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REVIEW

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Establishing symbiotic nitrogen fixation in cereals and other non-legume crops: The Greener Nitrogen Revolution

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Abstract

Haber's invention of the synthesis of ammonia from its elements is one of the cornerstones of modern civilization. For nearly a century, agriculture has come to rely on synthetic nitrogen fertilizers produced from ammonia. This large-scale production is now supporting nearly half of the world's population through increased food production. But whilst the use of synthetic nitrogen fertilizers brought enormous benefits, including those of the Green Revolution, the world needs to disengage from our ever-increasing reliance on nitrogen fertilizers produced from fossil fuels. Their pollution of the atmosphere and water systems has become a major global environmental and economic concern. Naturally, legume crops such as peas and beans can fix nitrogen symbiotically by interacting with soil nitrogen-fixing rhizobia, bacteria that become established intracellularly within root nodules. Ever since this was first demonstrated in 1888, consistent attempts have been made to extend the symbiotic interaction of legumes with nitrogen-fixing bacteria to non-legume crops, particularly cereals. In 1988, a fresh impetus arose from the discovery of *Gluconacetobacter diazotrophicus* (*Gd*), a non-nodulating, non-rhizobial, nitrogen-fixing bacterium isolated from the intercellular juice of sugarcane. Subsequently, strains of *Gd* inoculated under specific conditions were shown to intracellularly colonize the roots and shoots of the cereals: wheat, maize (corn) and rice, as well as crops as diverse as potato, tea, oilseed rape, grass and tomato. An extensive field trials programme using a seed inoculum technology based on *Gd* (NFix®) indicates that NFix® is able to significantly improve yields of wheat, maize, oilseed rape and grasses, in both the presence and absence of synthetic nitrogen fertilizers. Evidence suggests that these benefits are accruing through a possible combination of intracellular symbiotic nitrogen fixation, enhanced rates of photosynthesis and the presence of additional plant growth factors. Here, we discuss the research events that have led to this important development and present results demonstrating the efficacy of NFix® technology in non-legume crops, in particular cereals.

Keywords: *Gluconacetobacter diazotrophicus*, Nitrogen fertilizer, Nitrogen fixation, Intracellular colonization, Yield benefits

A brief overview of nitrogen fertilizer production and pollution

The Haber–Bosch process for industrial ammonia production is considered by some to be the most important invention of the twentieth century. This important industrial development is partly responsible for the world's population explosion from 1.6 billion in 1900 to 6 billion in 2000 [1]. It has also been claimed that the use of 500 million tonnes of N fertilizer produced from ammonia each year, utilizing 1% of the world's energy and 3–5% of gas production [2], sustains around 40% of the global population [3]. However, the environmental costs of using ammonia-based nitrogen fertilizer across the globe are no longer considered sustainable. Nutrient excesses are particularly large in China, northern India, the USA and Western Europe [4]. In the UK, agricultural nitrogen fertilizer use accounts for 66% of nitrous oxide emissions [5], whilst in Europe 5% of the population are exposed to drinking water contaminated with unsafe levels of nitrate [6].

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In North America, agriculture is responsible for half of the nitrogen loading in Canadian surface waters [7–9], 75% of nitrous oxide emissions in the USA and for more than 1.5 million Americans drinking well water contaminated with nitrate [10]. Two-thirds of the coastal systems of the USA are moderately to severely impaired due to nutrient loading, with around 300 low-oxygen zones along the USA coastline. For example, the Mississippi River dumps 1.5 million metric tonnes of nitrogen into the Gulf of Mexico every year.

Addressing the pollution problems and damage caused by nitrogen fertilizer use is currently expensive making it unsustainable. Removing nitrates from water in Europe costs taxpayers between £130 and 650 per year. The economics of fertilizer use in European agriculture is questionable, given that the overall nitrous oxide damage costs £60–80 billion per year, a sum more than double the extra income gained from using nitrogen fertilizers in European agriculture [11].

The need for a sustainable alternative

There has been little argument that there is a need for more sustainable solutions to nitrogen fertilizer use. However, there is a shortage of genuine technical solutions available to address this global problem; hence, it remains difficult for governments to tackle the nitrogen issue.

