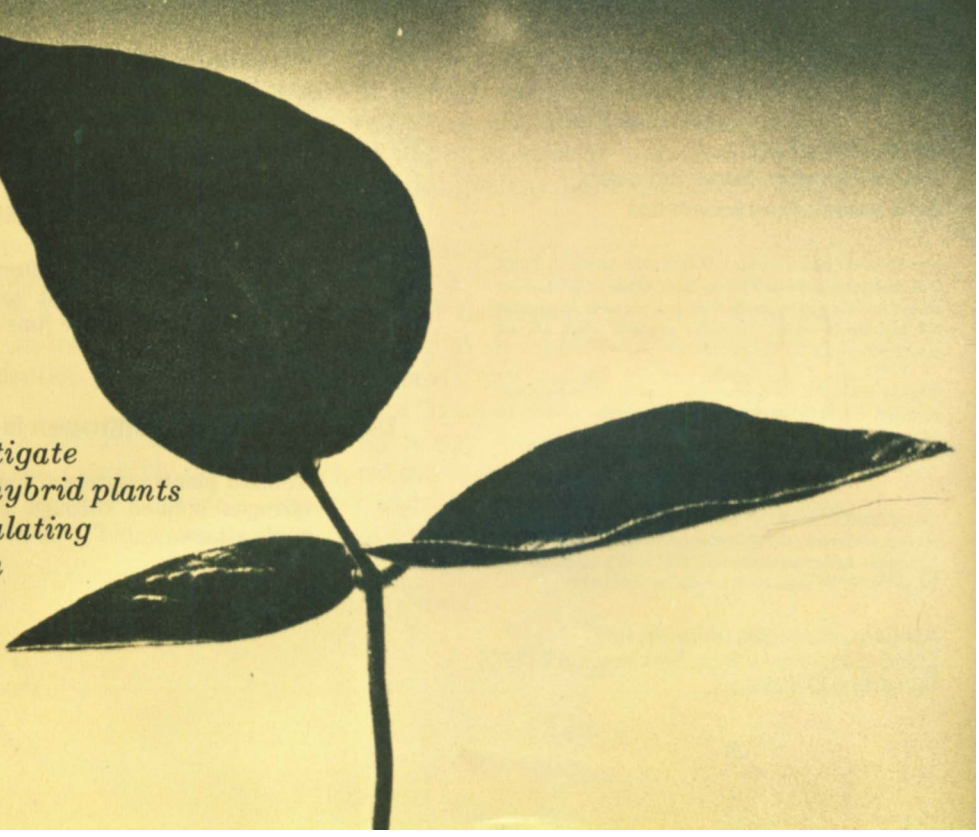
Genetic engineering with nitrogen fixation

Man has harnessed nitrogen-fixing microorganisms through the culture of soybeans and other leguminous crops. If we are to genetically engineer new hybrid plants self-sufficient for nitrogen, we must learn to manipulate the hereditary traits responsible for this process.

A major advance was made in 1971 by Stanley Streicher, a graduate student in the laboratory of R. C. Valentine, then at the Department of Biochemistry, U.C., Berkeley. Streicher discovered the Nif genes on the chromosome of a single-celled soil organism, *Klebsiella pneumoniae*, which may inhabit the human colon and actually fix some nitrogen in the human gut.

The *Klebsiella* chromosome is believed to be a circular DNA molecule. The *Klebsiella* chromosomal map seems to be similar to that of a related organism, *Escherichia coli*.

Mapping of the Nif genes, a crucial step in creating new nitrogen-fixing hybrids, was accomplished using a transducing virus, P_1. This virus, by mistake,



Research is under way to investigate the possibility of creating new hybrid plants self-sufficient for nitrogen, regulating and enhancing nitrogen fixation by legumes, and introducing a nitrogen-fixing symbiosis to grasses and cereals.

packages in the head of the virus small bits of the chromosome of the cell it has previously infected. (Normally the virus does not pack the host chromosome.) Among the progeny viruses, one could find a percentage of Nif-containing virus particles. Normal particles kill their host, but the Nif particles are harmless and may supply their new host with the ability to fix atmospheric nitrogen.

Transducing viruses thus transfer nitrogen-fixation hereditary traits from cell to cell. New hybrids are formed when the hereditary strand carrying the Nif trait introduced by the virus becomes permanently inserted into the host chromosome. This was the method first used to transfer the Nif trait. Mutant strains of the bacterium, *Klebsiella*, unable to fix nitrogen because of genetic lesions (nitrogen-fixation-defective mutants, Nif$^-$), were converted to active nitrogen fixers after infection with viruses grown on wild-type strains.

