The prospect of N_2-fixing crops galore!

Edward Cocking and David Dent (University of Nottingham and the University of East Anglia, UK)

The impact of carbon on our climate has been of major concern for a number of years. However, we are now learning to be equally concerned about the next element in the periodic table, nitrogen, and the consequences of using synthetic nitrogen fertilizers in agriculture that pollute our planet and its atmosphere.

Using nitrogen-fixing bacteria isolated from sugarcane we can produce intracellular and systemically colonized crops that fix nitrogen symbiotically, and can now begin to free ourselves from this pollution and ensure more sustainable agriculture.

Nitrogen is one of the most important elements in biological systems and is an essential macronutrient for plants. Nitrogen gas (N_2) comprises 78% of the atmosphere. However, in the biological world, the ability to convert the inert triple bonded molecules of N≡N gas into a metabolically active form is limited to certain bacteria and archaea prokaryotes (diazotrophs, N_2 eaters). These nitrogen-eating prokaryotes possess the complex metalloenzyme nitrogenase that fixes nitrogen at ambient temperature and pressure by reducing N≡N to reactive ammonia. All plant and animal eukaryotes lack the nitrogenase enzyme and are therefore unable to carry out this environmentally benign, biological nitrogen fixation. However, legume crops are able to biologically fix nitrogen by forming nodules in the cells in which they symbiotically fix nitrogen *via* diazotrophic rhizobia bacteria.

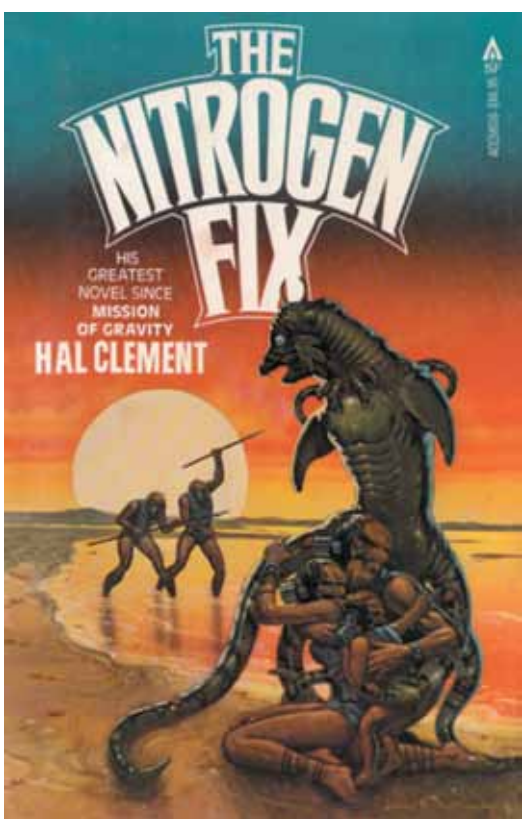

Figure 1. *The Nitrogen Fix*
The front cover illustration by David B. Mattingly of a novel of the ultimate ecological disaster (Reproduced with permission).

Nitrogen is crucial to crop yields but cereals and other non-legume crops do not symbiotically fix nitrogen with rhizobia. This inability leaves agriculture increasingly reliant on the use of synthetic ammonia-based nitrogen fertilizers. These are produced chemically from nitrogen and hydrogen gas at high temperature and pressure in the catalytic Haber–Bosch process, the industrial equivalent of bacterial nitrogen fixation. Chemical fixation of nitrogen can also result naturally from the action of lightning on oxygen and nitrogen gases in the atmosphere yielding nitric acid. There is a thought-provoking science-fiction novel, *The Nitrogen Fix* by Hal Clement (1980), which is based on the proposition that the production of polluting nitric acid has used up all of the oxygen in the air, so that humans have to live in artificial environments protected from the all-encompassing acid (Figure 1). Although not as extreme, the overuse of synthetic nitrogen fertilizers has resulted in the pollution of aquatic systems by soluble nitrates with associated eutrophication and health hazards. In addition, the atmosphere has been polluted by ammonia and nitrous oxide, the latter being a potent greenhouse gas associated with photochemical smog, fine particulate pollution, ecosystem acidification and climate change. The environmental, energy and economic costs of producing and using ammonia-based fertilizers globally are no longer considered sustainable; if all crops were nitrogen fixing, then nitrogen sustainability of agriculture would be assured.