There has been much support for developing technical solutions, not the least by the late Norman E. Borlaug, The Father of the Green Revolution. Hopes of biologically fixing nitrogen in cereals have been raised since the 1970s [12, 13], although these have yet to even be partially realized. Hence, an understandable reticence and caution has enveloped the whole field such that new developments are viewed with great scepticism, and even a reluctance to respect and accept results reflecting common standards of proof for the discipline. Such disparities in both interpretation and approach are indicative of an emerging paradigm shift [14–16].

Background to symbiotic nitrogen fixation in cereals

The introduction of symbiotic biological nitrogen fixation into cereals and other major non-legume crops would be regarded as one of the most significant contributions that biotechnology could make to agriculture. However, this has been recognized for many years as a major research challenge [17]. Currently, there are two strategic approaches used in attempts to achieve this long-standing aspiration. One is a long-term synthetic biology GM approach, engineering a nitrogen-fixing symbiosis from existing signalling and developmental mechanisms, to provide a suitable environment for rhizobial nitrogenase activity in the plant nodule [18]. The other, much shorter-term and simpler approach builds on the discovery that a non-rhizobial, naturally occurring nitrogen-fixing bacterium that fixes nitrogen in sugarcane is able to intracellularly colonize the root systems of cereals and other major crops [19]. In this approach, which is now at a field trial evaluation stage (Dent and Cocking in preparation), an adequate level of bacterial intracellular colonization and nitrogen fixation can be established throughout the plant without any need for nodulation. In such symbiotic nitrogen fixation, nitrogen-fixing bacteria establish an intracellular symbiosis with plants in which they fix nitrogen inside the cells of their host utilizing energy supplied by plant photosynthesis.

The need for intracellular colonization

Nodulated legumes in endosymbiosis with rhizobia are amongst the most important nitrogen-fixing systems in agriculture. In nodular cells, intracellular rhizobia, surrounded by a membrane derived by endocytosis from the plant plasma membrane, symbiotically fix nitrogen in cytoplasmic vesicles (symbiosomes). It is only by such intracellular colonization of nitrogen-fixing bacteria in these membrane-bound compartments (a state in which the bacteria are referred to as bacteroids) in the cytoplasm, that the prerequisites for symbiotic nitrogen fixation can be fulfilled. In symbiosomes, there is a reliable supply of metabolic substrates from plant biosynthesis which provides (a) sufficient energy and reducing conditions, (b) protection against elevated oxygen concentrations that inhibit nitrogen fixation, (c) a pathway for the transport of nitrogen fixation products to the plant host, (d) development of membrane systems for bidirectional transport between the host and bacteria and endosymbiont and (e) protection against competitive or antagonistic bacteria in the environment [20].

However, the actual formation of nodules is not an essential requirement for the establishment of intracellular plant endosymbiotic nitrogen fixation. In non-legume angiosperm *Gunnera* plants, the nitrogen-fixing cyanobacterium, *Nostoc*, enters mucilage-secreting glands found on the stem in the axis of the leaves. It invades the gland cells and becomes intracellular in membrane-bound vesicles in the cytoplasm, similar to symbiosomes in rhizobium–legume symbiosis but without nodule formation [21, 22]. The cyanobacteria divide within the intracellular vesicles and differentiate into heterocysts. These heterocysts possess a double-layered envelope of polysaccharide and glycolipids which act as a barrier to oxygen diffusion. The quantity of nitrogen fixed by the intracellular heterocyst symbiont and transferred to the *Gunnera* host plant is sufficient to satisfy its nitrogen requirements [23].

Bacteria which can colonize plants intracellularly have now been identified in a wide range of plant species including non-nodulating legumes colonized by rhizobia [24], peach palm [25], scotch pine [26], Engelmann spruce and limber pine [27], banana [28, 29] and cactus, four of which include nitrogen-fixing bacteria [27, 30].

