Establishing intracellular symbiotic nitrogen fixation in crop plants for reduced inputs of synthetic nitrogen fertilizers

Prof Edward Cocking FRS
University of Nottingham, UK

The oil crisis of the early 1970s highlighted the ever increasing cost of synthetic nitrogen fertilizers for crop production, and their pollution of the atmosphere and water systems has become a major environmental concern throughout the world. Since then in the Centre for Crop Nitrogen Fixation at the University of Nottingham we have sought ways to find a biological symbiotic substitute for synthetic nitrogen fertilizers in agriculture. Our aim was to match Haber's ingenuity, in producing NH_3 from N_2 and H_2 at high temperature and pressure, by the use of naturally occurring nitrogen-fixing bacteria interacting symbiotically with crop plants. This would utilise their nitrogenase enzyme to reduce N_2 to NH_3 at ambient temperature and pressure and thereby grow and sustain agriculture without sacrificing the environment.

Legume crops such as peas and beans which already fix nitrogen from the air symbiotically, thereby eliminating the need for synthetic nitrogen fertilizers, are able to interact with nitrogen fixing rhizobia bacteria which become established intracellularly within nodules on their roots. Our approach for more than two decades was to attempt to imitate this nodulation interaction of legumes by inoculating cereals and other non-legume crops with a wide range of species of rhizobia in national and international collaborations with support from BBSRC, the Rockefeller Foundation, The Royal Society and DFID. We clearly established that in response to inoculation nodulation, which is genetically controlled by the plant, could occur but that there was no intracellular colonization of the cells of the nodules by rhizobia and therefore no resultant symbiotic nitrogen fixation.

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"The challenge for global agriculture is to grow more food on not much more land, using less water, fertilizer and pesticides than we have historically done"

Sir John Beddington FRS
UK Government Chief Scientific Adviser

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THE PATH TO SYMBIOTIC NITROGEN FIXATION IN NON-LEGUME CROPS

A. Our Earlier Attempts

- **Plant cell walls act as a major barrier to the invasion of plant cells by bacteria and other microorganisms.**

- **My development in 1960 (1) of an enzymatic method to degrade plant cell walls using cellulases resulted in the availability of plant protoplasts – plant cells lacking their cellulosic walls which could be used to investigate the extent they could be intracellularly colonized by bacteria, other microorganisms, viruses and also inert latex particles of various sizes.**

- **Interaction directly with the plasma membrane of protoplasts was readily possible, and uptake by endocytosis was extensively investigated by light and electron microscopy. This was in parallel with extensive studies on the fusion of protoplasts to produce novel plant somatic hybrids not possible by sexual hybridisation (2).**

- **What became clear from these investigations of endocytosis in protoplasts was that intracellular colonization of bacteria and viruses into membrane bound vesicles was taking place (3).**

- **My desire to focus on further investigations using nitrogen-fixing rhizobia, isolated from legume nodules, was enabled by a generous unsolicited project grant from Rank Hovis McDougall. This desire arose from my PhD on nitrogen metabolism in barley plants and being fascinated as to why legume crops, but not cereals such as barley, could establish symbiotic nitrogen fixation with rhizobia.**

- **A key feature of symbiotic nitrogen fixation in legume crops is the establishment of rhizobia in membrane bound vesicles in the cytoplasm of their nodule cells. My thinking was that this might now be imitated in non-legume crop protoplasts, but without the need for nodule formation, since we had developed the technology for**

protoplasts to re-form a cell wall, divide and regenerate into plants that would be systemically colonized by intracellular rhizobia in membrane bound vesicles fixing nitrogen symbiotically.