Thinking outside the nodule?

To enter legume nodule cells, rhizobia usually secrete cellulases that micropuncture the plant cell wall to form

Figure 2. The Brazilian scientist Professor Johanna Döbereiner (1924–2000) who discovered *Gluconacetobacter diazotrophicus* in sugarcane in Alagoas, Brazil, jointly with Vladimir Cavalcante. (Reproduced with permission from Brazilian Academy of Sciences Archive.)

a portal of entry large enough for bacterial penetration, becoming intracellular *via* endocytosis into membrane-bound nitrogen-fixing symbiosomes. Rhizobia are then trapped inside nodule cells and are unable to become systemic in the crop, and hence are unable to utilize the cells of the root, stem and leaves outside of the nodule as a habitat for symbiotic nitrogen fixation. Other endophytic microorganisms are more versatile. In the leaf glands of the non-legume garden plant *Gunnera* the nitrogen-fixing cyanobacterium *Nostoc* establishes intracellular symbiotic nitrogen fixation without any nodule formation; and in the intracellular root symbioses of legumes and non-legumes, including cereals, with phosphate-acquiring arbuscular mycorrhizal fungi there is no nodulation. Establishing symbiotic nitrogen fixation in crops, without nodulation, will require not only extensive systemic intracellular colonization by diazotrophic bacteria but also the protection of their nitrogenases from inactivation by oxygen. For instance, the nitrogenase complex of rhizobia is readily inhibited by oxygen, and rhizobia are now regarded as being unable to establish symbiotic nitrogen fixation in non-legume crops; regulating the flux of oxygen to endosymbiotic rhizobia has been suggested to be an insuperable challenge to the dream of effectively introducing rhizobial nitrogen-fixing symbiosis into non-legume crops.

Why are there no nitrogen-fixing crops?

Legume crops such as peas and beans are often said to be able to fix nitrogen but of course they do not. These plants actually form symbiotic associations with nitrogen-fixing rhizobia which supply fixed nitrogen as ammonia to the crop for its growth and development. Microbiologists and plant physiologists are agreed that, in terms of energetics, this bacterial nitrogen fixation should present no evolutionary obstacle to plants. During biological nitrogen fixation, eight molecules of ATP are required for every half N_2 molecule converted to NH_3 by bacterial nitrogenase, and an additional six ATP are required as a reductant, resulting in the need to produce 14 ATP/NH_3 molecule. However, crops mainly take up their nitrogen from the soil as nitrate which first has to be reduced by the plant to ammonia before it can be metabolized. This reduction of NO_3^- requires 12 molecules of ATP to provide one molecule of NH_3; thus, nitrogen fixation is only marginally more demanding than nitrate reduction in terms of energy consumption. Consequently, crops would not be likely to suffer any significant energy penalty if they were nitrogen fixing, and in legumes there is no evidence to support any suggestion that nitrogen fixation reduces yield.

It is an evolutionary enigma that endosymbiosis of diazotrophs in plants cells has not yet resulted in the generation of a nitrogen-fixing organelle and the formation of an autonomous nitrogen-fixing plant. Mitochondria and chloroplasts of eukaryotic cells are key organelles for respiration and photosynthesis, and are thought to have resulted from an ancient endosymbiosis in which bacterial-like cells were engulfed by endocytosis into an ancient eukaryotic-like cell. Nitrogen fixation remains an evolutionary option for plants. It is surprising that no plant has yet followed the easiest path to become independent of bacteria by forming a nitrogen-fixing membrane-bound symbiosome, a 'diazoplast', a new nitrogen-fixing organelle analogous in origin to a mitochondrion or chloroplast.