The ability of nitrogen-fixing bacteria, other than *Nostoc*, to endosymbiotically fix nitrogen in symbiosomes in non-legumes, without the need for nodulation is of major evolutionary interest. Also the ability of rhizobia to fix nitrogen in 13 species of non-nodulating legumes (species of the subfamilies Caesalpinioideae, Papilionoideae and Mimosoideae [31, 32]) has important implications from an evolutionary perspective [32]. There is sufficient evidence available reporting that nodule formation is not a necessary prerequisite for intracellular plant nitrogen fixation, even in the rhizobia–legume symbiosis. The Leguminosae nodulation seems to occur amongst the more specialized subfamilies. It is important to note that the host plant itself encodes the genetic developmental programme responsible for the development of the nodule tissues and for regulation of the process, with organogenesis being triggered by the rhizobial micro-symbiont [33]. This raises questions in regard to the means of cell entry in non-nodular and non-leguminous symbioses.

Entry of nitrogen-fixing bacteria

Until now, it has been unclear as to how nitrogen-fixing bacteria are able to breach the cell walls of plants to establish endosymbiosis; more is known about the uptake of rhizobia. They are endocytosed into plant cells in which they become surrounded by a peribacteroid membrane to create the symbiosome structure referred to above. Endocytosis is only possible after bacteria have penetrated the cell wall and are able to interact with the plant cell plasma membrane. Interestingly, forms resembling bacteroids have long been reported inside the roots of non-nodulating *Gleditsia* species [34–36]. This finding is supported by work using scanning electron microscopy in *G. triacanthos* and other species [31]. Penetration of these species (*G. triacanthos*, *Cassia fistula*, *C. grandis* and *Senna tora*) cell wall is most likely to occur through the formation of infection threads in the root hairs [31, 37], whereas other mechanisms of entry are also evident with rhizobia. Particularly pertinent in this regard is the production and isolation of cell-bound cellulases of white clover rhizobia that can erode a complete hole, slightly larger than the bacterial cell, that traverses the non-crystalline wall region of white clover root hair apices [38]. Alternatively, plant cell wall-degrading enzymes induced in response to rhizobia may be involved.

Further insights into the entry of nitrogen-fixing bacteria into plant cells and the establishment of intracellular colonization have been obtained from an extensive range of experiments extending between 1960 and the mid-1990s, using enzymatically isolated plant protoplasts. The isolation of plant protoplasts using cellulases to digest the cell wall opened a whole new range of studies in plant cellular and molecular biology. What made the isolated protoplast so powerful as an experimental system was that the enzymatic removal of the cell wall left the cell surface membrane (the plasma membrane) fully exposed as the only barrier between the external environment and the interior of the cell. The accessibility of the plasma membrane meant that experiments could be designed to investigate and manipulate the properties of this membrane, in a way that was not possible with walled cells [39]. It was therefore possible to demonstrate the endocytotic uptake of rhizobia into pea leaf protoplasts [40]. This seminal finding showed clearly the need to select bacteria for symbiotic interactions that are both nitrogen fixing and cellulase producing. The origin of the membrane-bound vesicles containing rhizobia was directly analogous to that of vesicles enclosing rhizobia, which enter root nodule cells via the normal infection thread. This highlighted the importance of cell wall degradation, without loss of cell viability, to establish the cell as a habitat for nitrogen-fixing bacteria in membrane-bound vesicles destined to become symbiosomes.

The ability to isolate nodule protoplasts containing symbiosomes with bacteroids [41], coupled with the ability to fuse these protoplasts with protoplasts isolated from non-legumes, provided another way of establishing rhizobial symbiosomes in non-legume crops for the production of nitrogen-fixing hybrid cells, possibly leading to the formation of nitrogen-fixing somatic hybrid plants. A wide range of heterokaryons between legumes and non-legumes was produced [42]. However, it proved impossible to maintain the ongoing viability and cell division of these heterokaryons.