- **In extensive investigations we demonstrated the uptake of rhizobia by non-legume protoplasts (4), and also the uptake by endocytosis of other nitrogen-fixing microorganisms (5). But regenerating plants from these protoplasts was not possible, probably due to negative effects of endocytosis on cell division and the difficulty of regenerating plants from cultured protoplasts.**

- **Because of these difficulties our approach shifted to attempts to stimulate nodulation of cereals and other major non-legume crops by inoculation with a range of rhizobial strains, coupled with various chemical and enzymatic treatments. We successfully nodulated rice, wheat, maize and oilseed rape and collaborated in an international programme supported by the Rockefeller Foundation, but we could not get the rhizobia to intracellularly colonize nodule cells and establish symbiotic nitrogen fixation (6, 7). We abandoned any approach to further obtain nitrogen fixing nodulation when other researches showed that all genes for nodulation were plant genes, and that nodulation could occur in the absence of rhizobia. It was clear that the basic key requirement was the intracellular colonization of plant cells by nitrogen-fixing bacteria to achieve symbiotic nitrogen fixation, and that nodulation was not an essential requirement.**

- **The findings in Brazil that in sugarcane colonization of xylem and intercellular spaces by nitrogen-fixing bacteria provided fixed nitrogen suggested to us another approach to establishing symbiotic nitrogen-fixing interaction with rhizobia. This resulted in our undertaking an extensive investigation of the internal colonization of rice, wheat, maize, oilseed rape and the model non-legume Arabidopsis by a wide range of labelled rhizobia (8, 9) in the presence of compounds likely to stimulate entry and internal**

colonization. We observed rhizobia growing in xylem and in intercellular spaces and some suggestions of nitrogen fixation – but it was clear that intracellular colonization of living cells by rhizobia was not taking place. All the indications were that rhizobia were not suitable for the establishment of the intracellar colonization of living cells of the roots and shoots of these non-legumes and that a totally fresh approach was required using nitrogen-fixing bacterial species other than rhizobia.

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B. <u>More Recent Investigations</u>

In 1988 a new species of a non-nodulating, non-rhizobial nitrogen-fixing bacterium (Gluconacetobacter diazotrophicus, G.d) was isolated in Brazil from the intercellular juice of sugarcane (non-legume) stems, and it was shown that the bacterium grew in the sucrose-rich intercellular environment and could supply 50% of the plant's overall fixed nitrogen. Somewhat similar results were obtained with Mexican sugarcane varieties. We thought that in other non-legumes, with much lower levels of intercellular sucrose, that inoculation with G.d might enable even more effective nitrogen fixation if G.d could become intracellular in roots and stems rather than just growing in the sucrose-rich intercellular spaces as in sugarcane. This would imitate the intracellular colonization of legumes resulting in symbiotic nitrogen fixation, but without the need for actual nodule formation. Our discovery that this is indeed possible using a Mexican strain of G.d is now enabling symbiotic nitrogen fixation to be established in a range of non-legumes, and legumes.

We have shown in patents granted in the US and EU, and in subsequent primary publications, that interaction of G.d with maize, rice, wheat, oilseed rape, tomato and the model non-legume Arabidopsis results in extensive intracellular colonization of roots and shoots and into seed progeny. We demonstrated the generality of intracellular colonization in this wide range of plant species (both non- legumes and legumes), its stimulation by sucrose and the need for interaction with very few G.d bacteria. To visualise in sections the bacteria unambiguously within membrane bound compartments in the cytoplasm, G.d labelled with a blue histochemical marker gene was used to inoculate seedlings (Fig 1). In maize for instance we demonstrated that 7 days post inoculation 91% of inoculated plants had intracellularly colonized root tips as in Fig 2. 21 days post

inoculation G.d had spread into the shoot system and blue-stained G.d could be detected in chloroplast-containing leaf cells (Fig 3). In divisional US and EU patents we showed microscopically and by molecular characterisation of seed progeny from inoculated seedlings that G.d in membrane bound cytoplasmic compartments could be transmitted to seed progeny.