Streicher found most Nif genes to be grouped in a cluster adjacent to a well-charted region of the chromosomal thread. Dixon, in Postgate's laboratory at the ARC-unit on nitrogen fixation in England, was then able to transfer the Nif genes into the common gut bacterium, *Escherichia coli*. This experiment may be the first step in genetically engineering new, agronomically important, nitrogen-fixing hybrids.

Regulating nitrogen fixation

Nitrogen-fixing bacteria have evolved an elaborate chemical sensing device for monitoring the nitrogen available in their environment. For example, when nitrogen-fixing bacteria, such as *Klebsiella pneumoniae*, are grown in the presence of available pre-formed nitrogen, the biosynthesis of nitrogen-fixing enzymes is completely repressed. When nitrogen reserves become low, the nitrogen-fixation genes are "switched on" (induced), permitting the cell to convert atmospheric nitrogen gas to ammonium ion for building cell protein.

Recently, Shanmugam and Andersen of the Plant Growth Laboratory and the Department of Agronomy and Range Science, U.C., Davis, genetically altered the regulatory genes of nitrogen fixation, leading to the construction of an "artificial nodule" useful for studying several concepts of nitrogen fixation.

Some of the key considerations for microbial production of N fertilizer as determined from these studies are: (1) the nitrogen fixation genes must be switched on in both the presence and absence of N fertilizer; (2) bacterial genes responsible for using N fertilizer as ammonium ion must be switched off; (3) large quantities of energy are needed; (4) considerable genetic variability has been observed among NH_4^+-exporting bacterial strains; (5) the best bacterial strains in terms of longevity of export of fixed nitrogen appear to be those capable of maintaining a slow rate of growth.

Similar experiments with bacteria concentrated by the billions in the root nodules of plants, such as alfalfa, have led to a new concept of symbiotic nitrogen fixation. The nature of the regulatory signal(s) that switch on the Nif genes and permit most of the fixed ammonium to be partitioned to the plant is a fascinating question from the standpoint of the basic biology of symbiotic N_2 fixation and also may have important practical applications. For example, the development of active nodules may be inhibited by available nitrogen (NH_4^+ or NO_3^-) in the soil. It would be of considerable interest to construct "derepressed" strains no longer subject to nitrogen inhibition.

Enhancing nitrogen fixation by legumes

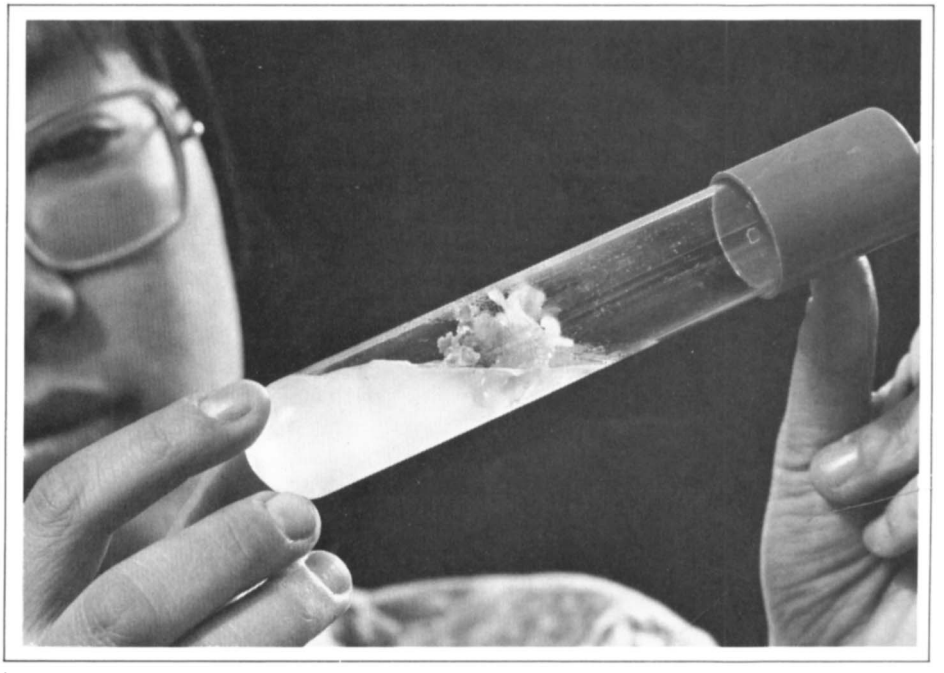
Legumes are high in protein, as compared with cereals, and they can be used in both human and animal diets. Somewhere in the future lies the discovery of alternate technology that will enable cereals to provide their own nitrogen needs. However, our best alternative for the near future seems to be to expand the use of the legumes presently available and concomitantly to seek ways to improve them.