Increasing the host range of rhizobia

Establishing intracellular colonization and symbiosome formation in non-legume crops could result from increasing the host range of rhizobia. In the soil–root surface, the rhizosphere, developing root hairs play an important role in symbiotic recognition. Rhizobia, and especially the Nod factors (signal lipo-chitooligosaccharides) they secrete, stimulate the re-orientation of root hair cell wall growth, resulting in curled root hairs. Nod factors promote the formation of infection threads, and it is through these tubular structures that rhizobia enter most legumes. For temperate legumes such as clover, there is a very tight control of Rhizobium specificity. A barrier to this specificity could be removed by enzymatically degrading the cell wall at the apices of root hairs in

white clover, using a cellulase–pectolyase enzyme mixture. Subsequent inoculation with rhizobia in the presence of polyethylene glycol enabled rhizobia to induce the formation of nitrogen-fixing nodules on white clover (a non-host). This cellulase–pectolyase enzyme treatment is also known to release protoplasts from the tips of root hairs of legume and non-legume crop plants and provides an opportunity for rhizobial endocytosis via root tip interaction, thus identifying the apical cell wall of root hairs as a target site for the nodulation of cereals and other non-legume crops [43].

In rice, it was shown that rhizobia could induce formation of nodule structures provided the rice seedling root hairs were treated with cell wall-degrading enzymes and polyethylene glycol. These nodule structures consisted of several cell layers enclosing scattered centrally located cells, with rhizobia mainly located in spaces between the cell layers, and in dead cells of the central region, there was negligible nitrogenase activity [44]. Similar enzyme treatment and inoculation of oilseed rape also produced nodular structures morphologically resembling clover nodules. However, only dead cells were invaded by rhizobia and there was only a very low nitrogenase activity [45]. From these attempts, it became progressively evident in the 1990s that it was not possible, using these novel inoculation procedures to imitate in non-legume crops the organized nodule development pattern, characteristic of legume nodulation by rhizobia. Nor was it possible to obtain the membrane-bound rhizobia colonization of the living nodular cells required for endosymbiotic nitrogen fixation.

This failure to achieve effective nodulation of non-legume crops by rhizobia led to a search for a non-nodular niche for endophytic nitrogen fixation. This was comparable to the situation arising from the naturally occurring non-nodular colonization of intercellular spaces and xylem of sugarcane by endophytic nitrogen-fixing bacteria. Some sugarcane varieties are able to obtain over 60% of their nitrogen from plant associated biological nitrogen fixation [46].

The use of other nitrogen-fixing bacteria

Rhizobia are now regarded as being inadequate to establish symbiotic nitrogen fixation in non-legume crops, because they do not colonize living cells and their nitrogen-fixing capability is inhibited by oxygen [47, 48]. Regulating the flux of oxygen to endosymbiotic rhizobia has been suggested to be an almost insuperable challenge to the dream of introducing rhizobial nitrogen-fixing symbiosis effectively into non-legume crops [48]. The use of other nitrogen-fixing bacteria such as *Azoarcus* spp., an endophyte of Kallar grass, and *Klebsiella* spp., an endophyte of maize, highlighted the readiness with which endophyte colonization of intercellular spaces and dead xylem cells can occur following their inoculation of non-legume crops. However, like with rhizobia in non-legumes, there was no evidence for any intracellular colonization of living cells in any of the plants investigated and therefore no opportunity for the establishment of symbiotic nitrogen fixation [49].

Nitrogen fixation in sugarcane

The long-term continuous cultivation of sugarcane in Brazil uses low N fertilizer inputs without apparent depletion of soil N response. This led to the suggestion that nitrogen-fixing bacteria associated with the sugarcane might be the source of the agronomically significant nitrogen inputs to this crop [50]. One of these was a new species of nitrogen-fixing non-rhizobial, aerobic bacterium that had been isolated in 1988 from Brazilian sugarcane roots and stems [51]. The same species of bacteria was later extracted in large numbers from the xylem of Mexican sugarcane cultivars, which are also colonized intercellularly [52]. This bacterium, later renamed *Gluconacetobacter diazotrophicus* (*Gd*), has many characteristics that made it attractive for the establishment of intracellular symbiotic nitrogen fixation, without the need for nodulation, paralleling the situation in the angiosperm *Gunnera* with *Nostoc* [21]. *Gd* is known to produce cellulases, hemicellulases (xyloglucanases) and pectinases, which could facilitate the penetration of plant cell walls [53], all of which are formed in the presence of sucrose which is involved in the establishment of the Gunnera–Nostoc endosymbiosis [54].