It has been shown that G.d is able to excrete the nitrogen it fixes from the air in a form (NH_3) that is potentially available to plants. In all the non-legume crops investigated, using inoculation with G.d carrying a nitrogenase gene promoter linked to the blue histochemical gene marker, expression of this construct to turn the G.d bacteria blue demonstrated that intracellular conditions in the cytoplasmic compartments containing G.d were suitable for nitrogenase gene expression and nitrogen fixation. This was confirmed by direct measurement of the nitrogenase enzyme activity of G.d inoculated wheat seedlings using the acetylene reduction gas chromatography assay during 24 hours, and an activity level per wheat seedling of 50% of that of clover inoculated with rhizobia was recorded. Additionally this was also confirmed in maize intracellularly colonized by G.d, using the heavy isotope of nitrogen (^{15}N) to show that up to 30% of total nitrogen per plant was derived from atmospheric nitrogen by symbiotic nitrogen fixation in young plants.

Further proof of concept has been obtained in oilseed rape seedlings inoculated under controlled growth room conditions and systemically intracellularly colonized by G.d. Inoculated seedlings were deprived of any inputs of fixed nitrogen synthetic fertilizer but nevertheless they were able to grow for many months forming green shoots and leaves (Fig 4), proving that oilseed rape plants, initially colonized in their root meristem cells (Fig 5) can obtain all their required nitrogen from atmospheric nitrogen as a result of systemic intracellular colonization and resultant symbiotic nitrogen fixation.

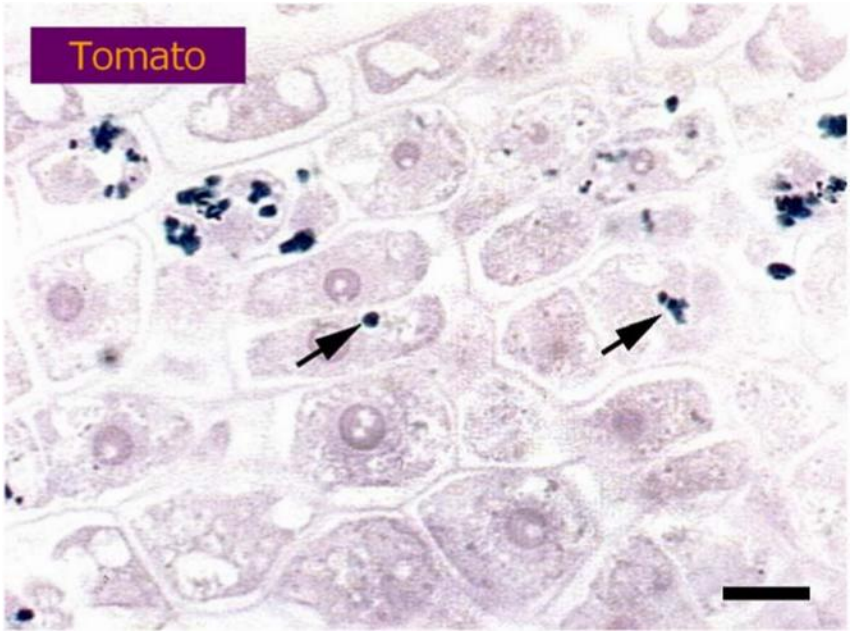

Fig 1

Section of region of tomato root tip showing blue-stained *G. diazotrophicus* bacteria (arrows) within cells (scale bar = 10 µm).

