FROM PLANT PROTOPLASTS TO DIAZOPLASTS: THE CHALLENGE OF ESTABLISHING SYMBIOTIC NITROGEN FIXATION IN CEREALS

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It is a fitting testimonial to the unique scientific contributions, in the disparate fields of electromagnetic waves and plant life, of Sir Jagadis Chandra Bose FRS that his bust commemorating his 150th Birth Anniversary now stands alongside that of Charles Darwin FRS in the garden of Christ’s College Cambridge. It is also a fitting testimonial that latest developments in the plant sciences from plant physiology to plant biology should be highlighted in this 150th Birth Anniversary Commemorative International Symposium; Bose was unusual as a physicist in taking up biological materials for his investigations.

The Challenge

When it was established at the end of the 19th century that legume nodules fixed atmospheric nitrogen (N2), attempts were made to extend this symbiotic interaction with nitrogen-fixing bacteria (rhizobia) to a wider range of crops. This was primarily to reduce the dependence of cereal production on natural resources of reactive nitrogen, particularly of Peruvian guano and Chilean saltpetre (sodium nitrate) then under threat of being exhausted. However, this interest in extending the range of crop symbiotic biological N2 fixation declined steadily after Fritz Haber filed his patent on the “Synthesis of ammonia from its elements” in October 1908. Haber had discovered how ammonia (NH3), a chemically reactive highly usable form of nitrogen, could be synthesized by reacting atmospheric dinitrogen (N2) with hydrogen in the presence of a catalyst (iron) at high pressures and temperatures1. Haber’s invention of industrial ammonia synthesis is one of the cornerstones of modern civilization and for more than 100 years agriculture has progressively come to rely on synthetic nitrogen fertilizers produced from ammonia. The annual world consumption of synthetic nitrogen fertilizers manufactured with fossil fuels by the Haber-Bosch process has averaged 85 million Mg N in recent years, with nearly 60% of that amount applied to cereal crops; application rates in North America are 112 kg N/ha, 155 kg N/ha in East Asia but only 9 kg N/ha in Africa2. There are major detrimental effects on the environment resulting from the over-use of synthetic nitrogen fertilizers in agriculture and the resulting pollution of the atmosphere by oxides of nitrogen and water by nitrates3. A large share of the nitrogen applied in agriculture is lost to the environment; this is a major contributor to photochemical smog, fine particulate pollution, ecosystem acidification and fertilization, coastal eutrophication and global warming. The overall central challenge is how to optimise the use of fixed nitrogen to not only produce enough food to meet demand from population increase, but also to minimize the impacts of chemically fixed nitrogen on the environment and human health — a key need in this respect is to establish symbiotic nitrogen fixation in cereals and other major non-legume crops to minimise the use of synthetic nitrogen fertilizers.

In his Nobel Peace Prize lecture in 1970 Norman Borlaug4 also highlighted the need to extend the symbiotic nitrogen fixation of legumes with rhizobia to

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the world’s major cereals, maize, rice and wheat to sustain the Green Revolution. “In my dream I see green, vigorous, high-yielding fields of wheat, rice, maize, sorghum and millet which are obtaining, free of expense, 100 kilogram of nitrogen per hectare from nodule-forming, nitrogen-fixing bacteria. This scientific discovery has revolutionized agricultural production for the hundreds of millions of humble farmers throughout the world, for they now receive much of the needed fertilizer for their crops directly from these little wondrous microbes that are taking nitrogen from the air and fixing it without cost in the roots of cereals, from which it is transformed into grain”. He acknowledged that even though high-yielding dwarf wheat and rice varieties were the catalysts that ignited the Green Revolution, chemical fertilizers, particularly synthetic nitrogen fertilizers produced from fossil fuel by the Haber-Bosch process were the fuel that enabled its forward thrust. Haber himself anticipated such a possible development in his 1920 Nobel Prize acceptance speech “it may be that this solution is not the final one. Nitrogen bacteria teach us that nature, with her sophisticated forms of the chemistry of living matter, still understands and utilises methods which we do not as yet know how to imitate”. Additional impetus to try to establish nitrogen-fixing (diazotrophic) symbioses in the cells of non-legume crops has come from the realization that the amount of energy, from plant photosynthesis, required for the reduction of N₂ to ammonia in the symbiotic nitrogen fixation of plants with diazotrophic bacteria is similar to that required for the formation of ammonia by the reduction of nitrate, the main form of nitrogen assimilated by plants. Consequently, cereals would not be likely to suffer any significant energy penalty if they were nitrogen fixing.