Gluconacetobacter diazotrophicus (*Gd*), 'this most extraordinary diazotroph' [55], is a small, 0.7–0.9 by 1–2 μm, motile, gram-negative aerobic rod showing pellicle formation. Best growth occurs with high sucrose or glucose concentrations (10% w/v) [56]. It secretes an enzyme, levansucrase, which hydrolyses sucrose and catalyses the formation of the exopolysaccharide fructan levan [57][1] which limits oxygen diffusion. This protects nitrogenase activity from excessive oxygen flux, enabling the bacteria to fix nitrogen even if the pO_2 is not much lower than tropospheric levels [58]. Detoxification of reactive oxygen species may also occur [59]. *Gd* possesses no nitrate reductase, and nitrogen fixation is not inhibited by high concentrations of nitrate (25 mM). The nitrogenase activity of *Gd* is only partially inhibited by ammonium, implying that *Gd* can continue fixing nitrogen inside the plant even when

[1] With the recent discovery of nitrogen fixation in sugarcane, the development of nitrogen fixation in other grasses now seems within our grasp in the near future. This development will likely not be based on the introduction of *nif* genes into grasses or on the induction of nodules on grass roots that harbour diazotrophs. This development will most likely be based on the successful inoculation of diazotrophs that infect and inhabit the roots and stems of grass species in a manner similar to the sugarcane system.

nitrate (and ammonium)-containing fertilizers are applied. Another unique feature of this bacterium is its ability to excrete almost half of the fixed nitrogen as ammonium, which becomes potentially available to plants [60].

The intracellular establishment of *Gd*

The primary cell walls of plant meristems consist of β-1,4-glucan-linked chains of cellulose that coalesce via hydrogen bonding to form polysaccharide microfibrils [61]. The action of *Gd* cell wall-degrading enzymes might weaken the primary cell walls of root meristems sufficiently, enabling *Gd* to interact directly with the plasma membrane of root cell protoplasts. Endocytotic uptake of *Gd* into membrane-bound vesicles in the cytoplasm could then result in the intracellular establishment of *Gd* in symbiosomes, similar to rhizobia in root nodule cells [19]. Using seedlings of maize, rice, wheat, oilseed rape, tomato, white clover and Arabidopsis grown aseptically in sucrose-containing culture media, inoculation with very low numbers of *Gd* has resulted in extensive intracellular colonization of root meristems within membrane-bound vesicles [19]. *Gd* subsequently spreads intracellularly from the root tip meristem into cells of the elongating region of the root and into leaf cells of the shoot system in which *Gd* associates closely with chloroplasts. This raises the possibility that energy might be supplied directly from chloroplasts to nitrogen-fixing endosymbiotic bacteria within the same cell.

The generality of the intracellular colonization of this wide range of plant species by *Gd*, contrasting with the high degree of rhizobial strain specificity for most legume nodule interactions, may be due to the very close similarity in primary wall composition of root meristems throughout the plant kingdom [19]. This discovery of plant systemic intracellular colonization by *Gd* sets the stage for investigating their possible utilization to reduce synthetic nitrogen fertilizer application used in agriculture, and any additional effects on intrinsic yield potential.

Implications for evolution

The ability of *Gd* to systematically colonize both non-legumes and legumes intracellularly and without nodulation could provide new insights into the evolutionary origin of symbiotic nitrogen fixation in plants. Postgate [62] has posed the question as to why there is no such thing as a nitrogen-fixing plant and why do plants have to form a symbiotic association with nitrogen-fixing bacteria to gain access to atmospheric nitrogen? In terms of energetics, nitrogen fixation should not present an evolutionary obstacle to plants. Eight molecules of ATP are required for every 1/2 molecule of N_2 reduced to NH_3 by the bacterial nitrogenase. Additional six ATP molecules are required as a reductant, resulting in a need of 14 ATP molecules per NH_3 produced. Plants usually assimilate their nitrogen by reduction of nitrate that 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.