An extraordinary diazotroph

Such a diazoplast would require an initial association of the crop with a symbiotic nitrogen-fixing bacterium that has a highly developed intracellular capability; a characteristic that is closely correlated with genome size in bacteria. Interestingly, among a range of plant endosymbionts the nitrogen-fixing bacterium *Gluconacetobacter diazotrophicus* (Gd for short) emerged as having a sufficiently small genome to place it firmly in the facultative intracellular colonizer category. This same species is also known to have a respiratory system that enables the bacterium to survive in a wide range of oxygen concentrations and to respire at rates among the highest ever reported for aerobic bacteria. These are characteristics that, within an evolutionary timescale, place Gd among a number of other candidate species for the role of potential proto-mitochondria.

Gd was discovered in sugarcane in 1988 by Johanna Döbereiner and Vladimir Cavalcante, with the bacteria reportedly fixing around 60% of the plant's nitrogen needs (Figure 2). The species was discovered in the sugar-rich xylem vessels and was evident intercellularly throughout the stems and leaves and was thus, non-nodule forming. Gd is a Gram-negative, non-spore forming endophyte belonging to the family of bacteria called Acetobacteraceae—the acetic acid bacteria; a family that has been used extensively in food and cosmetic production.

Research has shown that Gd fixes nitrogen over a broad range of oxygen concentrations and excretes about half of

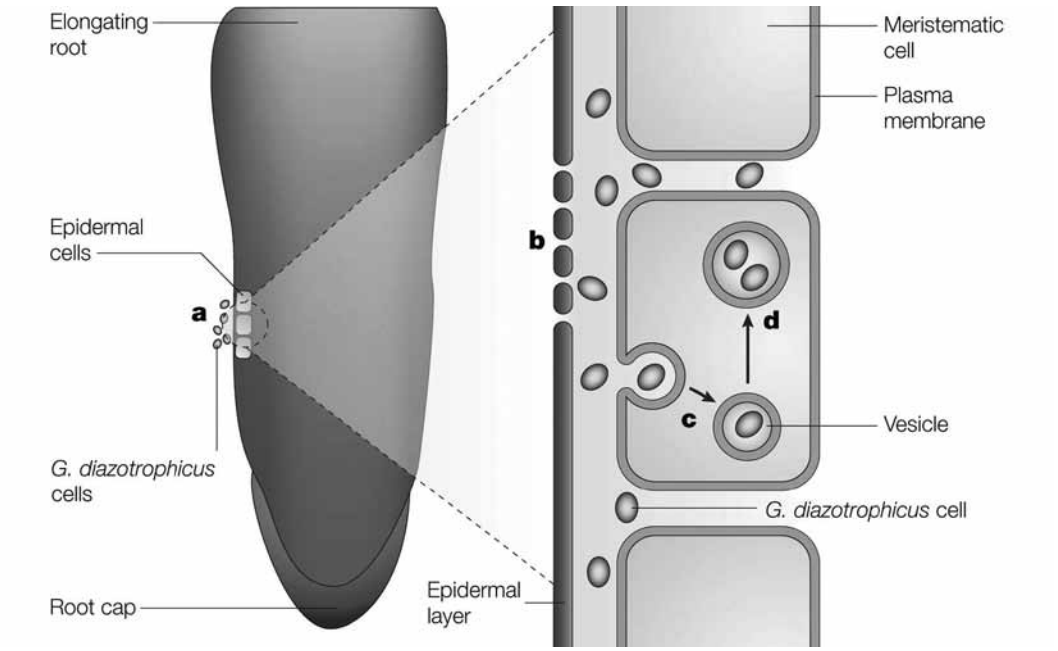

Figure 3. Schematic uptake of Gd with cereal roots. **(a)** Meristematic zone of the elongating root, **(b)** Gd penetrates the epidermal cell wall by secreting cellulase enzymes, **(c)** the plasma membrane pinched off via endocytosis forms a membrane surrounding vesicles containing Gd, **(d)** vesicles with Gd are surrounded by a membrane analogous to the symbiosome membrane of rhizobia. (Reprinted with permission, Chapter 3 in Agronomy Monograph No. 52, Nitrogen Fixation in Crop Production, D.W. Emerich and H.B. Krishnan (Editors), American Society of Agronomy, Crop Science Society of America, Soil Science Society of America, 2009)

the nitrogen it fixes as ammonia. It also produces plant growth hormones and is able to cope with the osmotically stressful conditions of high sucrose concentrations, low oxygen levels and acidic conditions. A range of differences between strains have been identified, including expression of cell wall degrading enzymes, intracellular (as opposed to intercellular) colonization, responses to nitrates, as well as siderophore and bacteriocin production: a range of attributes that very early-on earned this bacteria the title of 'this most extraordinary diazotroph' (Figure 3).