Features of Existing Symbiotic Nitrogen Fixation

The most efficient N₂-fixing bacteria establish an intracellular symbiosis with plants in which they fix nitrogen inside the cells of their host utilising energy for nitrogen fixation supplied by plant photosynthesis. Within their host cells bacteria are separated from the plant cytoplasm by a membrane derived from the plant cell plasma membrane. In legume nodule cells rhizobia become internalised by a process resembling endocytosis (Fig. 1) in membrane-bound vesicles (secondary vacuoles) within the cytoplasm. In these symbiosomes the N₂-fixing bacteria are surrounded by a plant-derived membrane, the symbiosome membrane. Whilst intracellular colonization of nodules occurs in legumes, nodule formation as such is not an essential requirement for the microbial symbiotic intracellular colonization of plants. In angiosperm Gunnera spp. the cyanobacterium Nostoc establishes intracellular symbiotic nitrogen fixation without 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. Fully efficient use of atmospheric nitrogen (N₂) for plant growth can only be expected in endosymbiotic systems since only here can the prerequisites for symbiotic nitrogen fixation be fulfilled: reliable supply of metabolic substrates by the host photosynthesis providing sufficient energy and reducing conditions, protection against too high oxygen concentrations, transport of the N₂-fixation products to the host, development of membrane systems for bi-directional transport between host and endosymbiont and protection against competitive or antagonistic bacteria in the environment.

Intracellular Colonization of Protoplasts

Protoplasts can be readily and rapidly isolated from the root hairs of crop plants, including the cereals, by enzymatic degradation of the cell wall of root hair apices (Fig. 2). The first report of the uptake of rhizobia by plant protoplasts set the stage for a better understanding of the uptake of rhizobia into nodule cells and the central role of endocytosis in both systems. It was shown in protoplasts...
that rhizobia were taken up into vesicles formed by invagination of the plasma membrane. Since the membranes of the vesicles containing bacteria are derived from the plasma membrane (Fig. 1) they have an origin directly analogous to that of the membrane enclosing bacteria which enter root nodule cells. It is now generally recognised that endocytosis is an essential cellular process occurring in all eukaryotic cells, both animal and plant.

Establishing a N₂-Fixing Symbiosis in Cereals

The challenge of establishing a N₂-fixing symbiosis in cereals and other major non-legume crops is seen as basically the challenge of establishing an adequate level of bacterial intracellular colonization and nitrogen fixation without necessarily any need for nodulation. But why is there actually no such thing as a N₂-fixing plant and why do plants have to form a symbiotic association with N₂-fixing bacteria to be N₂ fixing? As highlighted by Postgate, in terms of energetics, nitrogen fixation should present no evolutionary obstacle to plants. Eight molecules of ATP are required for every half N₂ molecules converted to NH₃ by bacterial nitrogenase and an additional 6 ATP are required as reductant making a need of 14 ATP per NH₃ produced. However, plants usually assimilate their nitrogen by the reduction of nitrate which requires 12 molecules of ATP to provide one molecule of NH₃; thus, nitrogen fixation is only marginally more demanding than nitrate reduction, in terms of energy consumption. Physiologically, the extreme oxygen sensitivity of nitrogenase proteins ought not to present a serious difficulty to plants. Anaerobic membrane-bound vesicular compartments are generally present in plant cells, and mitochondria have a low redox potential and adequate supplies of ATP. Indeed nothing in our present understanding of both the physiology and genetics of nitrogen fixation appears to raise any serious obstacle to the evolution of an autonomous nitrogen fixing plant. Postgate has commented that “it remains surprising that no plant followed what seems to be the easiest path to independence of bacteria: to exploit bacterial solutions to both the genetic and physiological problems of nitrogen fixation by way of a ‘diazoplast’, a new organelle analogous to a chloroplast, acquired in a like manner by accretion of an endosymbiotic prokaryote into the plant’s genome. The genetic obstacles to the emergence of autonomous nitrogen-fixing plants seem like the physiological obstacles, to be minor. So why has none appeared?” He suggests that plants never experienced the necessary selective pressure. He sees the requirement experimentally for the production of an autonomous nitrogen-fixing plant as “giving evolution a push in a direction in which it is already poised to go”. A first step towards a N₂-fixing symbiosis in cereals, with the possibility of diazoplast formation, can therefore be seen as finding a bacterium that will establish itself intracellularly within plant membrane-bound vesicular compartments fixing nitrogen symbiotically.

Gluconacetobacter Diazotrophicus

The N₂-fixing bacterium *Gluconacetobacter diazotrophicus* was originally isolated in 1988 from within sugarcane plants in Brazil. This endophytic, non-rhizobial bacterium colonizes sugarcane roots and shoots intercellularly and also the xylem, without nodulation and it has been demonstrated by ¹⁵N₂ incorporation that *G. diazotrophicus* fixes nitrogen inside sugarcane plants.