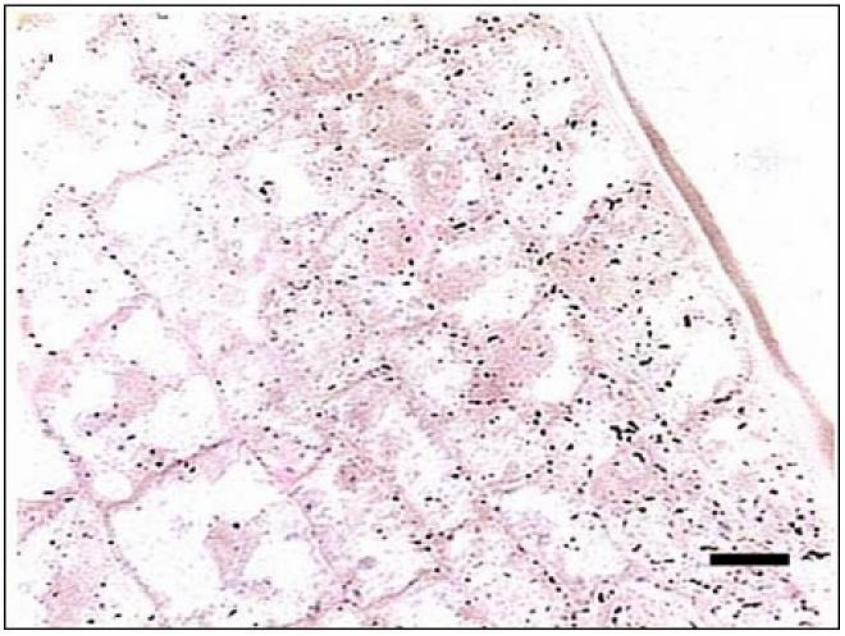

Fig 2

Section of edge of maize root tip showing blue-stained *G. diazotrophicus* bacteria within cells (scale bar = 10 µm).

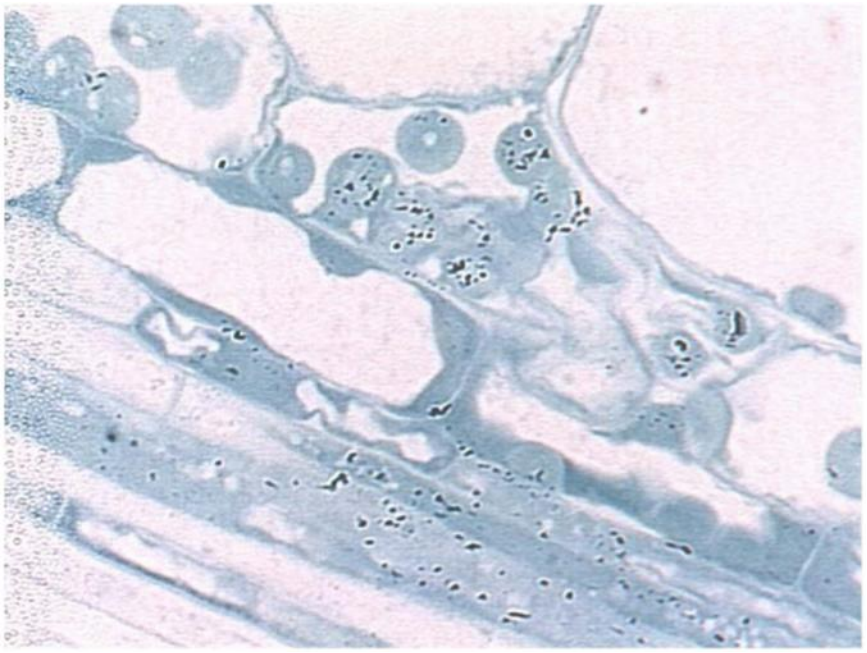

Fig 3

Section of region of maize leaf showing blue-stained *G. diazotrophicus* bacteria within cells and closely associated with chloroplasts.

Fig 4

Oilseed rape, inoculated with *G. diazotrophicus* and systemically intracellularly colonized, growing for many months without any inputs of fixed nitrogen synthetic fertilizer, and forming green shoots and leaves.