Having an isolate of Gd with the ability to intracellularly and systemically colonize a range of crop species and in doing so, fix nitrogen, is crying out for further development and commercialization. The successful use of rhizobia as legume seed inoculants provides a route map for the development and delivery of an extensive symbiotic nitrogen fixer.

The first step of such a development, i.e., a seed inoculant formulation, is to determine the optimum dose and form of the bacteria to be used. This can then be combined with a surfactant to help spread the bacteria onto the surface, an adhesive to hold the bacteria in place and an energy source to keep the bacteria alive. Following seed treatment and bacterial colonization of the plant (assessed using direct or indirect quantitative and qualitative methods), a range of laboratory techniques can be used to measure the amount of nitrogen being fixed by the bacteria. The traditional technique is the acetylene reduction assay, however more recently, greater emphasis has been given to the use of mutant bacteria lacking nitrogen fixation genes, so called *nif*- mutants, and comparing the extent of fixation relative to the wild-type bacteria. More recent still, nanoSIMS microscopy with $^{15}N_2$ has been used to visually identify the extent and location of intracellular nitrogen fixation resulting from Gd colonization. If the fixed nitrogen is being utilized by chloroplasts then this may have a positive impact on the efficiency of photosynthesis, a phenomenon that has been observed experimentally in Arabidopsis with Gd.

Every technique used to measure the field efficacy of legume rhizobial inoculants has also been used to determine the effectiveness of Gd inoculants, with similar positive results. These include increases in indices of crop greenness, levels of leaf nitrogen, increases in crop yield (including biomass in grass) and in grain protein content in wheat. The yield impact has also been demonstrated to be additive with respect to nitrogen fertilizer use, thereby allowing farmers to make choices about balancing the rate of synthetic nitrogen fertilizer use and crop yield benefits.

The greening of agriculture

Utilizing energy from photosynthesis, cells of the roots and shoots of a steadily increasing range of crops intracellularly colonized by Gd, can now be used as biological nitrogen-fixing factories for the synthesis of ammonia from nitrogen and hydrogen. The very close association of Gd with chloroplasts in leaf cells, resulting in the formation of diazo-chloroplasts, could increase crop photosynthetic efficiency with consequent increases in crop yields by decreasing the photorespiration activity of RuBisCO, the most abundant plant enzyme. This greener nitrogen revolution will be a key component of the 'Greening of Agriculture', enhancing crop productivity in perpetuity, minimizing the use of environment-polluting synthetic nitrogen fertilizers and ensuring the sustainability of agriculture. ■

Edward Cocking is a research Professor Emeritus and presently Director of the Centre for Crop Nitrogen Fixation at the University of Nottingham. Trained in plant physiology and biochemistry, cell biology and bacteriology, he has extensive experience of research group planning, training and laboratory organization, particularly in projects relating to plant protoplasts and tissue culture, their fusion for plant somatic hybrid production and in crop systemic intracellular colonization by nitrogen-fixing Gluconacetobacter diazotrophicus *bacteria. He is currently establishing symbiotic N_2-fixing crops in agricultural practice for enhanced food security and environmental protection.*
Email: Edward.Cocking@nottingham.ac.uk

David Dent is the Royal Society Entrepreneur in Residence at the School of Biological Sciences, UEA and Founder Director and former Chief Technical Officer of Azotic Technologies Ltd established to develop and commercialize intracellular nitrogen fixation by Gluconacetobacter diazotrophicus. *Trained as an agricultural ecologist he has experience managing international science-based and technology commercialization projects, establishing, raising funds for and working in spin-out companies, as well advising governments and business on science, technology and innovation. David is currently a Visiting Professor at the University of Surrey Business School and a Special Advisor to the UK's Parliamentary and Scientific Committee.*
Email: david.dent@uea.ac.uk

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