It is surprising that no plant has yet become independent of bacteria in relation to N uptake [62]. The development of a 'diazoplast', a new organelle analogous to a chloroplast, would help plants overcome the genetic and physiological bacterial-dependent nitrogen fixation problems. The diazoplast could be acquired similarly to a chloroplast by an accretion of an endosymbiotic prokaryote into the plants genome. The inoculation of plants with *Gd*, in the presence of sucrose, results in its establishment intracellularly in root meristems and then systemically in roots and shoots within plant membrane-bound symbiosome-like vesicular compartments in the cytoplasm. These structures are likely the location for the formation of diazoplasts and the emergence of autonomous nitrogen-fixing plants by 'giving evolution a push in a direction in which it is already poised to go' [62]. It would now seem likely that the independent evolution of intracellular non-nodular nitrogen fixation, thought at present to be restricted to the Gunneraceae [63], is not unique but that such non-nodular nitrogen fixation can be extended to a very wide range of plant families [25–27, 32]. Interestingly the phylotype of some conifers has been found to be related to *Gd* and other nitrogen-fixing acetic acid bacterial endophytes including *Acetobacter*, *Acidomonas*, *Kozakia* and *Asaia* [27]. This suggests that non-nodular, endocytotic, intracellular meristem cell colonization by a nitrogen-fixing and cellulase producing bacterium, such as *Gd*, is the actual single evolutionary innovation that drove the deep evolution of symbiotic nitrogen fixation in plants [64].

Agricultural value of *G. diazotrophicus*

The criteria for evaluating the practicality of using any nitrogen-fixing bacterium in agriculture need to at least match those applied for the assessment of the efficacy of rhizobia/legume associations. Leaving aside the need for colonization, intracellularity and the development of symbiosomes (considered above), BNF studies to demonstrate nitrogen fixation should look for increases relative to controls associated with the following criteria (expanded on below):

- The measurement of foliage greenness/chlorophyll content
- The measurement of the percentage N in the biomass
- Labelled N studies
- *Nif* minus mutants

- The measurement of nitrogenase activity by e.g. ARA
- Demonstration of field efficacy and yield benefits in nitrogen poor soils in the absence and/or presence of a nitrogen fertilizer

Foliage greeness/improved chlorophyll content: There is a very close link between chlorophyll and nitrogen content [65–67]. Studies with legume/rhizobia associations have reported enhanced leaf chlorophyll content in chickpea [68], cowpea [69] and common bean [70] when the associations were established. Similarly, chlorophyll levels have been demonstrated to increase in sorghum colonized by *Gd* [71] and leaf greenness increased in *Lolium perenne* with *Gd* IMI501986 relative to uninoculated controls (Patent Number: WO2016/016629).

Percentage N in leaves: A 40% increase in leaf N in sorghum has been demonstrated when co-inoculated with AM fungi [71]. Trials by Azotic Technologies have shown a 43% increase in leaf N of inoculated *L. perenne* relative to uninoculated field plots (Dent and Cocking in preparation).

Nitrogenase activity demonstrated by ARA: The acetylene reduction assay (ARA) has been used for many years to demonstrate nitrogen fixation by rhizobia in root nodules. For *Gd*, similar assays have successfully focused on nitrogen fixation capability of bacteria in culture [72, 73], but when attempted for inoculated maize plants there was no evidence of nitrogen fixation [74].

Nitrogen fixation demonstrated by *Nif* minus studies: The ability to use *Nif* mutants in a bacterium and therefore the loss of nitrogen-fixing ability is the key indicator of nitrogen fixation *in planta*. For *Gd*, this was achieved by demonstrating that the shoot nitrogen content of sugarcane plants inoculated with wild-type Pal5 was significantly higher than in uninoculated control plants, or plants inoculated with the *Nif*-mutant Mad3A in nitrogen-limiting conditions [75].