Soybeans, the world's major grain legume, provided 47 percent of total world grain legume production in 1972; 67 percent of the soybean production was in the United States. Alfalfa, which ranks second to soybeans in total acreage grown in this country, is a highly productive plant that is an essential feed for much of our cattle and poultry.

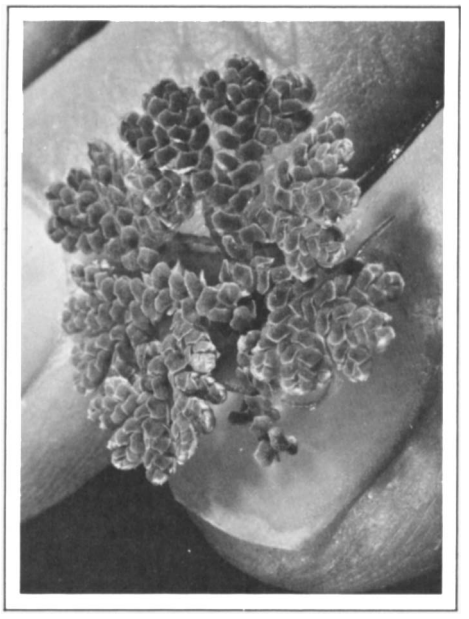
In spite of the agronomic importance of these crops, they have not risen sharply in productivity as have cereals, which, for example, respond dramatically to applied nitrogen fertilizer. However, recent advances in understanding the mechanism of nitrogen fixation may provide a basis for increasing legume yields.

For example, studies of the biochemistry of nitrogen fixation have led to the development of an easy, effective method of measuring the amount of nitrogen actually fixed in the field. Rather than laboriously measuring the nitrogen converted to ammonia, this method monitors a parallel reduction—that of acetylene (C_2H_2) to ethylene (C_2H_4) by the enzyme nitrogenase. One technician, using a gas chromatograph, can measure up to 200 samples a day in the soybean field.

Measurement of nitrogen-fixation rates under different field conditions led



New plant being grown from cell culture in test tube.



Tiny aquatic fern, Azolla, lives in symbiosis with blue-green algae and may be useful as a source of nitrogen for rice crops.

Hardy and co-workers at DuPont to conclude that the supply of photosynthate, the energy-rich product of photosynthesis in the green leaf, is the rate-limiting factor in soybean production of solar protein. The DuPont workers are currently experimenting with ways of increasing energy production by the soybean leaf to raise the ammonium production level in the energy-hungry nodules. The idea is to somehow make the carbon-dioxide-trapping machinery of the leaf more efficient, thus providing more sugar, which serves as fuel for ammonium ion production in the nodule.

To increase the photosynthate supply, the DuPont team used a technique they refer to as carbon dioxide fertilization—the technique greenhouse operators have been using for more than a decade in tomato and lettuce production. Here, fertilizing with carbon dioxide to overcome a deficiency in the air was done as one fertilizes with nitrogen, phosphorus, or potassium to overcome a deficiency in the soil.

In the DuPont experiment, carbon dioxide fertilization increased nitrogen fixation from 80-100 pounds of nitrogen per acre to more than 400. The soybean plant fixed more nitrogen during one week with carbon dioxide fertilization than non-carbon-dioxide-fertilized plants fixed during the complete growth cycle. This was a result of increasing the mass of root nodules and causing each gram of nodules to fix twice as much nitrogen. Total nitrogen in the mature plant approached the amount required for a yield of 100 bushels per acre—with more than 80 percent coming from nitrogen fixation and less than 20 percent from soil nitrogen.

Since it is not yet practical to "fertilize" air in the field with carbon dioxide, other approaches for achieving the same end need to be explored, such as breeding more efficient plants. There is a limit to just how much of a single enzyme protein can be packed into a plant cell, and this limit (20 to 50 percent) may have been reached for the carbon-dioxide-fixing enzyme.