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Fig. 2 (A) Protoplast release from tip of rice root hair after a 5-min enzyme treatment. (B) Protoplast release from tip of barley root hair after a 5-min enzyme treatment. (C) The same as B after a 10-min enzyme treatment. (D) Protoplast release from tip of lettuce root hair after a 5-min enzyme treatment: note nucleus (arrow). (E) Protoplast release from very young root hair of tobacco after a 2-min enzyme treatment. (Adapted from Cocking).
However, there was no evidence for any intracellular colonization of living sugarcane cells. This bacterium has many novel features that would facilitate its effectiveness in symbiotic nitrogen fixation if it could be established intracellularly. Compared with most other microaerobic diazotrophs, its ability to fix nitrogen is more tolerant to oxygen since it can achieve a flux of oxygen that maintains bacterial aerobic respiration, while not inhibiting nitrogenase activity, by utilising the path length of colony mucoid levan polysaccharide between the atmosphere and the bacteria. It lacks nitrate reductase and can fix N2 in the presence of 20mM NO3−, and NH4+ causes only partial inhibition of its nitrogenase. It has a capability to excrete almost half of the fixed nitrogen in a form that is potentially available to plants. Moreover, the UAP5541 strain of *G. diazotrophicus* is known to produce endoglucanase, endopolymethylgalacturonase and endoxyglucanase constitutively when grown on sucrose which could facilitate bacterial penetration of the primary cell wall of root meristematic cells. It seemed likely that in plants such as the cereals and other crops, with much lower concentrations of intercellular sucrose than the 10-12% in sugarcane, *G. diazotrophicus* might seek to establish itself intracellularly by enzymatic cell wall penetration and uptake into endocytotic vesicles rather than by restricting its colonization to sucrose rich intercellular spaces as in sugarcane.

**Inoculation of Cereals with *G. Diazotrophicus***

We investigated the interaction of *G. diazotrophicus* (UAP5541) with seedlings of the cereals maize, rice and wheat. An aqueous suspension of *G. diazotrophicus* (5 colony forming units) was used for the inoculation of surface sterilised seeds germinated aseptically on Murashige and Skoog medium containing 3% (w/v) sucrose and vitamins but lacking growth...
regulators. Following inoculations with *G. diazotrophicus* containing a constitutive expressed *gus* A gene, the bacterial colonization of roots was visualised by light microscopy of the dark blue precipitate resulting from the degradation of the histochemical substrate X-GLUC by bacterial ß-glucuronidase encoded by the *gus* A gene. For observations on sections of roots, samples exhibiting blue-staining GUS activity were fixed in glutaraldehyde, dehydrated with ethanol and embedded in LR White acrylic resin. Sections of 1 µm were cut and counterstained with safranin (0.01% w/v) for light microscopy. Following inoculation of maize, wheat and rice, *G. diazotrophicus* was detected microscopically intracellularly within the cytoplasm of the cells of the root tips of all these inoculated non-legume crops at 7 days post inoculation. A schematic representation of the interaction is shown in Fig. 3. In maize, for example, dark blue stained *G. diazotrophicus* was clearly visible within the cytoplasm of meristematic cells of the elongating region of the root, and also within their cell walls (Fig. 4)\(^1\). Electron microscopy of ultrathin sections of the same roots confirmed that the *G. diazotrophicus* bacteria appeared to be within membrane-bound vesicles in the cytoplasm. Similar results using inoculation with *ni*/*H* promoter-GUS-labelled *G. diazotrophicus* showed blue-stained *G. diazotrophicus* within the cytoplasm of root cells indicating that intracellular conditions were suitable for the expression of nitrogenase, the bacteria enzyme complex that forms ammonia from gaseous nitrogen (N\(_2\)) and hydrogen.

The fact that *G. diazotrophicus* is able to become established in symbiosome-like compartments in the meristematic root cells of cereals and other non-legume crop (Fig. 3) indicates that there is likely to be no need for nodulation to achieve symbiotic nitrogen fixation. If stably transmitted from cell to cell fixing nitrogen, these symbiosome-like membrane-bound compartments containing *G. diazotrophicus* could become diazoplasts, a new type of organelle.

**Future Needs**

We are investigating by nitrogen balances and 15N methodology whether this non-nodular intracellular colonization by *G. diazotrophicus* results in symbiotic nitrogen fixation of benefit to plant growth. Field trials will need to be performed under a range of environmental and soil conditions to establish reductions possible in synthetic nitrogen fertilizer, while maintaining or increasing yields. There is an ever increasing urgency to do so not only to mitigate increases in reactive nitrogen depositions but also for Global Food Security. The Royal Society Report\(^16\) on Reaping the Benefits: Science and the Sustainable Intensification of Global Agriculture has highlighted the need for research on high-return topics such as nitrogen fixation in cereals to provide the dramatic, but sustainable, improvements in crop production that are urgently required. Food Security and the challenge of feeding 9 billion people requires a multifaceted and linked global strategy including how to optimise the use of nitrogen to not only produce enough food to meet the demand from population increase and the expansion of biofuel production, but also to minimise the impacts of synthetic nitrogen fertilizers on the environment and human health\(^17\).

This survey from plant protoplasts to diazoplasts parallels well the extensive investigations of J. C. Bose FRS on the behaviour of plant tissues under different modes of stimulation, with the idea of showing the exact parallelism in the behaviour of animal and plant tissues. We know that plant protoplasts can be fused with animal cells to produce heterokaryons that can synthesise both haemoglobin and cellulose\(^18\); and endocytosis, which occurs extensively in plant and animal cells\(^10\), now enables intracellular colonization of plant cells by N\(_2\)-fixing bacteria, resulting in the formation of diazoplasts. The vision of J. C. Bose FRS is still with us today.

**References**