Nitrogen fixation demonstrated by labelled nitrogen ($^{15}N_2$) studies: A $^{15}N_2$ incorporation experiment demonstrated that *Gd* Pal5 wild-type strains actively fixed nitrogen inside sugarcane plants, whereas *Nif* mutants did not [75]. Another $^{15}N_2$ incorporation experiment also demonstrated nitrogen fixation by *Gd* in sugarcane [73].

Plant growth and yield benefits: Plant growth and yield parameters (seed yield, plant size, flowering times) have been shown to be markedly improved in rhizobia inoculated versus uninoculated legume crops for soybean [76, 77], pea and lentils [78], common bean [79], chickpea [80], mung bean [81] and cowpea [69]. Similarly with *Gd*, tomato plants inoculated with the Pal5 strain significantly increased both number and weight of tomato fruit production as compared to non-inoculated controls [82]. Biomass accumulation following inoculation with 14 strains of *Gluconacetobacter* spp. was well matched with corresponding nitrogen fixation in all tissues of sugar beet [83]. Inoculation with two strains of *Gd* (Pal5, UAP5541), which colonized the roots and shoots of sorghum and wheat, increased both the shoot and root dry weight of sorghum, whereas they had no effect on either shoot or root dry weight of maize [84, 85]. Interestingly, Pal5 inoculation was also unable to relieve the N-deficiency symptoms of unfertilized maize in either the field or the greenhouse [86]. However, recent independent field trial research utilizing proprietary NFix® technology (Patent Number: WO2016/016629) based on *Gd* demonstrated significant yield improvements of around 1 tonne per hectare in both maize and wheat (Figs. 1, 2, for raw data see Additional file 1).

Photosynthetic rates: The rhizobia–legume symbiotic association is known to contribute benefits to the plant greater than just nitrogen fixation. Indeed, even with the cost to the plant of reduced photosynthate by as much as 14% [87], rhizobia have been demonstrated to increase the rate of photosynthesis and other associated measures in common bean (intercellular CO_2 concentration and the transpiration rate) [70], peas [88] and soybean [87]. Rhizobial colonization of the non-legume crop rice increased photosynthetic rate, stomatal conductance, transpiration velocity, water utilization efficiency and flag leaf area. Furthermore, it was shown that higher levels of the growth hormone indole-3-acetic acid (IAA) and gibberellic acid (GA) were accumulated [89]. The

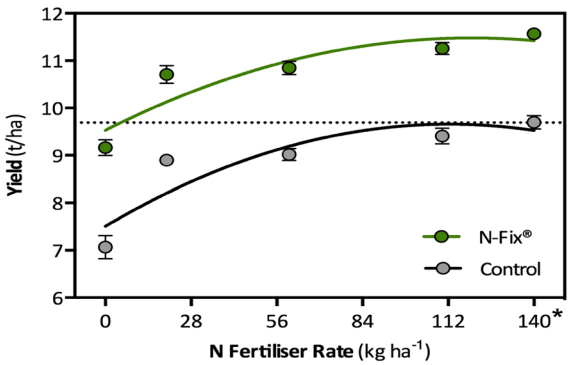

Fig. 1 Yield of maize inoculated with NFix® against uninoculated controls in an independent N fertilizer trial. This N fertilizer trial was conducted by independent third-party contract researchers on maize variety PR39F58 in Germany. The untreated seed control was compared with the NFix® treatment at five different levels of N fertilizer application. *Bars on each point* represent standard errors of the means of yields from six plots (60 m^2) in a split-plot design (main plot: seed treatment; subplot: nitrogen fertilizer). The *dotted line* indicates when the yield of the full recommended rate of N fertilizer* according to the total available N in the soil pre-sowing is equivalent to the yield of the NFix® treatment