If we cannot breed a plant with more, could this carbon-dioxide-fixing enzyme be genetically engineered to do its job with greater efficiency, so that the same amount of enzyme produced more sugar? In fact, a number of workers believe that the carbon-dioxide-fixing system of soybean and other plants may be wasteful, destroying sugar, even as it is made, by a process called photorespiration. Can a plant be genetically engineered with a more efficient carbon-dioxide-trapping system—one perhaps that does not destroy as much sugar? The chemist might approach this problem by constructing a molecule, to be sprayed on the green foliage, which specifically inhibits photorespiration.

Other workers believe more efficient nodules might be created to use the available sugar more efficiently, or might be engineered to accommodate increasing energy production geared to the hypothetical "high sugar" plants.

New nitrogen-fixing plants

The discovery of an association between nitrogen-fixing bacteria and corn may be one of the most promising developments for enhancing biological nitrogen fixation. Brazilian scientists Von Bulow and Dobereiner have recently reported that corn as well as certain tropical grasses in loose symbiotic association with free-living, nitrogen-fixing bacteria may fix significant quantities of nitrogen.

The tropical grasses do not form nodules, although an organism (*Spirillum*) thought to be the symbiotic agent has been isolated from infected plants. The Brazilian workers think that the tropical grass system may be an intermediate form between completely independent nitrogen-fixing bacteria and nodule-forming symbionts like *Rhizobium*. They suggest that this kind of symbiosis may be a good model for the development of symbiosis between nitrogen-fixing bacteria and grasses or cereal grain crops, which are much more efficient at trapping solar energy and producing photosynthate than are the legumes. Although, theoretically, this system seems promising, there are still a number of hurdles to overcome.

Von Bulow and Dobereiner report that corn may fix up to 2 kg of N_2 per hectare per day. This figure is obtained by extrapolating from nitrogen-fixation measurements on root fragments of corn, under conditions which, as the result of proliferation of *Spirillum* at the expense of decaying plant tissue, might give exaggerated high values. However, even if the actual rates turn out to be lower by an order of magnitude or two, this system is of great interest, because it may be possible to genetically engineer either the plant or *Spirillum*, or both, to enhance N_2 fixation.

In other words, despite the lack of solid experimental evidence to support yield increases in field crops attributable to *Spirillum* infection, the possibility remains that an effective symbiosis might be gained through genetic manipulation of this system. *Spirillum* clearly is capable of association with root systems of grasses and cereals such as corn, perhaps actually "infecting" the root tissue. Thus, *Spirillum* seems a logical choice of microbial vector for introduction of an N_2-fixing symbiosis to new plants.

Rice, one of the world's most important cereal grains, is grown in markedly different ways in different regions. For example, in California, large quantities of N fertilizer are applied, leading to extremely heavy yields. In contrast, much of the rice in the Orient receives only sparse nitrogen treatment because of the cost and unavailability of fertilizer. Although the yields of nonfertilized plots are lower than those achieved through heavy fertilization, year after year a consistent crop of up to 1.5 tons per acre has been harvested at the International Rice Research Institute, in the Philippines. There is increasing evidence that nitrogen-fixing microorganisms may contribute nitrogen in the rice paddy, although there is uncertainty regarding both the levels fixed and the dominant N_2-fixing species.

One of the most promising systems in rice involves a tiny aquatic plant (*Azolla*) and an alga (*Anabaena*). The nitrogen-fixing status of the fern, *Azolla*,

is due to "infection" with blue-green algae, which contribute this trait to the fern in exchange for still unknown materials supplied by the host plant. As in the case with the root nodule bacteria of leguminous plants, the nitrogen-fixation genes of symbiotic blue-green algae are probably derepressed, leading to the production of N fertilizer for the host plant. Nitrogen levels fixed by *Azolla* have been reported to be more than 600 kg N per hectare per year in the long tropical growing season.

As *Azolla* are currently managed, this nitrogen only becomes available to the rice plant indirectly through manuring of the decaying *Azolla* bloom. On the other hand, it may be possible in the future to genetically engineer the *Azolla*-blue-green-algae system to export N fertilizer in a form that could be used directly by the plant. Although this system of N input would not be as synchronized to the nitrogen needs of the rice plant as the root nodule system, where the nodule is physically attached, it has the advantage to the rice plant of using radiant energy directly from the sun for production of N fertilizer. So long as some light penetrates the canopy, *Azolla* can be grown simultaneously with rice plants. In more dense plantings, *Azolla* may be grown in a small block of open paddy and transferred as green manure to surrounding paddies.

Indeed, one of the most interesting aspects of the *Azolla* system is that N_2 fixation may not be limited by available energy as in the case for legumes. An abundance of energy might account for the prolific rates of N_2 fixation reported for *Azolla*.