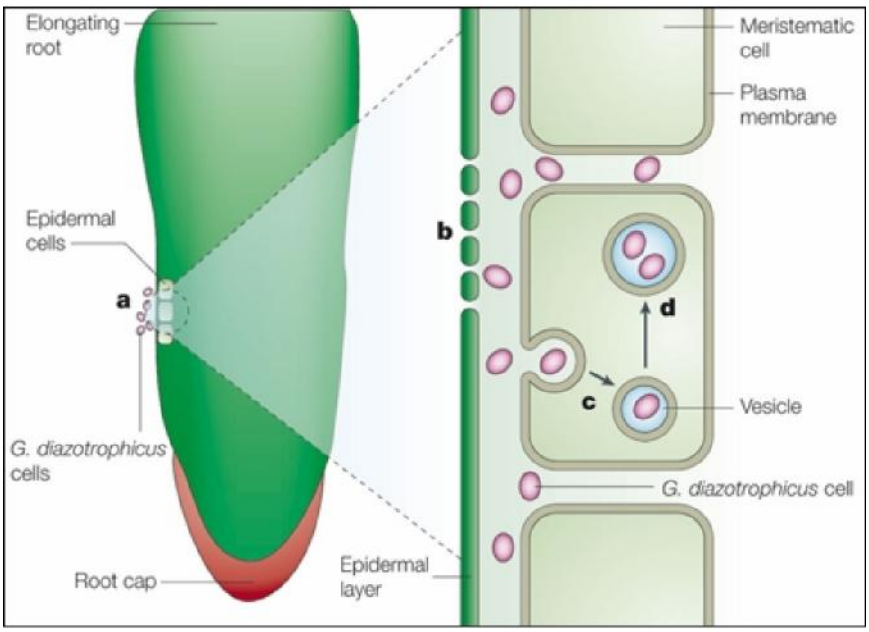

Fig 5

Schematic representation of the interaction of *G. diazotrophicus* with roots: (a) meristematic zone of the elongating root, (b) *G. diazotrophicus* penetrates the epidermal cell wall by secretion of cellulase enzymes, (c) the plasma membrane pinched off via

endocytosis forms a membrane surrounding vesicles containing *G. diazotrophicus*, (d) vesicles with *G. diazotrophicus* are surrounded by a membrane analogous to the symbiosome membrane of rhizobia.

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Prof Edward Cocking FRS

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The Company
Azotic Technologies Ltd was established in January 2012 to develop and commercialise a coating technology, which is a natural nitrogen technology providing a sustainable solution to fertiliser overuse and nitrogen pollution. This technology is unique to Azotic. It is patent protected, providing proof of concept and enabling commercial development. Azotic has a highly experienced management team with a very strong track record in international agriculture.

Product
Azotic's coating technology is based on a beneficial bacteria Gluconacetobacter diazotrophicus for coating plant seeds in order to create a symbiotic relationship within the plant enabling it to substitute the nitrogen it normally takes up from the soil with atmospheric nitrogen – thus reducing dependency on nitrogen fertilisers.

Azotic's patented technology has the following features:

- It is environmentally friendly and is ubiquitous to all crops.
- It provides every cell in the plant (leaves, stems and roots) with the ability to fix its own nitrogen.
- It can provide approximately 50% of the plant's nitrogen needs.
- It is neither genetic modification (GM) nor bio-engineering.
- It offers a significant cost benefit to the grower through reduced fertiliser costs.

Azotic Technologies is currently generating new IP based on further research and extension of the current patents. Two new patent applications have been filed:

- Seed germination at low temps
- Novel approach to perennial crops

This allows for market segmentation and will generate multiple products which will address a growing focus in agriculture.

Market Potential
The accessible markets available to Azotic Technologies are liquid inoculants, freeze dry powders and seed coatings on all crop species.

Azotic Technologies has achieved a world first covering the following:

- The seed coating technology applies to all crops.
- Proof of concept of colonised crop seedlings on grass, wheat, corn, rice, tomato and canola.
- Proof of atmospheric nitrogen fixation.

Commercialisation
We have a positive commercial strategy with options on various routes to market. It is anticipated that our nitrogen fixing products will be market ready by late 2014 or early 2015. The potential for sales in the first two years is increased by focusing on key crops in key countries. Our initial focus, based on current financial resources, is on grass, wheat, canola, corn and potatoes. Our priority markets are the UK, Europe, North America and Mexico.

Azotic is currently working on field trials in order to produce robust efficacy data. This will be followed by seeking regulatory approval in the UK, Europe, USA, Canada and Brazil. Although the Company has made substantial progress it is still raising investment monies in order to fully achieve its target milestones.

Tel: + 44 (0) 1257 226590| DD: 44 + (0) 20 8446 8000 | E: allen@azotictechnologies.com |www.azotictechnologies.com