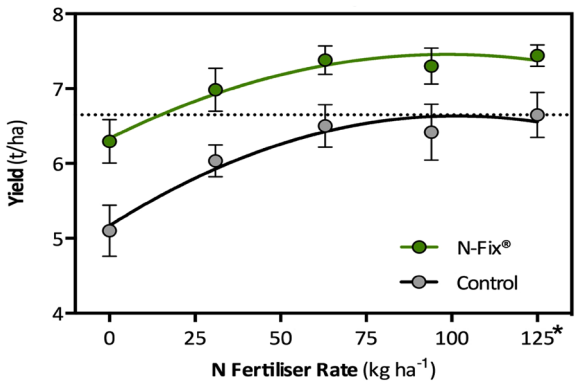

Fig. 2 Yield of wheat inoculated with NFix® against uninoculated controls in an independent N fertilizer trial. This N fertilizer trial was conducted by independent third-party contract researchers in the UK on wheat variety Mulika. The untreated seed control was compared with the NFix® treatment at five different levels of N fertilizer application. *Bars on each point* represent standard errors of the means of yields from four plots (24 m^2) in a split-plot design (main plot: seed treatment; subplot: nitrogen fertilizer). The *dotted line* indicates when the yield of the full recommended rate of N fertilizer* according to the total available N in the soil pre-sowing is equivalent to the yield of the NFix® treatment

only research that has been conducted in this field with *Gd* has involved the inoculation of *Arabidopsis thaliana*. In this study, *Gd* had beneficial effects on photosynthesis [90]. Whole-canopy photosynthesis levels were 68% higher in inoculated plants compared to uninoculated plants. However, transpiration rates were 15.5% lower. As a consequence of greater carbon uptake and lower water loss through transpiration, inoculated plants showed increases of 94.5% in whole-canopy water-use efficiency compared to non-inoculated plants. These results suggest that *Gd* positively affects plant physiology by increasing the photosynthetic capacity and improving water-use efficiency [90], which goes some way to explaining the parallel yield relationships across all levels of N fertilizers, shown in Figs. 1 and 2.

Conclusions

The need for an improved means of delivering nitrogen to cereals and other non-legume crops is crucial for the future of sustainable agriculture, including the reduction of ammonia, nitrate and nitrous oxide pollution but ensuring food security [91].[2] The promise of biological nitrogen fixation for cereals proffered in the 1980s may now be realized, however, not as originally envisaged through rhizobial association with genetically manipulated root nodules on wheat for instance, but rather through appropriate application of the naturally occurring nitrogen-fixing endophyte *Gd* to the plant using products, such as NFix®.

The necessary ability for intracellular colonization was established initially in 2006 with *Gd* in a range of crop species [19]. This has now been independently validated with a number of nitrogen-fixing bacteria for a range of plants (e.g. [27]), and the enzymes required for cell entry determined. Measures used to establish nitrogen fixation and plant growth benefits in rhizobia–legume symbiosis have been successfully applied to *Gd*, similarly demonstrating nitrogen fixation and significant crop yield benefits, the latter even in the presence of full recommended rates of nitrogen fertilizer. The development of a nitrogen-fixing endophytic bacterium that can be applied to all staple food crops and substitute for mineral nitrogen fertilizers, whilst delivering yield benefits, is a significant major development and heralds the prospect of a *Greener Nitrogen Revolution*!

Additional file

Additional file 1.

Abbreviations

Gd: *Gluconacetobacter diazotrophicus*; GM: genetically modified; N: nitrogen; ATP: adenosine triphosphate; ARA: acetylene reduction assay; BNF: biological nitrogen fixation; AM fungi: arbuscular Mycorrhizal fungi; IAA: indole-3-acetic acid; GA: gibberellic acid.

Authors' contributions

DD and EC wrote the manuscript. Both authors have read and approved the final manuscript.

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Dedicated to the pioneering research and memory of José de Jesús Caballero-Mellado (1953–2010).

Competing interests

DD is a Founder Director of Azotic Technologies Ltd. and Chief Technology Officer and hence has been responsible for the development of the NFix® formulation cited in the text and the patent WO2016/016629.

Availability of data and materials

All data generated or analysed during this study are included in this published article [and its supplementary information files].

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[2] Creating new strains of rice, wheat and corn that fix their own nitrogen could achieve in the twenty-first century is what the Haber–Bosch breakthrough managed for the twentieth and without the serious environmental drawbacks of industrial ammonia production. Environmentalists should not be scared of this prospect; they should welcome it. There can be no more important task than feeding people whilst protecting the planet. We must use the best of science and technology to help us to achieve this vital aim.

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