In the future, it may be possible to use *Azolla* for ambient production of N fertilizer adaptable to a variety of cropping systems in California. Genetic variants of *Azolla*-blue-green algae that export fixed nitrogen may be cultivated in a separate paddy (or, in the case of rice, within the same paddy) connected to the irrigation system. Fixed nitrogen may be flushed from the *Azolla* paddy and continually (or batchwise) transferred to the field or orchard via the irrigation water.

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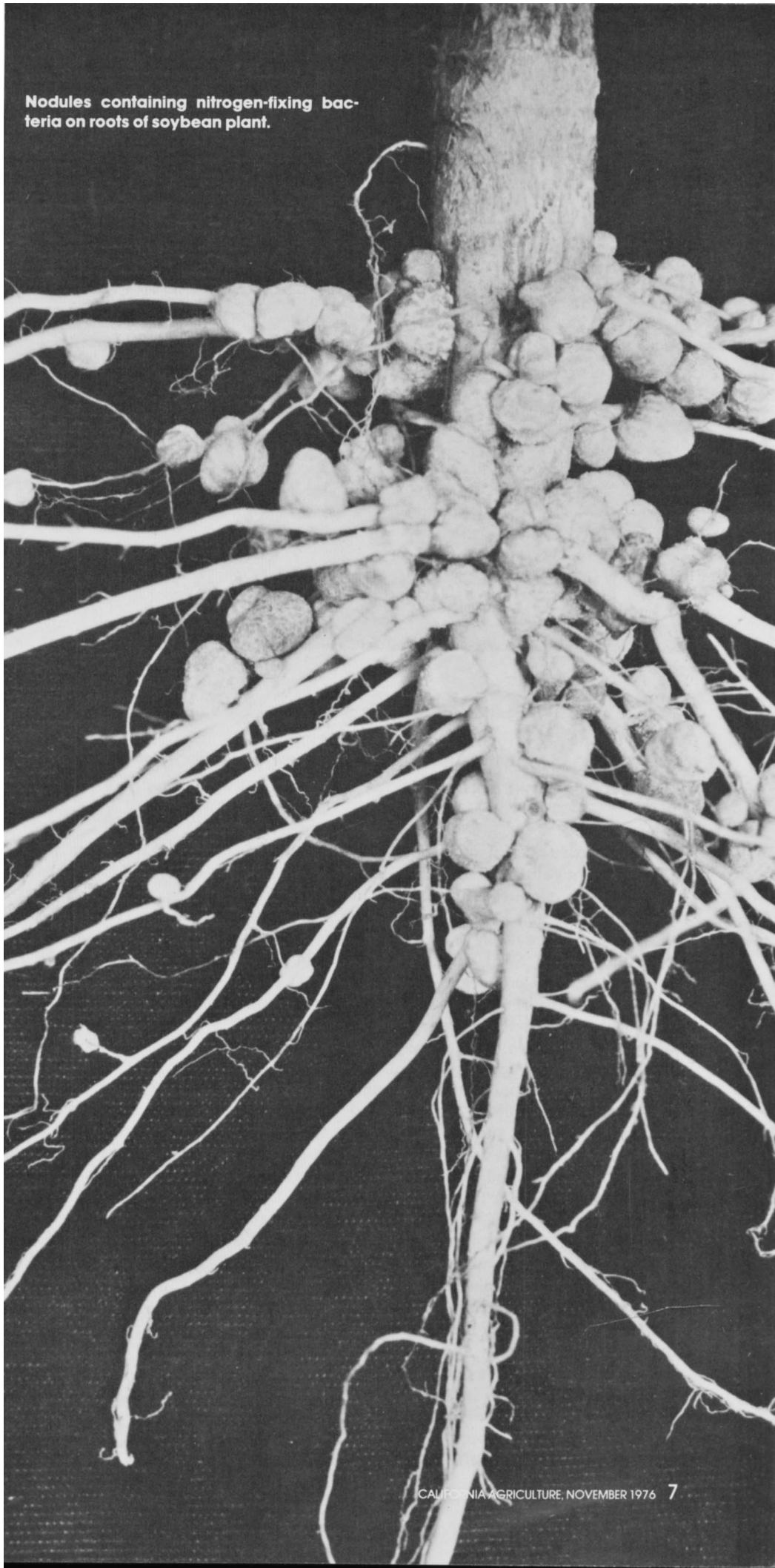

Nodules containing nitrogen-fixing bacteria on roots of soybean plant.